How Biology Handles Nitrite

ARTICLE *in* CHEMICAL REVIEWS · APRIL 2014

Impact Factor: 46.57 · DOI: 10.1021/cr400518y · Source: PubMed

2 AUTHORS:

Luisa Maia
New University of Lisbon, Portugal
19 PUBLICATIONS **220** CITATIONS
SEE PROFILE

José J G Moura
New University of Lisbon
572 PUBLICATIONS **12,037** CITATIONS
SEE PROFILE

How Biology handles nitrite

Journal:	*Chemical Reviews*
Manuscript ID:	cr-2013-00518y
Manuscript Type:	Review
Date Submitted by the Author:	20-Sep-2013
Complete List of Authors:	Maia, Luisa; REQUIMTE/CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Moura, José; REQUIMTE/CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa,

Manuscript ID: cr-2012-00412w

Manuscript title:"How Biology handles nitrite"

Journal: Chemical Reviews

Proposal ID: cr-2012-00412w

Author(s): Luisa Maia, José J. G. Moura

How Biology handles nitrite

CONTENTS

1. INTRODUCTION

Nitrite is one of the players of the broad nitrogen biogeochemical cycle. This nitrogen oxo-anion is involved in key pathways crucial to life on Earth and to the planetary "recycling" of nitrogen. From a more "human" perspective, nitrite (and nitrate) is an important food preservative, used during the last five millennia.[1] The successfully nitrite "human history" was, however, overshadowed in the 1970s, when it was suggested that nitrite might increase the incidence of cancer, through the formation of N-nitrosamines.[2-8] Recently, another twist took place, and nitrite is now being rediscovered as a beneficial molecule (endogenously formed or therapeutically added) involved in cell survival during hypoxic events (as will be here discussed).

space (= blank line)

In this article, we will review the nitrite physiological role on the nitrogen biochemical cycle (sections 2.) and on the particular "scenario" of mammalian and plant signalling pathways (sections 3.1. and 3.2), including a very brief description of potential bacterial signalling (section 3.). On the *"Nitrite on the nitrogen cycle"* chapter, the main, well established, pathways will be briefly described, with emphasis on the nitrite-mediated reactions. On the *"Nitrite on signalling pathways"* chapters, the nitrite-mediated reactions will be discussed with a deeper detail: the nitrite-mediated signalling and damaging pathways are a (comparatively) recent and controversial area and the review will be oriented to discuss the feasibility of these *novel* pathways mainly from the chemical point of view.

As will be described, the living organisms use nitrite for remarkably different purposes, oxidising and reducing it. After identifying each type of nitrite reaction, key reaction mechanisms will be analysed at a molecular level of detail (sections 4.) and structure/activity relationships will be (as much as possible) systematically explored to discuss the mechanistic strategies that Biology developed to handle nitrite.

The global aim is to review the present functional, structural and mechanistic knowledge of nitrite reduction/oxidation, to assess in what extent we understand how nitrite is handled by living organisms (nitrite formation is outside the scope of this review). This knowledge is essential for the comprehension of the global nitrogen biochemical cycle and, consequently, of the impressive changes

the human activities are introducing on the cycle. Furthermore, the wealth of information gathered and discussed enables to further evaluate the feasibility and physiological significance of the presently accepted "avenues" of NO formation in humans and plants and to foresee new potential pathways.

2. BIOLOGICAL FATE OF NITRITE - NITRITE ON THE NITROGEN CYCLE

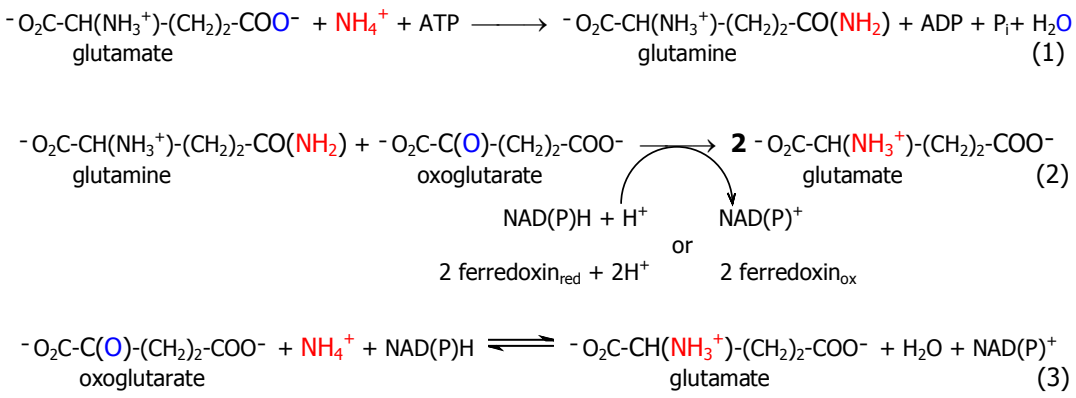
Nitrogen is absolutely essential for life, being the fourth most abundant element in living organisms (behind hydrogen, oxygen and carbon).[9] Nitrogen is used by ***all*** organisms for the biosynthesis of amino acids, nucleosides and other fundamental compounds, and two nitrogen **assimilatory pathways** were developed to provide the necessary reduced nitrogen; this is, then, recycled in an universal "organic nitrogen pool". In addition, ***some*** organisms also use nitrogen compounds as substrates for "respiration" and for that purpose several nitrogen **dissimilatory pathways** have also evolved. The nitrogen biochemical cycle (Fig. 1) keeps this element in forms available to support live on Earth, "starting" with fixation from the atmosphere (the largest nitrogen source), and "recycling" it through the dissimilatory pathways.[10-17]

2.1. NITROGEN ASSIMILATORY PATHWAYS

Two nitrogen assimilatory pathways provide the reduced nitrogen (ammonium) essential for biosynthetic purposes (Fig. 1, yellow and orange arrows). The organisms capable of dinitrogen fixation (some free-living archea and bacteria, *e.g.*, *Azotobacter*, and symbiotic bacteria, *e.g.*, *Rhizobium*) possess a unique pathway, where the atmospheric dinitrogen is directly reduced to ammonium, in a reaction catalysed by molybdenum/iron-dependent nitrogenases[18] (Fig. 1, yelow arrow).[22-33]. All the other organisms, prokaryotic and eukaryotic, depend on environmental (soils, oceans and crust) available ammonium and nitrate/nitrite. The nitrate assimilation is dependent on two sequential reactions (assimilatory ammonification, Fig. 1, orange arrows): first, the reduction of nitrate to nitrite (catalysed by molybdenum-dependent nitrate reductases (NaR)) and, then, the reduction of nitrite to ammonium (catalysed by sirohaem-containing nitrite reductases (CSNiR); described in section 4.1.2.).[34-41] The electron source to carry out the nitrite reduction to ammonium is (i) the photosynthetically reduced ferredoxin, in photosynthetic organisms (*e.g.*, cyanobacteria and chloroplasts of photosynthetic eukaryotes[36,39,42]), or (ii) the reduced pyridine nucleotide pool, in most

heterotrophs, but also in some phototrophs (*e.g.*, non-photosynthetic tissues of higher plants[42] and *Rhodobacter capsulatus*[43,44], respectively). For that reason, there are two types of assimilatory CSNiR: ferredoxin-dependent and NAD(P)H-dependent enzymes. Within each type, the enzymes share sequence and structural similarity, regardless of their prokaryotic or eukaryotic origin. Both types hold a sirohaem, where the nitrite reduction takes place, and an iron-sulfur centre (Fe/S), but NAD(P)H-dependent enzymes also contain a FAD domain that is involved on the NAD(P)H binding and oxidation.[34-41]

Ammonium, then, enters the "organic nitrogen pool" (Fig. 1, pink arrows) in the form of two amino acids, glutamine and glutamate, through the concerted action of glutamine synthase (eq. 1) and glutamate synthase (eq. 2) present in bacteria, fungi and plants. Ammonium can also be directly incorporated into glutamate, *via* the glutamate dehydrogenase enzyme found in all forms of live (eq. 3). However, under most physiological conditions, glutamate dehydrogenase catalyses instead the reverse reaction (in amino acid catabolism), yielding ammonium. Hence, the key enzyme that controls the entrance of the "organic nitrogen pool" is the glutamine synthase, not surprisingly one of the most complex regulatory enzymes. Besides this crucial role, glutamine synthase is also of main importance in animals, where it is responsible for the removal of toxic ammonium.



The organic nitrogen, in the form of amino and amide groups, can now be transferred to other amino acids and several other nitrogen-containing biomolecules, supporting the vital biosynthetic needs. The organic nitrogen, thus formed, is recycled between all living organism, through chain foods, waste products and organic decay. Eventually, the organic nitrogen is converted back to ammonium

(in particular through organic decay, by bacteria and, in some cases, fungi) and returned to the environment, in a process called mineralization, thus closing the "organic" part of the nitrogen cycle ((Fig. 1, pink arrows).

2.2. NITROGEN DISSIMILATORY PATHWAYS

The biochemical cycle of the nitrogen continues with four main ("classic") nitrogen dissimilatory pathways, where the nitrogen compounds are used as electron donors/acceptors to derive energy: (i) nitrate is anaerobically reduced to dinitrogen (denitrification - Fig. 1, blue arrows), or (ii) to ammonium (dissimilatory nitrate reduction to ammonium (DNRA) - Fig. 1, green arrows), (iii) while ammonium is aerobically oxidised to nitrate (nitrification - Fig. 1, black arrows), or (iv) anaerobically oxidised to dinitrogen (anaerobic ammonium oxidation (AnAmmOx) - Fig. 1, gray arrows).

The denitrification[45,47] is found in a wide range of organisms, from archaea to eubacteria and even in some eukaryotes (*e.g.*, fungi[48], protozoa and benthic Foraminifera and *Gromiida*[54,56-60]), in both autotrophic and heterotrophic organisms, coupled to the anaerobic oxidation of organic carbon. It encompasses the anaerobic nitrate reduction to molecular dinitrogen, through four sequential steps (eq. 4; Fig. 1, blue arrows), involving three one-oxygen atom abstractions and one N-N bond formation ($NO \rightarrow N_2O$), catalysed by specific metalloenzymes dependent on molybdenum, iron and copper. The nitrite reduction to nitric oxide radical[61] (NO), in particular, is catalysed by two different types of enzymes: iron-dependent and copper-dependent nitrite reductases (denitrifier organisms have either one of these enzymes, but not both). The iron-dependent enzyme arbours *c* and d_1 haems (d_1 haem-containing nitrite reductase (Cd_1NiR), discussed in section 4.2.1.) and obtains the necessary electrons from a range of structurally different soluble electron carriers (pseudo-specificity), such as *c* type cytochromes (c_{550}, c_{551}, c_{554}) and copper proteins (azurins and pseudoazurins).[62] The copper-containing nitrite reductases (CuNiR, discussed in section 4.2.2.) are classified into two sub-groups depending on their colour being blue (*e.g.*, from *Alcaligenes xylosoxidans*[63]) or green

(*e.g.*, from *Alcaligenes faecalis* or *Achromobacter cycloclastes*). The CuNiR are more widespread (although less abundant) and less promiscuous with the electron donors than the Cd_1NiR, being pseudo-specific in their interaction with azurin and *c* type cytochrome (c_{551}, blue CuNiR) or pseudoazurin (green CuNiR).[65-67]

$$NO_3^- \longrightarrow NO_2^- \longrightarrow {}^{\bullet}NO \longrightarrow N_2O \longrightarrow N_2 \qquad (4)$$

The DNRA (Fig. 1, green arrows) is a different strategy to reduce nitrate, employed to grow anaerobically. On this pathway, nitrite is directly reduced to ammonium (dissimilatory ammonification) with a multi-*c*-haem-containing enzyme (*c* haem-containing nitrite reductase (CcNiR), discussed in section 4.1.1.) and the necessary electrons are derived from the anaerobic oxidation of organic carbon through the membrane quinone pool.[68]

Conversely, the nitrifiers (nitrification - Fig. 1, black arrows) use of the aerobic oxidation of ammonium to nitrite, and then to nitrate, for the chemoautotrophic fixation of inorganic carbon.[69,70] (DNRA and nitrification can be regarded as short-circuits that by pass the vast dinitrogen reservoir.) Aerobic ammonium-oxidising bacteria (*e.g.*, *Nitrosomonas europaea*) use ammonium almost exclusively as the electron donor in "respiration" and oxidise it to hydroxylamine (catalysed by ammonium monooxygenase) and, then, to nitrite (catalysed by hydroxylamine oxidoreductase (HAOR))[71]. In addition, also archaea (from marine and soil environments) seem to be able to oxidise ammonium for chemolithoautotrophic growth.[91-95] Subsequently, nitrite-oxidising bacteria (*e.g.*, *Nitrobacter* or *Nitrospira* species) are responsible for the final oxidation of nitrite to nitrate ("closing" nitrification and being he principal source of nitrate), in a reaction catalysed by a molybdenum-containing nitrite oxidoreductase (MoNiOR, described in section 4.3.1.). The nitrite-oxidising bacteria are chemolithoautotrophs, phylogenetically heterogeneous, that derive energy from the nitrite oxidation to nitrate, in a strictly aerobic process, where all the carbon needs can be satisfied with carbon dioxide assimilation (via the Calvin cycle).[96-102]

Ammonium can also be anaerobically oxidised in an exergonic pathway (AnAmmOx - Fig. 1, gray arrows) used for the anaerobic chemoautotrophic growth of planctomycetes (like "*Candidatus*

Kuenenia stuttgartiensis" or "*Candidatus* Scalindua profunda").[103-112] In this pathway, ammonium is first oxidised by NO (instead of dioxygen) to yield hydrazine, in a reaction catalysed by the di-haem-*c*-containing hydrazine synthase; hydrazine is, then, oxidised to dinitrogen by the octa-haem-*c*-containing hydrazine oxidoreductase.[105,106,110,113,114] The required NO is formed via nitrite reduction by the Cd_1NiR enzyme (an enzyme associated with the "classical" denitrification), coupling, in this way, the anaerobic ammonium oxidation to the nitrite reduction. (In this context, it is worth mentioning that an AnAmmOx bacterium (strain KSU-1) was hypothesised to have a CuNiR enzyme, suggesting that the enzymatic "machinery" of the AnAmmOx organisms might be more "flexible" than initially thought.[115]) According, AnAmmOx likely provided the first complete "recycling" of fixed nitrogen to dinitrogen and fulfilled this role until the emergence of the copper-containing nitrous oxide reductase (on the oxic era) that presently completes the denitrification.[105,116] Operating in marine (where it is responsible for 30-70% of global dinitrogen formation), freshwater and terrestrial ecosystems, AnAmmOx is presently one of the major enigmas of the nitrogen biochemical cycle.

2.3. *NEW* PATHWAYS

In addition to those well recognised four nitrogen dissimilatory pathways, several new processes are being identified, including the [117]anaerobic phototrophic nitrite oxidation[118], [119]microaerobic nitrification/AnAmmOx coupling[91,120-123] or the hyperthermophilic dinitrogen fixation/methane production[124].

Particularly remarkable is the novel "denitrification/intra-aerobic methane oxidation" pathway that links the nitrogen and carbon cycles (Fig. 1, violet arrows). Very recently, it was described a new anaerobic bacterium -"*Candidatus* Methylomirabilis oxyfera"- that couples the reduction of nitrite to dinitrogen (denitrification) with the oxidation of methane.[125-132] Methane is one of the least reactive organic molecules and, so far, no known biochemical mechanism has been able to explain its activation in the absence of oxygen (or reverse the last step of methanogenesis), what poses a problem for an anaerobic organism. In spite of that, the anaerobic "M. oxyfera" encoded, transcribed

and expressed the well-established aerobic pathway for methane oxidation to carbon dioxide (particulate methane monooxygenase complex).[131] On the other hand, "M. oxyfera" lacked the known gene cluster necessary to produce the enzyme that reduces nitrous oxide to dinitrogen, questioning how the organism produces the dinitrogen. Yet, "M. oxyfera" expresses a Cd_1NiR enzyme.[131] To solve this conflict, a new pathway was proposed in which nitrite-derived Cd_1NiR-catalysed NO is converted into dinitrogen and dioxygen by a NO dismutase enzyme (eq. 5; Fig. 1, violet arrows).[127] This key NO dismutase was not yet identified, but two of the "M. oxyfera" most abundant proteins seem to be to good candidates: these proteins are supposed to be (by genome analysis) "strange" quinol-dependent NO reductases that apparently are unable to accept electrons or uptake protons and have a different catalytic site **-**features that compromise their function as nitric oxide reductases (see eq. 7), but would not hamper a dismutase activity[127,129]. In this new "version" of denitrification, the last two steps of "classical" denitrification (NO and nitrous oxide reduction - Fig. 1, blue arrows) would be replaced by the NO dismutation, to yield, not only dinitrogen, but also dioxygen. The dioxygen formed intracellularly through this pathway would be responsible for the intra-aerobic oxidation of methane, with the remaining dioxygen being used in "respiration" by terminal "respiratory" oxidases.[127,133,134] (Remarkably, in spite of the "M. oxyfera" capacity to generate its "own supply" of oxygen, exposure the organism to microoxic conditions has an overall detrimental (oxidative stress) effect.[134]) The "denitrification/intra-aerobic methane oxidation" pathway would have enabled the organisms to thrive on the abundant methane in the Archaean atmosphere without direct dependence on oxygenic photosynthesis.

$$2^{\bullet}NO \longrightarrow N_2 + O_2 \qquad (5)$$

Noteworthy, it was also suggested that a similar mechanism is possibly used by the unrelated γ-proteobacterial strain HdN1 to synthesise oxygen, from nitrate or nitrite, for "intra-aerobic" hexadecane oxidation.[129,135,136] If confirmed, the "oxygenic denitrification" could be more widespread than initially thought and nitrite could became a relevant dioxygen precursor[137] **-**further highlighting the biological relevance of nitrite.

2.4. NITRIC OXIDE AND THE NITROGEN "RECYCLING"

This section can not be concluded without emphasising the NO relevance to the biological nitrogen "recycling": the complete biological "recycling" of nitrogen requires the re-formation of a N-N bond (to ultimately produce dinitrogen), what is accomplished **-**as far as is presently known**-** only with the oxidising power of NO.

The only recognised enzymes capable of bonding two nitrogen atoms are the hydrazine synthase (eq. 6; AnAmmOx - Fig. 1, gray arrows) and NO reductase (eq. 7; denitrification - Fig. 1, blue arrows) and both use the oxidizing power of NO. In addition, also CcNiR (DNRA - Fig. 1, green arrows)) was described to catalyse the nitrous oxide formation from NO (eq. 8; see section 4.1.1.2. for details). Moreover, the suggested NO dismutase (eq. 9), crucial for the "denitrification/intra-aerobic methane oxidation"(Fig. 1, violet arrows), is thought to use NO to form dinitrogen directly. This consensus supports the hypothesis that NO may have been the first deep electron sink on Earth, before the emergence of dioxygen.[110,148] Because NO is formed from the nitrite reduction, these NO roles ("general" oxidant and N-N bond "former") further emphasise the biological importance of nitrite. Furthermore, as will be discussed in the following sections (3.1.1. and 3.2.1.), the nitrite reduction to NO is also employed by mammals and plants to sustain the NO-dependent signalling pathways under hypoxic/anoxic conditions.

$$^{\bullet}NO + NH_4^+ + 2H^+ + 3e^- \xrightarrow{\text{hydrazine synthase } (c \text{ haem})} {}_2HN{\equiv}NH_2 + H_2O \quad (6)$$

$$2\,^{\bullet}NO + 2H^+ + 2e^- \xrightarrow{\text{NO reductase } (b \text{ haem + non-haem iron})} N{\equiv}NO + H_2O \quad (7)$$

$$2\,^{\bullet}NO + 2H^+ + 2e^- \xrightarrow{\text{CcNiR } (c \text{ haem})} N{\equiv}NO + H_2O \quad (8)$$

$$2\,^{\bullet}NO \xrightarrow{\text{NO dismutase (haem + non-haem iron?)}} N{\equiv}N + O_2 \quad (9)$$

2.5. NITROGEN CYCLE - CONCLUDING REMARKS

The assimilatory and *old* and *new* dissimilatory pathways "close" the nitrogen biochemical cycle (Fig. 1), with the fixed nitrogen being lost to the atmosphere (in the form of dinitrogen), but also to the soils, crust and oceans.

Over the last years, the discovery and addition of *new* organisms involved in the "classic" processes and the scrutiny of *new* ways to transform nitrite add more complexity to the system, unrevealing an intricate network of pathways. The regulation and control of these complex pathways is still a challenging problem, and future research will certainly change some of the presently established dogmas.

3. BIOLOGICAL FATE OF NITRITE - NITRITE ON SIGNALLING PATHWAYS

Apart from the most obvious molecules, like amino acids and nucleosides, nitrogen is also necessary for the biosynthesis of another essential molecule: the NO. NO is a signalling molecule involved in several physiological processes, in both prokaryotes and eukaryotes, and, as will be described in this section (subsections 3.1.-3.3.), nitrite is an important source of NO. Nitrite is also converted to nitrate and nitrogen dioxide radical and those oxidative -signalling and deleterious- pathways will also be addressed (sections 3.1.2. and 3.2.2.).

3.1. NITRITE ON SIGNALLING PATHWAYS IN MAMMALS

3.1.1. Nitrite reduction in mammals

In mammals, NO controls a plethora of functions, including vasodilation (through the well-known activation of guanylate cyclase), neurotransmission, immune response, platelet aggregation, apoptosis, gene expression and mediates a wide range of both anti-tumour and antimicrobial activities.[149] Nevertheless, its overproduction (and of other reactive nitrogen species, in particular peroxynitrite, formed through the reaction of NO with superoxide radical[150]) has been implicated in several pathological conditions, such as chronic inflammation and infection conditions, septic shock syndrome, diabetes and Parkinson's and Alzheimer's diseases.[151] All these functions made the NO metabolism the focus of a huge interest by the medical scientific community. In 1992, NO was designated "Molecule of the Year" by the Science magazine[152] and, in 1998, the Nobel Prize in Physiology or Medicine awarded the discovery of NO as the "endothelium-derived relaxing factor"; since then the reactive nitrogen species never stopped to be an "hot topic" in biomedical research.

Three tissue-dependent isoforms (neuronal, endothelial and inducible) of NO synthase (NOS) catalyse the formation of NO from dioxygen and the guanidinium nitrogen atom of L-arginine (eq. 10-11).[153-155]

NOS are complex homodimeric enzymes, constituted by one flavinic reductase C-terminal domain and one haemic oxygenase N-terminal domain. During catalysis, the electrons from NADPH are transferred through the reductase domain to the *b* haem iron of the oxygenase domain; on the haem, the dioxygen is activated to hydroxylate L-arginine (eq. 10); the N^{ω}-hydroxy-L-arginine, thus, formed is, then, oxidised to yield L-citrulline and NO (eq. 11).

$$\underset{\text{(L-arginine)}}{>C=N^{+}H_2} + O_2 + NADPH \longrightarrow \underset{\text{(N^{ω}-hydroxyl-L-arginine)}}{>C=N\text{-}OH} + H_2O + NADP^{+} \quad (10)$$

$$\underset{\text{(N^{ω}-hydroxyl-L-arginine)}}{>C=N\text{-}OH} + O_2 + 1/2NADPH \longrightarrow \underset{\text{(L-citruline)}}{>C=O} + {}^{\bullet}NO + H_2O + 1/2NADP^{+} \quad (11)$$

The NO biological effects are accomplished, mainly, by posttranslational modification of transition metal centres (mostly haems and labile [4Fe-4S] centres) and of cysteine residues and other thiols, to form, respectively, nitrosyl (metal-N=O) and S-nitrosothiol (R-S-N=O; abbreviated as RSNO) derivates.[151,156-170] To control the specificity of NO signalling and to limit the NO toxicity, the NOS activity is tightly regulated and the NO life time is controlled through its rapid oxidation to nitrite (*e.g.*, by dioxygen[171-173] and ceruloplasmin[174]) and to nitrate (*e.g.*, by the well known reaction with oxy-haemoglobin and oxy-myoglobin (see section 3.1.1.1. for details)).

In fact, nitrate and nitrite were long regarded as "useless" end-products of the NO metabolism. As a result of this dogma, the human physiological role of nitrite was neglected until the end of the XX century, when (i) it was realised that nitrite can be reduced back to NO (eq. 12) under acidic, anaerobic conditions (see references throughout the following sub-sections) and (ii) re-discovered[175] that nitrite can be cytoprotective during *in vivo* heart, liver, kidney and brain ischaemia[176] and other pathological conditions (see references throughout the following sub-sections and, *e.g.*,[177-197]). Since then, a new concept began and, presently, blood and tissue nitrite are thought as a NO "storage form" that can be made available, by "simple" reduction, to ensure cell functioning under conditions of hypoxia/anoxia[198], when the oxygen-dependent NOS activity is impaired. The relevance of this nitrite-derived NO during ischaemia goes well beyond the obvious vasodilation. It extends to other roles of the NOS-catalysed NO, such as the regulation of smooth muscle proliferation[196],

angiogenesis[199], gene expression (*e.g.*, haem oxygenase-1 or heat shock proteins expression)[200], or, and most important, regulation of mitochondrial "respiration"[201] and energy production[208-210]. Through this nitrite "recycling" pathway, an organ under ischaemia can maintain (or even increase) the blood flow, modulate the dioxygen distribution and the reactive oxygen species formation (by controlled inhibition of mitochondrial "respiration" by NO) and, at the same time, maintain an anti-apoptotic and anti-inflammatory environment.

$$NO_2^- + 1e^- + 2H^+ \longrightarrow {}^{\bullet}NO + H_2O \qquad (12)$$

In this context, the question of "who" is reducing nitrite in mammals imposes itself. To date, no "dedicated" mammalian nitrite reductase was identified. On the contrary, the nitrite reduction to NO has been ascribed only to prokaryotic organisms (through Cd_1NiR and CuNiR enzymes, as described in section 2.2.). But, while the absence of a "dedicated" nitrite reductase enzyme was interpreted as "mammals do not need to metabolise nitrite", the correct question was (long) disregarded: are other mammalian proteins reducing nitrite?

In the recent years, several mammalian metalloproteins were shown to be able to reduce nitrite to NO (Fig. 2): the molybdenum-containing enzymes xanthine oxidase (XO) and aldehyde oxidase (AO) and a growing number of haem-containing proteins, where haemoglobin (Hb) and myoglobin (Mb) stands out in number of publications, but including also the neuroglobin (Nb), cytoglobin (Cb), cytochrome *c* (Cc) (see references throughout the following sub-sections) cytochrome P_{450}[211], cytochrome *c* oxidase (CcO)[212-214], NOS[215,216], among several other proteins[217,218]. (Although outside the scope of this review, it is very interesting that also nitrophorin 7, a salivary haemic protein from a blood-feeding insect, is able to reduce nitrite to NO.[219,220] Together with the haemic proteins of mammals and plants (discussed in the following section), this example strongly emphasises the physiological relevance of haemic proteins on the nitrite reduction in higher organisms.)

space (= blank line)

In order to restrict the scope of information presented to a manageable size, only some "non-dedicated" nitrite reductases will be here discussed: (i) Mb and XO, because they are the only

ones that have been (so far) identified as crucial for the cytoprotective action of nitrite *in vivo* or *ex vivo* (they will be discussed in section 3.1.1.1. and in 3.1.1.3., along with Hb and AO, respectively) and (ii) Nb, Cb and Cc (section 3.1.1.2.), because they constitute promising examples of how the nitrite reduction could be allosterically regulated, becoming a "tuneable" NO source. Moreover, only the molecular mechanism of XO-catalysed nitrite reduction will be discussed (section 4.2.3.). In addition, the protein-independent nitrite reduction and carbonic anhydrase-dependent NO formation will also be discussed (sections 3.1.1.4. and 3.1.1.5.). As will be described, the pathways reduce nitrite to NO under acidic, hypoxic/anoxic conditions, precisely the conditions under which a "salvage" pathway is needed to accomplish the NOS role.

3.1.1.1. Haem-dependent nitrite reduction - haemoglobin and myoglobin

A large number of studies have suggested that the mammalian nitrite reduction can be achieved by haemic proteins, with Hb (erythrocyte) and Mb (cardiac, skeletal[221] and smooth[222] muscle) being two of the most studied proteins.

To reduce nitrite to NO, the haem must be reduced and penta-coordinated to, in this way, provide the necessary electrons and have an "open site" to bind nitrite and carry out the oxygen atom abstraction and reduction (see section 4.). In accordance, the ferrous deoxy-haemoglobin and deoxy-myoglobin (deoxy-Hb/Mb; $(Hb/Mb)Fe^{2+}$) do reduce nitrite to NO, under anaerobic conditions, in a reaction that is pH-dependent (eq. 13; k(Hb, pH 6.5)≈$10 M^{-1}s^{-1}$ [223], k(Hb, pH 7.4)≈$0.1\text{-}1 M^{-1}s^{-1}$, k(Mb, pH 7.4)≈$6\text{-}12 M^{-1}s^{-1}$ [209,224-226]).[224,226-230] It is this *in vitro* anaerobic reaction that establishes the starting point for the proposed hypoxic/anoxic Hb/Mb-dependent NO formation *in vivo*. Moreover, this reaction could contribute to the NO formation in all situations/localisations where significant Hb deoxygenation occurs: it should be noted that, even under "normal" normoxic conditions, the oxygen concentration at the precapillary arterioles and capillary is sufficiently low (lower than 50μM[231]) to promote significant Hb deoxygenation[226,232-234]; therefore, the Hb-dependent NO signalling could also occur locally, under "normal" non-ischaemic conditions. This reasoning could explain why the *in*

vivo administration of nitrite leads to a decrease in blood pressure, not only in hypertensive patients, but also in normal individuals[187,189,191,225,235-240]. The Mb-dependent NO formation, on the contrary, is expected to be significant only under hypoxia or ischaemia (since sufficient Mb deoxygenation only occurs when the oxygen concentration decreases to values lower than 3-4μM).

Interestingly, nitrite also reacts with ferrous oxygenated Hb/Mb (oxy-Hb/Mb; $(Hb/Mb)Fe^{2+}\text{-}O_2$), but to be oxidised to nitrate (eq. 14; see section 3.1.2.1. for details). This oxidation reaction prevents the nitrite accumulation *in vivo* under normoxic conditions and is co-responsible for the NO life time control.[149,241,242] The oxygen-controlled reactivity of nitrite:

deoxy-Hb,Mb/reduction to NO *versus* oxy-Hb,Mb/oxidation to nitrate

could, thus, represent an ideal mechanism to control the NO formation throughout the entire physiological oxygen gradient, from normoxia to anoxia: under normoxic conditions, NOS synthesises NO and the nitrite (formed from the NO oxidation) is oxidised to nitrate; when the dioxygen concentration decreases, the deoxy-Hb/Mb reduces nitrite to NO to compensate the impairment of the NOS activity.

Regardless of the "beauty" of this mechanism, the ability of the Hb,Mb/nitrite-dependent NO to perform its action *in vivo* is controversial, because: (i) the NO should be rapidly ($k_{on}\approx10^{7}M^{-1}s^{-1}$ [243-252]) trapped in a stable complex with the deoxy-Hb/Mb (eq. 15; $K_d\approx10^{-12}\text{-}10^{-10}M$ [244,250,252-254]) [224,226,255-257] and/or (ii) be rapidly oxidised (eq. 16; $k\approx10^{7}\text{-}10^{8}M^{-1}s^{-1}$ [149,241,243,246,251,258-264]) to nitrate and met-haemoglobin/myoglobin (met-Hb/Mb; $(Hb/Mb)Fe^{3+}$) by the oxy-Hb/Mb molecules still present under non-anoxic conditions[265]. As a result of this well documented (*in vitro*) chemistry of the haem, the (*in vivo*) physiological role of these haemic proteins in the formation of bioactive NO has been greatly questioned.

$$NO_2^- + 2H^+ + (Hb/Mb)Fe^{2+} \longrightarrow (Hb/Mb)Fe^{3+} + {}^{\bullet}NO + H_2O \quad (13)$$

$$NO_2^- + H^+ + (Hb/Mb)Fe^{2+}\text{-}O_2 \longrightarrow (Hb/Mb)Fe^{3+} + NO_3^- + 1/2H_2O_2 \quad (14)$$

$$^{\bullet}NO + (Hb/Mb)Fe^{2+} \rightleftharpoons (Hb/Mb)Fe^{2+}\text{-}NO \quad (15)$$

$$^{\bullet}NO + (Hb/Mb)Fe^{2+}\text{-}O_2 \longrightarrow (Hb/Mb)Fe^{3+} + NO_3^- \quad (16)$$

In spite of these "pure" chemical constrains, several *in vivo*, *in situ* and *in vitro* experimental results indicate that red blood cells, isolated hearts, Hb and Mb, in the presence of nitrite, under hypoxia, do generate NO and can stimulate the NO signalling[179-181,187-189,191,209,225,226,234-240,274-293]. Particularly relevant are the studies showing that *in vivo* administration of nitrite generates NO, nitrosylates cardiomyocyte iron-containing proteins and reduces (by 60%) myocardial infarction in mice; on the contrary, in Mb knockout mice, nitrite has no protective effects and there is a decrease of the NO formation.[209,282,285,293] These studies provide insights on the mechanism of nitrite cytoprotection (via reduction to NO) and suggest an important role for the pair Mb/nitrite in myocardial ischaemia-reperfusion injury[294].

In light of all those *in vivo*, *in situ* and *in vitro* evidences, and to circumvent the NO scavenging by Hb/Mb, some authors argue that, even if the majority of NO is scavenged, enough NO would still be available to exert its role, since very low concentrations are needed to accomplish the physiological effects and due to the lipophilicity of the molecule.[149,305]

In the recent years, several mechanisms were proposed to explain how the nitrite reduction can yield bioactive NO to carry out its activity, without being scavenged by Hb or Mb. Before discussing those mechanisms, that are expected to be essentially similar in both Hb and Mb, it is worth mentioning that Mb has important features that distinguish it from Hb: (i) Mb has a lower P_{50} value (1.5-3μM *versus* $\approx$35μM, that determines that it only becomes significantly deoxygenated at lower dioxygen concentrations); (ii) Mb has a lower reduction potential; (iii) it is a monomer without allosteric behaviour; and (iv) it reduces nitrite faster than T-state Hb, at rates similar to R-state Hb. These properties must be kept in mind during the following mechanistic discussions that will be particularised for Hb for the sake of simplicity.

space (=blank line)

A possible mechanism to release NO involves the reactivity of nitrite with oxy-Hb itself. Several *in vivo*[274,306-308] and *in vitro*[309-313] observations suggest that, in the presence of oxy-Hb, the (Hb)Fe^{2+}-NO complex (eq. 15) is not an irreversible trap of NO. On the contrary, it was suggested that an intermediate formed during the nitrite reaction with oxy-Hb (see eq. 14, 37-40 in section 3.1.2.1.), possibly nitrogen dioxide radical ($^{\bullet}NO_2$), is able to oxidise the (Hb)Fe^{2+}-NO and promote the NO dissociation[251,313] **-**an oxidative denitrosylation (eq. 17)[313].

$$^{\bullet}NO + (Hb)Fe^{2+} \rightleftharpoons (Hb)Fe^{2+}\text{-}NO \quad (15)$$

$$(Hb)Fe^{2+}\text{-}NO + {}^{\bullet}NO_2? \longrightarrow ? \longrightarrow (Hb)Fe^{3+}\text{-}NO \longrightarrow ? \longrightarrow (Hb)Fe^{3+} + {}^{\bullet}NO \quad (17)$$

In accordance with this proposal, the nitrite reduction and NO release would be accomplished by the simultaneous reaction of nitrite with deoxy-Hb and oxy-Hb[313]: in the reaction beginning, deoxy-Hb is consumed much faster than oxy-Hb, as reaction 17 consumes intermediate(s) of the propagation phase of the oxy-Hb reaction (eq. 37-40). So, the nitrite reaction with oxy-Hb would be limited **-**it would not critically compete with the nitrite reduction**-** and, at the same time, would facilitate the NO release. The major conundrum of this mechanism is the *in vivo* small nitrite concentration (in the sub-micromolar range in the erythrocytes and plasma[167,314-317]) to feed all the reactions (the experimental setup[313] used to build up the model employed a large (reversed) nitrite/Hb ratio). Hence, to be feasible, this mechanism would require special spatial localisation conditions (at the cell membrane, as will be discussed below) to control the local concentrations of reactants[313]. Moreover, *in vivo*, the formation of the crucial nitrogen dioxide (or other oxidative intermediate) should be inhibited by several antioxidants (as will be discussed in section 3.1.2.1.). Therefore, while these reactions (eq. 13, 41, 17) demonstrate that Hb has the intrinsic ability to, *in vitro*, form and release NO, the physiological relevance of these observations remain unclear and controversial.

space (=blank line)

Another possible mechanism for NO not to be scavenged by Hb is through the nitrite conversion to dinitrogen trioxide (N_2O_3) (eq. 13→18→19 or eq. 13→20→21, both energetically feasible[318]), in a reaction model where Hb acts as a true catalyst (eq. 22)[276,312,319-327]. The dinitrogen trioxide is less reactive with the haem than the NO and is believed to be capable of crossing the cell membrane; thus, it would be, in principle, able to diffuse out of the red blood cell and reach the endothelium.[276,322,323] The bioactive NO is, subsequently, formed directly (eq. 23) or indirectly (eq. 24) from the dinitrogen trioxide (N_2O_3). However, this model presents several drawbacks. First, the formation of the $(Hb)Fe^{3+}\text{-}NO_2^-$ complex (eq. 18) in the presence of excess deoxy-Hb,

should not be favoured. The nitrite affinity for ferric haem is *ca* 100 times lower (1-5x10^{-3}M [328-330]) than for ferrous haem (K_d≈1-7x10^{-5}M [329]) and, even if the recently reassessed value[331] of 5x10^{-5}M is considered[323,330,332], the presence of excess of ferrous Hb dictates that the actual concentration of the (Hb)Fe^{3+}-NO_2^- complex would be in the nanomolar order. Second, this (Hb)Fe^{3+}-NO_2^- complex (present at a very low concentration) has to compete with readily available (Hb)Fe^{2+} (and (Hb)Fe^{2+}-O_2) for the reaction with NO (eq. 19 *versus* eq. 15/16). Even if the (Hb)Fe^{3+}-NO_2^- is considered to react as a (Hb)Fe^{2+}-$NO_2^{\bullet}$ radical species, at (very rapid) radical-radical reaction rates, the competition with the predominant (Hb)Fe^{2+} (and (Hb)Fe^{2+}-O_2) may not be favourable. Third, a similar reasoning applies to the alternative pathway (eq. 20→21), since the formation of the (Hb)Fe^{3+}-NO complex, in the presence of excess deoxy-Hb, is even more unfavourable (K_d(ferric complex, eq. F20)≈10^{-5}M *versus* K_d(ferrous complex, eq. F15)≈10^{-10}-10^{-12}M [223,251]). Regarding the competition for met-Hb (eq. 18 *versus* eq. 20), it should be noted that, although the NO affinity for met-Hb is *ca* 100 times higher than the nitrite one, both pathways are energetically feasible[318]. Fourth, the equilibrium 23 (K≈2-7×10^{-5}M [333,334]) is expected to be rapidly (k ≈$10^9$$M^{-1}s^{-1}$ [333]) dislocated towards the dinitrogen trioxide formation, with subsequent hydrolysis to yield nitrite (reverse of eq. 22)[151,334-336]. These constrains suggest that, *in vivo*, the dinitrogen trioxide formation should not be favoured or might require special spatial localisation conditions (at the cell membrane; see next paragraph) to overcome the competing reactions.[330] If formed, its reaction to yield RSNO (eq. 24)[337-340] could constitute a probable way for NO to avoid be trapped by the haemic proteins, as will described below.

$$NO_2^- + 2H^+ + (Hb)Fe^{2+} \longrightarrow (Hb)Fe^{3+} + {}^{\bullet}NO + H_2O \quad (13)$$

and

$$NO_2^- + (Hb)Fe^{3+} \rightleftharpoons (Hb)Fe^{3+}\text{-}NO_2^- \quad (18)$$

$$^{\bullet}NO + (Hb)Fe^{3+}\text{-}NO_2^- \longrightarrow (Hb)Fe^{2+} + N_2O_3 \quad (19)$$

or

$$^{\bullet}NO + (Hb)Fe^{3+} \rightleftharpoons (Hb)Fe^{3+}\text{-}NO \quad (20)$$

$$NO_2^- + (Hb)Fe^{3+}\text{-}NO \longrightarrow (Hb)Fe^{2+} + N_2O_3 \quad (21)$$

global reaction (13+18+19 or 13+20+21):

$$2NO_2^- + 2H^+ \longrightarrow N_2O_3 + H_2O \quad (22)$$

$$N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2 \quad (23)$$

$$N_2O_3 + RSH \longrightarrow RSNO + NO_2^- + H^+ \quad (24)$$

space (=blank line)

It has also been suggested that NO escapes the red blood cell by being formed locally in the cell membrane, under allosteric control. It was shown that the kinetics of the nitrite reaction with Hb is more complex than previously thought and, most important, is controlled by the Hb conformation[225,226,234,290,293,341-345]. First: the nitrite reduction to form an electron delocalised intermediate (eq. 13a→13b)[275,346,347] is faster (5 [223,290,345] to 50 times [226,313,341]) in the deoxy-haems of R-state Hb (k≈10$M^{-1}s^{-1}$ [226,290,345]), a conformation populated under normal dioxygen concentration. This behaviour is consistent with the known lower affinity of the T-state for ligands and can be explained by the R-state haem having a more negative reduction potential (*i.e.*, being a better electron donor) and/or having a more accessible haem pocket than the T-state.[234,326,342,344,348-350] Second: on the other hand, to proceed, this reaction (eq. 13a) needs the vacant ferrous deoxy-haems that predominate on the T-state. Third: although the intermediate formation is favoured in the R-state, the NO release from the intermediate (eq. 13c) is *ca* ten times faster in the haems from the T-state (k_{off}≈1s^{-1} [251,345]), a conformation populated under low oxygen concentration. This observation could be due to the conformational changes that take place during R to T conversion: modifications on the hydrogen bonds network of the distal haem pocket could affect the stability of NO on the haem[345,351] and facilitate its release. Therefore, the Hb conformation (dictated by the oxygen availability) affects all steps of nitrite reduction, but in opposite manners, resulting in a bell-shaped dependence of the nitrite reduction rate as a function of oxygen fractional saturation[226,290,234,343]: (i) at a fractional saturation of zero, Hb is in the T-state and the rate is low; (ii) as the oxygen fractional saturation increases, the rate rises in parallel with the increased R-state character of Hb (that reduces the nitrite faster); (iii) however, at higher fractional saturation, the T-state character and the concentration of deoxy-Hb (one substrate) decrease and, consequently, the rate diminishes. At intermediate fractional saturations, Hb will be comprised of both R- and T-state molecules and the

rate reaches maximum values around the Hb P_{50} (*i.e.*, around the oxygen concentration at which Hb is half-saturated, that is ≈35μM).

$$NO_2^- + 2H^+ + (Hb)Fe^{2+} \longrightarrow (Hb)Fe^{2+}\text{-}NO^+ + H_2O \quad (13a)$$

$$(Hb)Fe^{2+}\text{-}NO^+ \rightleftharpoons (Hb)Fe^{3+}\text{-}NO \quad (13b)$$

$$(Hb)Fe^{3+}\text{-}NO \rightleftharpoons (Hb)Fe^{3+} + {}^{\bullet}NO \quad (13c)$$

The fastest NO release from the T-state, a conformation (i) populated under low oxygen concentration, (ii) favoured at low pH and with (iii) an higher affinity for the cell membrane (within the context of a metabolon[352]), can potentially lead to the NO released out of the red blood cell, to diffuse to the vasculature[235,239,345]: (a) as the Hb begins to be deoxygenated, but still retains the R-conformation, the increased rate of the intermediate formation (eq. 13a→13b) contributes to the formation of a pool of potentially bioactive NO; (b) when Hb becomes further deoxygenated, the T-state is stabilised, its membrane-binding is enhanced and the NO release out of the red blood cell is feasible. However, the localised NO release does not avoid the "problem" of NO being trapped by the membrane-bound deoxy-T-state Hb itself. In addition, because the NO diffusion is directed by its concentration gradient[267,354,355], a significant fraction of this "membranar" NO should still diffuse towards the interior of the erythrocyte.

space (=blank line)

Regarding the NO diffusion towards the interior of the red blood cell, it was suggested that a high proportion of membrane-bound met-Hb[356], would provide a diffusional barrier that would "shield" the NO.[357] However, once again, the chemistry is not so simple, and NO is also able to bind to ferric haem, although at association rates lower than those for ferrous haem (k_{on}≈$10^7M^{-1}s^{-1}$ *versus* k_{on}≈$10^5M^{-1}s^{-1}$ [246]) and to form complexes less stable (K_d≈10^{-12}-10^{-10}M *versus* K_d≈10^{-5}-10^{-4}M [223,251]). But, if the $(Hb)Fe^{3+}$-NO complex is formed in some extent **-***i.e.*, if reaction 20 occurs in spite of the omnipresent reaction 15**-** it would provide a way for NO to avoid scavenging[311,312,327,346,358]: the reaction of NO with met-Hb results in the formation of the nitrosylating complex $(Hb)Fe^{2+}$-NO^+ (eq. 20→25b), which either can be hydrolysed to deoxy-Hb and nitrite (eq. 26) or it can transfer its

nitrosyl group to nitrosylate nucleophilic compound such as thiols (RS^-) (eq. 27)[249,319,359,360]; in addition, as described above, the (Hb)Fe^{3+}-NO complex can react with nitrite to eventually form RSNO as well (eq. 21→24). Furthermore, the met-Hb could also promote the formation of RSNO via reactions 18→19→24. Moreover, the initial reductive nitrosylation[361] was shown to be faster (rate constant at least 5 times higher)[363] in a subpopulation of β-subunit ferric haems, whose proportion is increased by T-state formation[311,327] **-**precisely the conformation with higher affinity for the cell membrane. So, in the presence of a high proportion of membrane-bound met-Hb, the NO formed, by the low proportion of deoxy-Hb, seems to have a chance to escape scavenging: formation of RSNO by locally concentrated met-Hb. In this context, it should be noted that met-Mb is nitrosylated only at pH>7; at lower pH values, it forms reversible complexes (contrary to met-Hb that reacts readily with NO at pH<6 to give the nitrosylating complex Fe^{2+}-NO^+).[360,364-366] Accordingly, it is controversial that Mb could contribute to the formation of RSNO through this mechanism.

$$^{\bullet}NO + (Hb)Fe^{2+} \rightleftharpoons (Hb)Fe^{2+}\text{-}NO \quad (15)$$

$$^{\bullet}NO + (Hb)Fe^{3+} \rightleftharpoons (Hb)Fe^{3+}\text{-}NO \quad (20)$$

$$(Hb)Fe^{3+}\text{-}NO \rightleftharpoons (Hb)Fe^{2+}\text{-}NO^+ \quad (25)$$

$$(Hb)Fe^{2+}\text{-}NO^+ + H_2O \longrightarrow (Hb)Fe^{2+} + NO_2^- + 2H^+ \quad (26)$$

$$(Hb)Fe^{2+}\text{-}NO^+ + RS^- \longrightarrow (Hb)Fe^{2+} + RSNO \quad (27)$$

$$(Hb)Fe^{3+}\text{-}NO + NO_2^- \longrightarrow (Hb)Fe^{2+} + N_2O_3 \quad (21)$$

$$N_2O_3 + RSH \longrightarrow RSNO + NO_2^- + H^+ \quad (24)$$

Concerning the formation of RSNO, note that their formation through reaction 27 could be initiated directly from ferrous Hb, without the NO release, via reaction 13a. This mechanism was proposed for the "self"-S-nitrosylation of Hb, as a strategy for the release of NO[225,310-312,322,367-371]: the delocalised intermediate (eq. 13a→13b) was suggested to be in equilibrium with a thiyl radical (eq. 28)[372] to which the NO is transferred, yielding the known β-Cys_{93} S-nitrosylated Hb (eq. 29); subsequently, the Hb/membrane-bound NO would be transferred to the endothelial cells through

transnitrosation of membrane thiol groups.

$$NO_2^- + 2H^+ + (Hb)Fe^{2+} \longrightarrow (Hb)Fe^{2+}\text{-}NO^+ + H_2O \quad (13a)$$

$$(Hb)Fe^{2+}\text{-}NO^+ + RS^- \longrightarrow (Hb)Fe^{2+} + RSNO \quad (27)$$

$$(Hb)Fe^{2+}\text{-}NO^+ \rightleftharpoons (Hb)Fe^{3+}\text{-}NO \quad (13b)$$

$$^-S(Hb)Fe^{2+}\text{-}NO^+ \rightleftharpoons {}^-S(Hb)Fe^{3+}\text{-}NO \rightleftharpoons {}^\bullet S(Hb)Fe^{2+}\text{-}NO \quad (28)$$

$$^\bullet S(Hb)Fe^{2+}\text{-}NO \rightleftharpoons ONS(Hb)Fe^{2+} \quad (29)$$

RSNO are more stable than NO and less susceptible to be trapped by Hb **-**two features that make them potential good NO reservoirs**-** and, most important, they are formed *in vivo* (as well as *in vitro*), not only in red blood cells, but also in other tissues, after nitrite administration.[167,181,200,225,282,311,312,322,323,373-375] To elicit the NO-dependent effects, the RSNO, would have to be, latter, activated through homolytic or heterolytic decomposition or transnitrosation reactions (*i.e.*, transfer of the NO^+ group) **-**bear in mind that the NO biological effects are accomplished, mainly, by posttranslational modification of thiol compounds and transition metal centres (see section 3.1.1.) and that S-nitrosation is presently recognised as a relevant cell signalling mechanism.[151,163,166-170,312,323,373]. However, the mechanisms for their formation and NO release/action *in vivo* are still controversial. (Note that a RSNO can be formed without the intervention of a haemic protein, via the NO addition to a thiyl radical ($RS^\bullet$) that would be formed by a radicalar process not related with nitrite.)

space (=blank line)

In summary, several *in vivo*, *in situ* and *in vitro* studies indicate that hearts, red blood cells, Hb and Mb do generate NO and stimulate NO signalling in the presence of nitrite, under hypoxia. However, the *in vitro* reactivity of haems with nitrite and NO involves a very intricate network of reactions (Fig. 3), making very difficult to foresee how the suggested *in vivo* outcome could be achieved. As a result, none of the mechanisms here discussed satisfactorily explains how NO avoids the *dogmatic* scavenging by ferrous haems. In this respect, the plausible allosteric and "spatial" control

(that "specifies" the reagents and determines their concentrations at the erythrocyte membrane) represent a fascinating and promising mechanism to facilitate the Hb-dependent nitrite reduction to bioactive NO. Nevertheless, it should be mentioned that, along with the several studies that suggest a major role for deoxygenated Hb in nitrite-mediated vasodilation, there are a number of evidences suggesting that tissues are more efficient than blood in reducing nitrite to NO[376-379] (and, in addition, have an higher nitrite concentration, up to 20μM [167,380,381] *versus* the sub-micromolar concentrations in erythrocytes and plasma[167,314-317]), thus, challenging the physiological relevance of Hb as a nitrite reductase.

In vivo, it is likely that a delicate balance between NO scavenging and NO formation takes place: while the scavenging occurs throughout the entire oxygen gradient (from normoxia to anoxia), the progressive deoxygenation promotes the nitrite reduction, eventually shifting the "scale" towards the NO generation. Taking the heart as an example to make this reasoning more clear: (i) Under normoxia, Mb would scavenge the NO (through reaction 16), thus avoiding, *e.g.*, the NO-dependent inhibition of CcO[269,270,297,382,383] or the inducible NOS-mediated nitrosative stress[384,385] (ultimately, protecting the mitochondrial "respiration" and the all cell from the deleterious effects of NO). (ii) As the oxygen concentration decreases, a mismatch between the oxygen supply and consumption is "translated" into an increase of the deoxy-Mb fraction; the nitrite reductase activity of Mb could, then, become significant, even though concurrently with the omnipresent NO scavenging activity (eq. 15); the bioactive NO formed would be responsible for a decrease in the oxygen consumption, reactive oxygen species formation and down-regulation of the cardiac energy status[209,282] (again, protecting the heart during intense muscle exercise, acute myocardial hibernation or infracted heart[282,386]).

This "activity switch" of Hb/Mb, from NO scavenger to NO generator, depending on the available oxygen concentration, reintroduces nitrite as a oxygen sensor molecule, although, certainly, through a more sophisticated mechanism than the plain

"*deoxy-Hb,Mb/nitrite reduction versus oxy-Hb,Mb/nitrite oxidation*"

mentioned in the beginning of this section. It also emphasises how mammals, that do not have a "dedicated" nitrite reductase, can reduce nitrite to NO: "reusing" proteins that *we attribute* to other functions and "switching" their activity when necessary. From a chemical point of view, mammals are

just doing a "substrate adaptation" to an available redox system to fulfil their needs; in this case, taking advantage of the potential haem redox chemistry of proteins "dedicated" to transport oxygen. (A haem is a perfect choice to reduce nitrite, as will become evident after comparing the mechanisms of reduction of several haemic proteins (sections 4.1 and 4.2.1.).)

One of the present major challenges is to discover how to connect the *in vitro* knowledge of nitrite reduction mechanisms to the *in vivo* observed nitrite effects: How can NO escape from the red blood cell? If RSNO are key NO reservoirs, which pathways can mediate nitrite-dependent S-nitrosation? Understanding the *in vivo* mechanisms by which Hb and Mb reduce nitrite to trigger the NO signalling remains, thus, an important goal for the future research efforts. Moreover, the knowledge gathered from the Hb/Mb-mediated nitrite reduction would certainly be relevant to the understanding of (i) other "non-dedicated" haem-containing nitrite reductases, (ii) as well as to the understanding of the NO release from haemic receptors (*e.g.*, guanylate cyclase) and transporters, all of which have to avoid being trapped by reduced haem iron.

3.1.1.2. Haem-dependent nitrite reduction - neuroglobin, cytoglobin and cytochrome *c*

Among the several haemic proteins proposed to act as nitrite reductases, the hexa-coordinated Nb, Cb and Cc deserve to be here considered in more detail. In order to carry out catalysis, or simply react with a molecule, the haem has to have a free coordinating position to which the substrate or molecule should bind (see section 4. for more details). In this context, the nitrite reductase activity of penta-coordinated enzymes or metabolite transporters (as Hb and Mb in the absence of dioxygen) is not so surprising: that activity could arise from a "substrate adaptation" to the well known redox chemistry of penta-coordinated haems. But the nitrite reductase activity of alleged hexa-coordinated haemic proteins raises two unavoidable questions: (i) where does nitrite bind to be converted into NO? (ii) why choose an hexa-coordinated protein?

3.1.1.2. (a) Neuroglobin and cytoglobin

Human Nb is a cytoplasmatic monomeric (≈17kDa) haemic globin (HG; see section 3.2.1.3. for more details about the HG protein family) present in nerve tissues, as its name indicates, namely in brain and retina.[387-392] Its amino acid sequence displays less than 25% of homology with other vertebrate Hb and Mb and reveals a very ancient origin: it was present long before the divergence of the genes encoding Mb and Hb and remained highly conserved throughout mammalian evolution, suggesting a strongly selected vital role.[387, 393-398] Nb possesses a bis-histidinyl (His_{F8} and His_{E4} [399]) hexa-coordinated *b* haem, in both iron oxidation states, in the absence of an "external" ligand[387,394,402-410] **-**as expected from its classification as a hexa-coordinated HG. However, the distal histidine coordination is reversible (eq. 30) and the penta-coordinated Nb is able to reversibly bind dioxygen, carbon monoxide, NO or, as recently described, nitrite. Remarkably, the hexa- to penta-coordination conversion is controlled (at least) through a redox mechanism that involves the formation (or cleavage, for the reverse conversion), of an intramolecular disulfide bond, which controls the distal histidine affinity[411] (K≈3300 *versus* 280 [412,413,423]). Hence, the Nb affinity for "internal" (histidine) *versus* "external" ligands is expected to be directly controlled by the redox status of the cell.

$$\text{(Protein) } Fe^{2+}\text{— His (Protein)} \rightleftharpoons \text{(Protein) } Fe^{2+} + \text{His (Protein)} \qquad (30)$$

Physiologically, Nb was initially suggested to play a role similar to the Mb one (facilitate the dioxygen diffusion to mitochondria, in order to increase the oxygen availability to the neurons)[387,427]. However, given its high dioxygen affinity and low dioxygen dissociation rate (k_{on}≈130-250$\mu M^{-1}s^{-1}$ and k_{off}≈0.3-0.8s^{-1} [394,403]; values that compare with k_{on}≈15$\mu M^{-1}s^{-1}$ and k_{off}≈13s^{-1} for sperm whale Mb)[387,394,403,428,429], Nb does not meet the equilibrium and kinetic requirements for functioning in oxygen transport by facilitated diffusion[430] under (the presently known) physiological conditions[394]. Furthermore, its (i) low tissue concentration (micromolar range, except in retina)[390,431], (ii) high auto-oxidation rate, (iii) low haem reduction potential[403,425,432] and (iv) redox-controlled

"internal" hexa-coordination support that Nb did not evolved to transport oxygen[425,431]. Instead, it is probable that Nb is involved in redox reactions, including (i) cellular redox state sensing or scavenging of reactive oxygen and nitrogen species[433-442], (ii) scavenging and/or sensing of dioxygen, carbon monoxide (another signalling molecule[443-446]) or NO[387,398,404,428,433,434,435,447-456], (iii) inhibition of Cc-induced apoptosis[457-459] ("resetting the trigger level" for apoptosis; see[554], in section 3.1.1.2.(b), for details), (iv) among several other functions[434,435,449,450,460-464]. In addition (and as has been suggested for Hb and Mb), there are several evidences (disribed below) that Nb can be involved in the generation of NO, through the nitrite reduction. In support of these roles, Nb has been linked to neuronal protection during ischaemia injury: Nb is induced by neuronal hypoxia *in vitro* and focal cerebral ischaemia *in vivo*, and its overexpression or knocking down, enhances or reduces, respectively, neuronal survival after hypoxia.[431,433-435,440,441,465-471]

The identification of Nb, with features similar to plant non-symbiotic HG (see section 3.2.1.3. for details about these plant proteins) instead of the obvious Mb, and associated with hypoxic conditions, triggered the search for other human globins, what culminated with the discovery of Cb.[429,447] Human Cb is a monomeric[471] (≈21kDa, although some evidences point towards a dimeric structure[425,472,473]) hexa-coordinated HG, present in the cytoplasm and nucleus of apparently all tissue types, although at varying concentrations (and, for that reason, was initially called histoglobin).[388,389,429,431,447,451,471,474-476] As Nb, it displays low amino acid sequence similarity with other vertebrate Hb and Mb[395,429,447], but Cb is phylogenetically "younger" (sharing a common ancestry with Mb)[447,461]. The Cb distal histidine coordination is reversible and can be also under the control of a redox mechanism, involving, *e.g.*, the cysteine residues B2 and E9. However, the Cb distal histidine affinity (K≈90-165, with k_{off}≈1-5s^{-1} [429,471,477]) does not change significantly with the disulfide bond cleavage or mutation of the Cys_{B2} and Cys_{E9} (K≈90-120, with k_{off}≈2s^{-1} [412,471]). Nevertheless, *in vivo*, other mechanisms could control the population of hexa- *versus* penta-coordinated Cb molecules. Noteworthy, although the Cb distal histidine affinity is lower, the Cb dioxygen dissociation rate is equally low as in Nb (k_{off}≈0.35-0.9s^{-1} [429,471]). Accordingly, and as has been suggested for Nb, Cb is believed to be involved in cytoprotection under hypoxia (when is up-regulated)[397,429,431,435,437,447,461,470,474-481] and oxidative stress conditions[423,479,482-488], among other roles[397,474,484,489,490]. Cb has been proposed to act as a NADH oxidase[473], a dioxygen sensor[389], NO

scavenger[476,480] and (obviously) as a nitrite reductase/NO synthase.

space (=blank line)

As expected from the previous discussion on Hb and Mb, the human ferrous deoxy-neuroglobin and deoxy-cytoglobin (deoxy-Nb and deoxy-Cb) do reduce nitrite to NO, under anaerobic conditions, in a reaction that is pH-dependent (equivalent to eq. 13; k(Nb, pH 6.5)≈1.2$M^{-1}s^{-1}$, k(Nb, pH 7.4)≈ 0.12-0.26$M^{-1}s^{-1}$ [491], k(Cb, pH 6.0)≈1.5$M^{-1}s^{-1}$, k(Cb, pH 7.0)≈0.14$M^{-1}s^{-1}$ [492]). However, contrary to the "open" (penta-coordinated) Hb and Mb, the Nb and Cb reactions are also dependent on the competition of nitrite with the distal histidine for binding to the haem. In the case of Nb, the nitrite reduction is further dependent on the redox state of the Cys_{CD7} and Cys_{D5}, whose binding controls the fraction of penta-coordinated haem-containing molecules. In accordance, the reduction of the disulfide bond or the mutation of the Cys_{CD7} and Cys_{D5} to alanine residues decrease the Nb reaction rate constant by a factor of two (k≈0.06$M^{-1}s^{-1}$ (pH 7.4)). Noteworthy, the replacement of the distal His_{E7} by a leucine or glutamine residue, that "locks" Nb in a penta-coordination state, leads to rates ≈2000-fold higher than the one of wild type Nb (259 and 267$M^{-1}s^{-1}$, respectively (pH 7.4))[491]; furthermore, and remarkably, under acidic conditions (pH 6.5), the rate constants of the His_{E7}Leu and His_{E7}Gln mutants increase above 2,500 and 2,000M^{-1} s^{-1}, respectively[491] **-**the higher reaction rates of nitrite with a mammalian HG ever reported. (In this respect, it should be noted a similar mutation of the Mb leads to a marked decrease in the nitrite reduction (1.8 and <0.5$M^{-1}s^{-1}$ for His_{E7}Ala and His_{E7}Leu Mb mutantes)[493,494].) Although these high values can not reflect the physiological NO formation, they confirm that the Nb-dependent NO generation is determined by the hexa- to penta-coordination conversion and, consequently, at least by the thiols redox state (or, by the redox status of the cell).

Unsurprisingly, the penta-coordinated deoxy-Nb/Cb also rapidly trap the NO (equivalent to eq. 15; k_{on}≈$10^8M^{-1}s^{-1}$ [435,449,477]) in a stable (Nb/Cb)Fe^{2+}-NO complex (k_{off}≈10^{-4}-$10^{-3}s^{-1}$ [435,449,495]), as Hb/Mb do. However, the competition of NO with the distal histidine for binding to the haem should result in a lower global NO affinity for Nb, comparatively to Hb/Mb. In addition, NO, as well as nitrite, can also be oxidised (scavenged) by oxy-neuroglobin[451] and oxy-cytoglobin[394,397,429,451,480] (oxy-Nb and oxy-Cb; equivalent to eq. 16 (k≈$10^7M^{-1}s^{-1}$ [477]) and 14, respectively). Once more contrary to the Hb/Mb reactions 16 and 14, the global rate of these Nb and Cb oxidations is dependent on the competition

between the dioxygen and the distal histidine (which controls the oxygenated protein concentration). The higher auto-oxidation rate (comparatively to Hb/Mb) also contributes to further decrease the oxy-Nb/Cb concentration available to react with NO and nitrite.[403,425,432].

Regardless of those "side" reactions (eq. 14-16) and of all the other possible haem reactions (Fig. 3), the *in vivo* nitrite reductase/NO synthase activity of Nb and Cb is supported by two lines of evidence: (i) Nb[431,433-435,440,441,465-471] and Cb[397,429,431,435,437,447,461,471,474-481,492] have been shown to mediate cytoprotective responses to ischaemic stress, promoting cell survival, and (ii) in the presence of nitrite, can stimulate the NO signalling and inhibit the mitochondrial "respiration", in a manner dependent on the pH, dioxygen concentration, cysteine redox state and haem coordination state[491,492].

Crucial for the *in vivo* significance of the Nb and Cb reactions (eq. 13, 14, 16) is the existence of regenerating systems that efficiently re-reduce the oxidised met-neuroglobin and met-cytoglobin (met-Nb and met-Cb), particularly because these proteins are present at low concentrations (micromolar range), comparatively to Hb and Mb (whose high concentrations allow them to effectively form and scavenge NO, in spite of relatively slow reduction mechanisms).[264,496] Although presently there are no known specific reductases, met-Nb and met-Cb can be rapidly reduced *in vitro* using conditions that mimic those existing within living cells[403,425,457] (met-Cb, *e.g.*, can be rapidly reduced by cellular reductants such as cytochrome b_5 and cytochrome b_5 oxidoreductase[476,477], cytochrome P_{450} reductase (NADPH; $k \approx 3X10^5 M^{-1}s^{-1}$ [477]), or even ascorbate ($k \approx 50 M^{-1}s^{-1}$ [477])). These "recycling" reactions (ferric to ferrous) would greatly increase the rate of NO formation, as well as of NO scavenging (under normoxia (eq. 16) and hypoxia (eq. 15)), allowing Nb and Cb to act as catalysts of the NO and nitrite metabolism at the low concentration that they are found *in vivo*.

space (=blank line)

In summary, the Nb and Cb-catalysed nitrite reduction to NO presents the same features and "weaknesses" as discussed for Hb/Mb, but with an additional level of complexity: the nitrite reduction is modulated by the redox state of key surface cysteine residues and by any other hypothetical "modification" that decreases the distal histidine affinity (*e.g.*, phosphorylation, protein-protein interaction, protein-lipid interaction, nitration). In accordance, these HG have been suggested to be allosteric-regulated nitrite reductases. In particular for Nb, the following mechanism has been

proposed: under normal conditions, the high cellular concentration of reduced thiols (*e.g.*, GSH ≈5mM, with GSH/GSSG >500) keeps the Nb cysteine residues reduced and "locks" the protein mainly in a "closed" bis-histidinyl-hexa-coordinated configuration, with low nitrite reductase activity; as oxidative stress[497] conditions develop (*e.g.*, after an ischaemic episode) and the concentration of reduced thiols decreases, the population of disulfide-containing penta-coordinated Nb increases, amplifying the NO formation[491]. (Subsequently, the NO formed can, for instance, inhibit the mitochondrial "respiration", limiting the dioxygen consumption and reactive oxygen species formation, thus preventing further oxidative stress damage.) Hence, the proposed mechanism allows the "translation" of the changes in the cellular redox status into a differentiated NO flux that would be, subsequently, "translated" into a biological response.

The existence of the equilibrium 30 explains how an alleged hexa-coordinated haemic protein can bind nitrite to convert it into NO: the hexa-coordinated protein is not a "rigid body" and the "internal" sixth coordination position can be "open" to allow for the binding of an "external" ligand. And the fact that this equilibrium can be modulated (changed in time) provides the answer for "why choose a hexa-coordinated protein?": to have a protein whose reactivity can be directly controlled by a selected stimuli, through a mechanism of the type "unready" *versus* "ready" (a regulatory strategy further discussed in section 4. and further exemplified with Cc and Cd_1NiR); a protein with such "tuneable" reactivity would allow the tissue to quickly respond towards different cellular conditions.

In this scenario, Nb seems to present the ideal characteristics to be a nitrite reductase: (i) it is present at low concentrations (more in line with a catalyst role)[431]; (ii) has a low haem reduction potential[403,425,432]; (iii) holds a large cavity ($120A^3$ [405]), connecting the haem with the bulk, that has no counterpart in vertebrate Hb or Mb and can function as a channel for substrates entrance/products release[405]; (iv) it is plausible to be involved in redox reactions, as discussed above; (v) it displays a redox-controlled "internal" hexa-coordination, linked with the cellular redox status; (vi) and it has been associated with hypoxia and oxidative stress conditions (when is up-regulated). In addition, Nb is of ancient evolutionary origin, having remained highly conserved throughout mammalian evolution[387, 393-398], and displays characteristics that are more in line with the bacterial and plant hexa-coordinated HG. The presence of these relatively similar proteins in so many different organisms suggests a strongly selected vital role. Nb may be a "molecular fossil" reminiscent of a redox catalytic

function of the HG family that was vital to preserve throughout the evolution[410]: a nitrite reductase/NO synthase or/and a redox sensor.

The major challenges concerning the nitrite reductase activity of Nb and Cb are essential the same as those discussed for Hb and Mb. Primarily, how can the NO formed avoid the *dogmatic* scavenging by oxy- and deoxy-Nb/Cb. Also of major importance is the identification of other allosteric regulators of the hexa- to penta-coordination conversion, which may reveal new mechanisms to control the nitrite reductase activity and, consequently, the NO signalling. In this respect, the interplay between nitrite reduction and cellular redox status, dioxygen and reduced/oxidised thiols should be to be further explored (if it is obvious the need of hypoxic conditions to decrease/increase the concentration of oxy/deoxy species, the relationship with the concentration of cellular oxidised thiols is not straightforward).

3.1.1.2. (b) Cytochrome *c*

Cc needs no introduction: discovered more than a century ago[499], it is a small (≈13kDa) globular haemic protein present (normally) in the intermembranar space of the mitochondria, where it transfers one electron from cytochrome bc_1 complex (Complex III) to cytochrome oxidase complex (Complex IV), in the mitochondrial electron transport chain responsible for the oxidative phosphorylation. However, besides this well known "respiratory" function, Cc is also an apoptotic signalling molecule: a variety of metabolic stimuli and insults, triggers the Cc release to the cytoplasm, where it participates in signalling pathways underlying apoptotic cell death, by binding to the apoptotic protease activating factor-1 and triggering activation of procaspase 9, in a complex called the apoptosome **-**remarkably, an action not redox-related.[500-512]. In addition, Cc is involved in oxidative and nitrosative stress responses: (i) it is an ideal antioxidant molecule to scavenge the superoxide anion radical[513] formed by the mitochondrial electron transport chain[516], but it is also (ii) a lipid peroxidation catalyst (namely of mitochondria cardiolipin and cytoplasm membrane phosphatidylserine (also associated with apoptosis)[517-519]), (iii) a peroxidase-like enzyme and

(iv) a nitrating agent (roles discussed under section 3.1.2.3.).

Cc holds a "closed" *c* haem, hexa-coordinated by His_{18} and Met_{80} (human numbering), as expected for an electron transfer protein. Nevertheless, and as discussed for Nb and Cb, the Met_{80} coordination is reversible and a penta-coordinated species is formed when the methionine is oxidised[520,521], Tyr_{67} is nitrated[522-526], or Cc interacts with anionic lipids, such as the mitochondrial cardiolipin[518,527-529]. The Cc interaction with negatively charged lipids/membranes (and *inorganic* electrode surfaces) has been subject to numerous studies[518,529-540] and shown to be accompanied, not only by protein conformational changes[530-534,537,538,541,542], but also by a significant decrease (≈300-400 mV [522,523,529,536,539,540]) of the reduction potential, what should facilitate the Cc participation in reduction reactions. Once the haem is "open" (becomes penta-coordinated), the Cc can bind "external" ligands (substrates) and the haem redox chemistry can be explored to catalyse several reactions, including the reduction of nitrite and hydrogen peroxide (peroxidase-like activity, discussed in section 3.1.2.3.). In this way, the Cc activity is "switched" from simple electron transfer to catalysis **-**ideal for the Cc roles in stress and apoptotic responses.

This *in vivo* Cc "function switching" **-**"respiration" into stress/apoptotic mediator**-** demands for regulatory mechanisms to control the "activity switching" **-**hexa-coordinated/electron carrier into penta-coordinated/catalyst. Once more (as in Nb and Cb), the regulatory mechanisms (oxidation, nitration, interaction with lipids) consist of posttranslational structural modifications that facilitate the cleavage of the methionine-iron coordination[518,517,542-545]. In this respect, it is noteworthy that, besides the Met_{80}, also the Tyr_{48}, Tyr_{67} and Lys_{72}-Lys_{73}, (the lysine residues are involved in interactions with anionic phospholipids) are highly conserved in Cc from different species, suggesting the existence of multiple conserved biological "switches". In particular during apoptosis, because both the peroxidase-like activity and the fraction of cardiolipin-bound Cc increase, it has been suggest that cardiolipin-binding is one of the main "switches".[529]

As it is by now expected, when under conditions that favour the haem penta-coordination, the Cc effectively catalyses the anoxic and acidic nitrite reduction to NO; the nitrite reductase activity of hexa-coordinated Cc is negligible[546]. A comprehensive kinetic characterisation was not yet undertaken, but 100μM Cc (a reasonable physiological value for cytoplasmatic Cc[547,548]), in the presence of negatively charged liposomes, can catalyse the formation of NO at an appreciable rate of $0.18 nMs^{-1}$

(pH 6.4)[546]. Also the Cc mutant $Met_{80}Ala$, that harbours a "locked" penta-coordinated haem, displays an electrochemical catalytic response towards nitrite reduction similar to the bacterial Cd_1NiR one.[549] Some what surprising, the NO scavenging by Cc (equivalent to eq. 15) is considerably slower than by the HG, with a $k_{on}\approx10$ and $10^3 M^{-1}s^{-1}$, for hexa- and penta-coordinated Cc, respectively[546,550-552], what could contribute to increase the bioavailable NO concentration. Nevertheless, once formed, the $(Cc)Fe^{2+}$-NO complex would be quite stable ($k_{off}\approx10^{-5}$ and $10^{-7}s^{-1}$ for hexa- and penta-coordinated Cc, respectively)[550-552]. In spite of the expected NO trapping, the Cc-catalysed NO formation can significantly inhibit the mitochondrial "respiration" through inhibition of CcO[546] and cause the guanylate cyclase activation (*in vitro*)[492].

The physiological significance of this Cc nitrite reductase activity would depend on the existence of regenerating systems that efficiently re-reduce the oxidised Cc. Because Nb binds[440,441,457,458,459,553] and reduces ferric Cc very rapidly ($k=2x10^7 M^{-1}s^{-1}$ [457,458,553]), this globin has been suggested to be one of potential Cc "co-substrates"[554]. If so, a Nb regenerating system must be in place to efficiently re-reduced the Nb present in tissues in low concentrations (see section 3.1.1.2.(a)).

space (=blank line)

In summary, Cc is able to reduce nitrite, to produce bioactive NO, under the conditions found during ischaemia injury and apoptosis (and others), when its haem may become penta-coordinated due to protein nitration, oxidation, interaction with anionic lipids (and others). Hence, the nitrite reductase activity of Cc can be of physiological relevance not only for hypoxia- and redox-dependent signalling, but also for the apoptotic process. If, on one hand, nitrite and low NO concentrations are known to prevent pore opening, lipid peroxidation and Cc release in isolated mitochondria after ischaemia[546,556], on the other hand, high NO concentrations or high nitrite reduction rates can lead to Cc self-nitrosylation and nitration, inhibition of CcO, leading to membrane depolarisation, Cc release and apoptosis[546,557-562]. How the posttranslational regulatory mechanisms are orchestrated to control the Cc "activity switch" into a nitrite reductase **-**and other "new" activities, *e.g.*, peroxidase**-** is one of the major challenges for future research. In particular, how are the nitrite reductase and nitrite oxidase (discussed in section 3.1.2.3.) activities articulated?

Of major importance is also the identification of other regulators of the hexa- to penta-coordination conversion that should control the Cc action in apoptotic and non-apoptotic cells. For example,

it would be of interest to study the effect of partial proteolysis, that increases the peroxidase and nitrating activities[563], and of phosphorylation, since Tyr_{48} phosphorylation impairs the caspase activation (an anti-apoptotic "switch") and inhibits the electron transfer process[564,565]. Also pertinent would be (i) the identification of physiological relevant regenerating systems that efficiently re-reduce the oxidised (Fe^{3+}) Cc, (ii) the comprehensive kinetic characterisation of the nitrite reductase activity, or (iii) the accurate description of the concentration and coordination state of cytoplasmatic and mitochondrial Cc, besides (iv) the comparative study of the equivalent "side" reactions described in Fig. 3.

To conclude, Cc is a "multi-task" protein, certainly involved in complex regulator mechanisms that control its "activity switching". The allosteric/redox-regulated nitrite reductase/NO synthase activity could play a relevant role in the Cc-mediated signalling pathways. Precisely how this activity will affect signalling in apoptotic and non-apoptotic cells remains to be truly explored.[546]

3.1.1.3. Molybdenum-dependent nitrite reduction - xanthine dehydrogenase/oxidase and aldehyde oxidase

Several studies have suggested that the mammalian nitrite reduction can also be achieved with the molybdenum-containing XO and AO.

Mammalian XO and AO are cytoplasmatic molybdoenzymes, belonging to the XO family (see section 4.), that are present in various tissues[566].[585-591] Noteworthy, besides the cytoplasm[592,593], XO was also described to be present on the outer surface of the cell membrane of endothelial and epithelial cells[594-601] and on the peroxisomes[602,603]. *In vivo*, XO exists predominantly as a NAD^+-dependent dehydrogenase, named xanthine dehydrogenase (XD).[585-591] Yet, XD can be rapidly converted into a "strict" oxidase form that reduces dioxygen instead of NAD^+ **-**the commonly studied and very well documented XO. This conversion can be reversible, through oxidation of the Cys_{535} and Cys_{992}, or irreversible, by limited proteolysis (the conversion details will be described in section 4.2.3.1.) **-**hence, XO/XD is the third protein type here described (after Nb and Cc) whose activity can

be "switched" by a posttranslational conformational modification. AO is structurally similar to XO/XD, but exists exclusively as an oxidase (reduces dioxygen, not NAD^+).[604,605] Physiologically, mammalian XO/XD is a key enzyme in purine catabolism, where it catalyses the hydroxylation of both hypoxanthine and xanthine to the terminal metabolite, urate, with the simultaneous reduction of dioxygen (XO) or NAD^+ (XD).[585-591] The physiological function of AO remains a matter of discussion, being a probable partner in the metabolism of xenobiotics, neurotransmitters and of retinoic acid.[605-608] However, XO/XD, as well as AO, catalyses also the oxidation of a wide variety of substituted pyridines, purines, pteridines, related compounds, including the NADH[567-573], and aldehydes[585-591,609,610]. Besides this broad specificity for oxidising substrates, these enzymes are also *promiscuous* with the reducing substrates, being able to catalyse the reduction of several sulfoxides and N-oxides, including nitrate and nitrite.[611-623] This unusual broad specificity has suggested the enzymes participation in other physiological pathways, including in the xenobiotic metabolism, but also in the activation of pro-drugs (*e.g.*, [605,607,624-625]) or toxic activation of azo dyes used as colorants in food and cosmetics (*e.g.*, [626]). In addition, their ability to catalyse the reduction of dioxygen has suggested their involvement in signalling pathways and, most important, in some reactive oxygen species-mediated diseases[627-635], including ischaemia-reperfusion injury[636-641] and ethanol hepatotoxicity[570,642-647]. Furthermore, XO/XD is also centre of development of new drugs against hyperuricaemia and gout. The proposed roles of both XO/XD and AO in a range of physiological and pathological conditions have resulted in a considerable and increasing medical interest in these enzymes. More recently, the demonstration that XO/XD and AO can also catalyse the nitrite reduction with NO formation contributed to further stimulate the interest in the catalytic properties of these versatile enzymes **-**changing the way theses enzymes are thought, from damaging to beneficial players.

space (=blank line)

In vitro, under anaerobic conditions, XO/XD and AO[615-623,648] catalyse the nitrite reduction to NO, in a reaction that is pH dependent (highly favoured at pH <7), with a molecular mechanism that will be discussed in section 4.2.3.. However, and as can be foreseen, dioxygen and NAD^+ (the "classic" oxidising substrates) act as strong competitive inhibitors of the nitrite reduction (by XO/AO and XD, respectively), "stealing" the electrons needed to reduce nitrite.[618,620,649] This *in vitro* anaerobic reaction

establishes the starting point for the suggested hypoxic/anoxic XO/XD/AO-dependent NO formation *in vivo*.

During ischaemia, several events occur that, in concert, can favour the nitrite reduction by XO/XD and AO. First, and obviously, the decrease in dioxygen concentration (hypoxia or even anoxia) and the resulting acidosis (pH values of 6.5 to 5.5). Second, in the course of ischaemia, the mitochondrial electron transfer chain would be disrupted and the ATP synthesis hindered; the subsequent ATP catabolism leads to an accumulation of hypoxanthine and NADH in tissues.[650-656] This increase in the concentration of two reducing substrates can "fuel" the enzymes with reducing equivalents to reduce nitrite. Third, as the ATP concentration decreases, the transmembranar ion gradients are dissipated, causing elevated cytoplasmatic calcium concentrations, which, in turn, activate calcium-dependent proteases that would convert the XD into the XO form.[601,657-663] In summary, during ischaemia: (i) the pH values are lower enough to provide the acidic conditions required for the nitrite reaction; (ii) reducing substrates are available to supply the necessary electrons; (iii) the formerly prevailing XD form (that reacts with NAD^+) can be converted into the "dioxygen-user" XO; (iv) the concentration of the competitive dioxygen is very low; (v) NAD^+ (regardless of its high concentration) would be no longer a competitive substrate of the nitrite reduction, because XO and AO do not react with it. Therefore, all the conditions seem to be gathered for nitrite to be reduced by XO and AO during *in vivo* ischaemia.

In accordance to the above reasoning, several *in situ* studies, validated with the employment of XO and AO specific inhibitors (allopurinol/oxypurinol and raloxifene, respectively, and also the general molybdenum-containing enzymes inhibitor sodium tungstate) suggested that these enzymes are acting as nitrite reductases *in vivo*, in models of ischaemia (and other types) injury in heart, liver, lung, kidney and vessels.[181,182,186,196,492,616,620-622,664-671] Particularly interesting is the demonstration of the XO protective role within the context of cardiac ischaemia in an isolated heart model.[664]

In spite of those *in situ* and *in vitro* studies, some authors argue that the high K_m values for nitrite (≈0.5-2mM [618,623], ≈3mM [622], for XO and AO, respectively), 1-2 orders of magnitude higher than the nitrite concentration in tissues (<20μM [167,380,381]), are a major limitation for the *in vivo* relevance of these molybdenum-dependent pathways. However, the kinetic parameters indicate that these enzymes can produce NO, with reasonable rates (k^{app}≈40$M^{-1}s^{-1}$ [623]), at conditions "fine-tuned" by the

availability of nitrite and dioxygen[618,623]. *I.e.*, by functioning in a concentration range well below the K_m value, the reaction rate is first order on nitrite (k^{app}), thus allowing the NO formation to be directly proportional to the nitrite availability and readily controlled by the strong competitor dioxygen, whose k_{cat}/K_m (pseudo-first order rate constant) is 2-3 orders of magnitude higher.[672] In this way, the concentration of the NO formed is kept within the characteristics of a local signalling molecule and controlled. In this respect, it should be emphasised that, *in vivo*, it is not conceivable to produce NO at micromolar or millimolar levels (the enzymes K_m order), if the NO performs its functions at nanomolar concentrations; at micromolar concentrations, it would not be achievable to control the NO specificity and toxicity (in fact, it is in situations of NO overproduction that its deleterious effects began, *e.g.*, in chronic inflammation where 2-4μM of NO were observed to be formed[151,339]). Thus, if these molybdoenzymes are to be physiologically relevant NO sources, they should not catalyse the formation of NO at the nitrite K_m concentration values.[623]

Another point against the feasibility of these pathways *in vivo* is related with the conversion of the *in vivo*-predominant XD into XO, whose extent and rate are a matter of great controversy: from no conversion at all (with XO being considered as an experimental artefact), to a small (20%) and slow conversion[577,632,657-659,661-663] and a conversion that is enhanced by hypoxic conditions and *in vivo* ischaemia[601,660]. The issue here is the competition between nitrite and NAD^+ to react with reduced XD. The NAD^+ concentration (≈0.5-1mM [653,654,675-678]), 2-3 orders of magnitude higher than the one of NADH, is not significantly decreased by the NADH accumulation during ischaemia[652-656]. As a result, if the conversion of XD into XO is not efficient (or does not occur at all), the NAD^+ reaction (with a k_{cat}/K_m 2-3 orders of magnitude higher[679]) would prevail over the nitrite reduction and the NO formation by this protein would be seriously compromised. Nonetheless, the AO would still be able to achieve the NO formation (since it does not react with NAD^+).

In addition, also the competition between nitrite and dioxygen is critical, and these pathways require substantial hypoxia or anoxia.[618,620,649] Moreover, in the presence of dioxygen (even at low concentrations; K_m for dioxygen is ≈10^{-5}M [672,680]), the superoxide radical formed would react with NO, to yield the strong oxidising peroxynitrite[150,151,339]. Thus, under non-anoxic conditions, the co-presence of superoxide dismutase is crucial for the NO signalling function. Besides those chemical (kinetics) constrains, the proposed role of XO/XD as a NO source faces another obstacle: for

long, countless studies pointed towards a beneficial clinical outcome through the inhibition of XO (reduction of symptoms by treatment with allopurinol).[636,637,641,681] How can those results be reconciled with a beneficial XO-mediated role? Clearly, another standpoint that would have to be "broken" to allow new, more comprehensive, studies.[682]

space (=blank line)

In summary, *in vitro*, under anaerobic conditions, the molybdenum centres of XO/XD and AO are able to reduce nitrite and, contrary to the haemic proteins so far discussed, to promptly release the NO formed (fully discussed in section 4.2.3.). With the molybdenum-containing proteins, the dilemma is related with the competition of nitrite with the "classic" oxidising substrates, dioxygen and NAD^+, for the electrons provided to the enzymes by the reducing substrates. *In vivo*, the molybdenum-dependent NO formation would be determined by the extension of ischaemia (hypoxia), the co-presence of superoxide dismutase and other antioxidants and by the availability of oxidising and reducing substrates. In the case of XD/XO, the NO formation is further dependent on an additional factor: its conversion to XO. Thus, a parallelism with Nb and Cc can be drawn and the XD/XO can be suggested to be an allosteric-regulated nitrite reductase controlled by the redox state of key cysteine residues or by limited proteolysis. As for Nb, it can be argued that, as oxidative stress conditions develop and the concentration of reduced thiols decreases, the fraction of XO increases and the NO formation is promoted. However, the relationship between the hypoxic conditions needed to achieve the nitrite reduction and the concentration of cellular oxidised thiols is not clear. On the other hand, the proteolysis-mediated conversion is easier to reconcile with an ischaemic event.

In light of the well known molybdenum oxo-transfer chemistry (discussed in detail in section 4.2.3.), the nitrite reductase activity of XO/XD and AO are not at all unexpected: once again, the mammalian cells are doing "substrate adaptations" to available redox systems and "switching" the systems activities in accordance with the cellular needs. In this context, the hypothesis of the XD nitrite reductase activity be further dependent on the conversion into XO adds an additional level of complexity (allosteric regulation) to the "activity switching".

One of the major challenges concerning the molybdenum-dependent enzymes (and not only the nitrite reductase activity) is to understand if, *in vivo*, the XD is converted into XO and, if it is converted, how, to what extent and when the conversion takes place. Is the conversion an

experimental artefact or a deliberated regulatory strategy? In fact, the question of the XD conversion goes well beyond the nitrite reductase activity, because the formation of XO has also been suggested to be responsible for the aerobic generation of reactive oxygen species that would participate in signalling cascades under oxidative stress conditions. However, in this respect, it should be emphasised that also the XD form is able to produce the reactive oxygen species.[571,572,680,683,684]. Hence, another question is also pertinent: how are the posttranslational regulatory mechanisms orchestrated to control the "switching" between all the purposed XD/XO activities? On the other hand, if the conversion of XD into XO does not occur *in vivo*, how can the XD avoid reacting with NAD^+?

Another challenge is related with the development of novel *in situ/in vivo* experimental approaches: most of the XO studies are validated with the employment of allopurinol/oxypurinol, which is a recognised reactive oxygen species scavenger and is known to interfere with alternative purine catabolism pathways (namely with adenosine).[685-688] Also raloxifene (estrogen receptor modulator) interferes with other vital pathways.[689] In this respect, the use of a new XO inhibitor, febuxostat (with a K_i value 3 orders of magnitude lower than the one of allopurinol), may reveal to be useful. Also valuable would be the obtainment of viable XO/XD and AO mutants.

The *in vivo* relative relevance of these enzymes on the total nitrite-dependent NO formation also needs to be revaluated in a tissue-dependent manner. For example, the XO/XD interaction with the vessel wall negatively charged glycosaminoglycans, in the context of the nitrite reduction, was poorly explored. It has been suggested that several pathologies (including liver and intestine ischaemia) causes the XO/XD release into the circulation[651,681,690-694], where it can bind to the endothelia glycosaminoglycans[594-601] to form a new complex (polysaccharide-XO/XD) that displays different kinetic properties towards xanthine and allopurinol[695]. How does this complex affect the nitrite activity of XO/XD? Is the enzyme reaching locations where it was not present before the injury and where the NO formation can be relevant?

3.1.1.4. Protein-independent nitrite reduction

Remarkably, mammals still can count on another mechanism to produce NO: the protein-independent nitrite reduction under acidic and reducing conditions. The non-proteic NO formation has been demonstrated in the stomach[696-703], skin surface[704,705], infected urinary tract[706] and oral cavity (even though the mouth higher pH (relative to the stomach one) dictates a lower NO formation)[707,708]. This protein-independent NO generation may play a decisive role, not only on the (obvious) control of vasodilation[709-711], but also in gastric mucosa formation[712] and in host defence (as bactericide in oral cavity[708], stomach[698,713-719], urinary tract[706] and even in skin[705]). In this context, it is worth mentioning that are the commensal bacteria of the oral cavity that reduce nitrate to the necessary nitrite for NO generation in the stomach (through the swallowed saliva[720])[703, 716-719,722-724]; moreover, the microbial communities of human dental plaque are capable of carrying out the complete denitrification pathway, mediating the nitrate reduction to NO, and further to nitrous oxide and dinitrogen[725] **-**an overlooked symbiotic interaction that may question the overuse of antibacterial mouthwash. On the other hand (and ironically), it is the nitrite production by some pathogenic nitrate-reducing bacteria that may cause their own destruction through the formation of cytotoxic concentrations of NO.[706]

space (=blank line)

The protein-independent NO formation relies on the nitrite decomposition, at acidic pH, to dinitrogen trioxide (eq. 31-32), which can, then, dismutate to NO and nitrogen dioxide radical (eq. 23) or be converted to a RSNO (eq. 24).[334,726,727]

$$2NO_2^- + 2H^+ \rightleftharpoons 2HNO_2 \quad (31)$$

$$2HNO_2 \rightleftharpoons H_2O + N_2O_3 \quad (32)$$

$$N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2 \quad (23)$$

global reaction (31+32+23): $2NO_2^- + 2H^+ \longrightarrow {}^{\bullet}NO + {}^{\bullet}NO_2 + H_2O$ (33)

The extent of this NO generation, however, is expected to be very small and limited to conditions of profound hypoxia and/or acidosis (specially the above mentioned stomach), because it depends on (i) the formation of nitrous acid (pK_a 3.1-3.4 for eq. 31) and (ii) on the dismutation of dinitrogen

trioxide, whose equilibrium (eq. 23) is expected to be rapidly dislocated towards the NO consumption (as was discussed in section 3.1.1.1.). Nevertheless, the rate and direction, at which the reactions 31→32→23 would be driven (*in vitro* and *in vivo*), depend, not only on the pH, but also on (i) factors that potentially shift the equilibriums by consuming NO (such as dioxygen (eq. 34), haem-containing proteins and thiol compounds) and (ii) on the presence of reducing compounds.[728] In its turn, the aqueous redox chemistry of nitrite is, once more, highly pH-dependent: the nitrite reduction to NO is proton-coupled ($2H^+/e^-$; eq. 12) and its reduction potential drops from 1.00, at pH 1, to 0.37V, at pH 7 (*vs* NHE).[728] Hence, on a laboratory scale, it is the reduction of acidified (pH 1) nitrite solutions with iodide that is used to rapidly and estequiometrically synthesised NO. In less harsh conditions, ascorbic acid, that acts as both a reducing and acidifying agent, rapidly reduces nitrite to NO (eq. 35).[7] In accordance, the NO generation was observed to be enhanced, in stomach and urinary tract, in the presence of vitamin C[7,706,711] and polyphenolic compounds[701,702,729,730]. In this respect, it is should be emphasised that eq. 31 plus 32 result in the global eq. 22, which was described to be catalysed by ferrous Hb (in section 3.1.1.1.) **-**once more, the dinitrogen trioxide formation being dependent on the presence of a reducer. In summary, the protein-independent NO formation is expected to be highly pH and reducer-dependent.

$$2^{\bullet}NO + O_2 \rightleftharpoons 2^{\bullet}NO_2 \qquad (34)$$

$$2HNO_2 + \text{ascorbic acid} \rightleftharpoons \text{dehydroascorbic acid} + 2^{\bullet}NO + 2H_2O \qquad (35)$$

Although the protein-independent NO generation had been demonstrated in the few above mentioned organs/localisations, it is noteworthy that this pathway is likely to occur at any site where nitrite is present under acidic and reducing conditions.[731,732] In particular, in any tissue under ischaemia, where the pH decreases to values as low as 5.5 and the reducing equivalents accumulate.[731-736] And, in fact, it was shown that the reducing (non-enzymatic) compounds present in homogenates of hearts subjected to ischaemia greatly (40-fold, at pH 5.5) increase the rate of NO formation.[731,736,737] Even so, it should be noted that this protein-independent, reducers-dependent NO formation was observed to be lower (<15% [377]) than the protein-dependent one (described on the previous

sections).[377,618]

space (=blank line)

Presently, little is know about the regulation and physiological/pathological significance of this protein-independent NO formation pathway, principally in the stomach, where its occurrence seems to be beyond any doubt **-**a challenge for future research.

3.1.1.5. Nitrous anhydrase

Besides the "non-dedicated" nitrite reductases so far discussed, also the zinc-containing ubiquitous carbonic anhydrase was shown to readily produce NO from nitrite.[738] Because Zn^{2+} (with its d^{10} electronic configuration) does not support redox chemistry, the carbonic anhydrase was suggested to act as a nitrous anhydrase (eq. 31→32), in a reaction that would be equivalent to the reverse of the carbon dioxide hydration (eq. 36); the subsequent dinitrogen trioxide dismutation would yield nitrogen dioxide radical and the aimed NO (eq. 23)[738] The feasibility of this mechanism is, however, questionable, because (i) the formation of dinitrogen trioxide (eq. 31→32) and (ii) the direction and rate of the equilibrium 23 have been subjected to controversy (as discussed in sections 3.1.1.1. and 3.1.1.4.). Nevertheless, the carbonic anhydrase-dependent NO was shown to induce the vasodilation of aortic rings and suggested to contribute to the regulation of the local blood flow in response to an increase in the tissue metabolic activity.[738] The carbonic anhydrase could "translate" the increased metabolic activity (equivalent to an increased carbon dioxide formation) into a decrease in local pH (through reaction 36); the local transient acidosis would, then, favour, not only the dioxygen delivery to the tissue (via the Bohr effect that decreases the Hb dioxygen affinity)[739], but also the NO formation (eq. 31→32→23), triggering the vasodilation, which further favours the dioxygen supply to the tissue. Noteworthy, and contrary to all the other pathways discussed, the carbonic anhydrase-dependent NO formation can occur before the dioxygen concentration decreases below critical values, even though it could take place under hypoxia as well.[738] However, the competition with carbon dioxide was not yet thoroughly explored.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \quad (36)$$

$$2NO_2^- + 2H^+ \rightleftharpoons 2HNO_2 \rightleftharpoons H_2O + N_2O_3 \quad (31\text{-}32)$$

$$N_2O_3 \rightleftharpoons {}^{\bullet}NO + {}^{\bullet}NO_2 \quad (23)$$

3.1.1.6. Nitrite reduction - summary

In summary, the mammalian nitrite-derived NO formation is, theoretically, quite simple (much simpler than the NOS-catalysed synthesis). It involves the one-electron reduction of nitrite by a redox active metalloprotein and requires just protons (acidosis), a low dioxygen concentration (both conditions created during ischaemia) and an electron donor (to reduce the metal). Therefore, from a chemical point of view, mammals, that do not have a "dedicated" nitrite reductase, can reduce nitrite by doing a "substrate adaptations" to an available redox metalloprotein. From a physiological standpoint, mammals are "reusing" metalloproteins, present in cells to accomplish other functions, and "switching" the proteins activity (to a nitrite reductase/NO synthase) when it is necessary to generate NO and the NOS activity is impaired. However, as was discussed, the *in vivo* nitrite "recycling" to NO is, actually, a very complex subject, further complicated by the (present) lack of knowledge to connect the *in vitro* understanding of nitrite reduction mechanisms with the *in vivo* observed nitrite effects.

Although the physiological relevance of each individual pathway (Fig. 2 and other proteins that will, probably, be identified in the near future) is currently being (and will be) debated, the fact that nitrite can be reduced to NO (i) by diverse metalloproteins (enzymes, metabolite transporters and electron transfers), (ii) with different cellular roles, (iii) tecidular and subcellular localisations and (iv) molecular features, suggests that nitrite does play a critical role in the mammalian cellular homeostasis. From a physiological point of view, the existence of several NO formation pathways

seems sensible. (i) It is not probable that Biology developed only one specialised enzyme to generate such a crucial molecule; certainly, it would be an advantage to have some "rescue" pathways to ensure the NO formation. (ii) These would be particularly relevant for mammals to be able to avoid the deleterious hypoxic/anoxic conditions, when the oxygen-dependent NOS activity would be impaired. (iii) In addition, the *promiscuous* function of an enzyme/protein can be reasoned as a "vestige" of the function of its ancestor[740]; the choice of nitrite as the NO source (instead of, *e.g.*, an amino acid) can be thought as a "vestige" of the pre-aerobic pathways[741,742].

Interestingly, some of those metalloproteins have a known oxygen-dependent or oxygen-related activity, but, under hypoxia, they apparently "switch" from their "classic" activity to a nitrite reductase activity. Furthermore, in a few of these proteins **-**Nb, Cc and possible XD/XO**-** the "activity switch" is allosterically regulated. These "activity switches" put forward the hypothesis that nitrite is acting, not only as a NO source, but also as an oxygen or redox sensing molecule. Accordingly to this hypothesis, *each* individual nitrite-mediated pathway would be activated when the oxygen concentration decreases below *its own threshold* of oxygen-dependent activity. In this way, all pathways would act in a concerted and self-regulated manner **-***i.e.*, "activity switching" triggered at different oxygen concentrations/redox conditions as a mechanism to orchestrate all the pathways.[743,744] Thus, each pathway would be relevant under different conditions and in different tissues. The "activity switching" hypothesis adds another level of complexity to the intricate puzzle of nitrite-mediated pathways and catapults the nitrite relevance to the entire physiological oxygen gradient, from normoxia to anoxia.

3.1.2. Nitrite oxidation in mammals

When compared to its reduction, the nitrite oxidation (Fig. 2) and its potential role in mammalian physiology and pathology have been overlooked.

Although no "dedicated" mammalian nitrite oxidase was yet described, several haemic proteins seem to be used to carry out the oxidase function, catalysing the nitrite oxidation, not only to nitrate

(a two-electron oxidation), but also to nitrogen dioxide radical (one-electron reaction). As described in sections 3.1.1.1.-3.1.1.2., the oxygenated HG are able to oxidase nitrite to nitrate (eq. 14). In addition, nitrite can also be oxidised by several haemic proteins, such as the peroxidases myeloperoxidase, lactoperoxidase, eosinophil peroxidase and catalase, but also by Cc, as well as by "inflammatory" oxidants, such as the hypochlorous acid (see references throughout the following sub-sections).

Unlike nitrate, the nitrogen dioxide is a powerfully oxidising and nitrating agent[745-751] that can nitrate, not only protein residues (considered to be a deleterious process that alters the protein function and targets it for degradation), but also fatty acids and guanine nucleotides (whose limited nitration has been shown to elicit protective responses against inflammatory tissue injury[752,753]). Moreover, are the nitration reactions that have been evoked to explain the loss of protection observed when higher doses of nitrite are administered in ischaemia-reperfusion conditions.[181,467]

3.1.2.1. Haem-dependent nitrite oxidation - globins

The nitrite oxidation by oxy-Hb is known for more than a century, with the initial studies endeavoured essentially to investigate the nitrite poisoning.[754-766] From this reaction results the formation of nitrate and met-Hb (eq. 14). However, the reaction kinetics is far more complex than just a simple bimolecular reaction (pH-dependent), with a single rate-limiting step. Instead, it was shown to be an autocatalytic radical chain reaction, described by reactions 14 and 37 to 40.[766]

initiation: $(Hb)Fe^{2+}\text{-}O_2 + NO_2^- + H^+ \longrightarrow (Hb)Fe^{3+} + NO_3^- + 1/2H_2O_2$ (14)

propagation: $(Hb)Fe^{3+} + H_2O_2 \longrightarrow (^{\bullet}Hb)Fe^{4+}{=}O + H_2O$ (37)

(where $(^{\bullet}Hb)Fe^{4+}{=}O$ is ferryl-haemoglobin molecule with a radical on an amino acid residue, formed by the two electrons reduction of $(Hb)Fe^{3+}$)

$(^{\bullet}Hb)Fe^{4+}{=}O + NO_2^- \longrightarrow (Hb)Fe^{4+}{=}O + {}^{\bullet}NO_2$ (38)

$(Hb)Fe^{4+}{=}O + NO_2^- + 2H^+ \longrightarrow (Hb)Fe^{3+} + {}^{\bullet}NO_2 + H_2O$ (39)

$$(Hb)Fe^{2+}\text{-}O_2 + {}^{\bullet}NO_2 \longrightarrow ({}^{\bullet}Hb)Fe^{4+}{=}O + NO_3^- \quad (40)$$

global reaction of the propagation cycle (37+38+39+40):

$$(Hb)Fe^{2+}\text{-}O_2 + 2NO_2^- + 2H^+ + H_2O_2 \longrightarrow NO_3^- + 2H_2O + ({}^{\bullet}Hb)Fe^{4+}{=}O + {}^{\bullet}NO_2 \quad (41)$$

The nitrogen dioxide and ferryl-haemoglobin radicals formed can initiate new propagation cycles, autocatalysing the reaction until all oxy-Hb is consumed. *In vivo*, under "normal" conditions, however, the presence of antioxidants should limit the propagation phase: (i) catalase and glutathione peroxidase should consume the initiator hydrogen peroxide, (ii) the erythrocyte-NADH-cytochrome b_5 reductase system reduces the met-Hb to ferrous Hb and (iii) small molecules antioxidants such as glutathione, urate and ascorbate should reduce the radical species that propagate the cycle. Other termination reactions include (i) the nitrogen dioxide dimerisation to dinitrogen tetraoxide (N_2O_4, eq. 42; $K_d \approx 10^{-5}$M [334]), (ii) its reaction with NO to yield dinitrogen trioxide (reverse eq. 23, eq. 44), both of which could be hydrolysed to yield nitrite and nitrate (eq. 43 and 45), and (iii) its participation in nitrating reactions[745-751]. Therefore, *in vivo*, the reaction is not expected to become autocatalytic (except for severe nitrite poisoning[767]) and the rate of nitrite oxidation by oxy-Hb is suggested to be determined only by reaction 14 and to be slow ($k \approx 0.5\text{-}1 M^{-1}s^{-1}$), under "normal" conditions[766].

$$2{}^{\bullet}NO_2 \rightleftharpoons N_2O_4 \quad (42)$$

$$N_2O_4 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+ \quad (43)$$

$${}^{\bullet}NO + {}^{\bullet}NO_2 \rightleftharpoons N_2O_3 \quad (44)$$

$$N_2O_3 + H_2O \longrightarrow 2NO_2^- + 2H^+ \quad (45)$$

Nevertheless, it should be noted that the reactions of the propagation phase are not driven only by the nitrite oxidation, but, of course, also by the presence of hydrogen peroxide (eq. 37) originated

from other sources, when the antioxidant defences are not able to efficiently remove it. Actually, the peroxidase-like activity of Mb[751,771-775] and Hb[776-778] is long known, resulting in the formation of ferryl- (eq. 46) and radical ferryl-haems (eq. 37). These oxidised proteins, in a situation of oxidative stress[497], could greatly amplify the oxidation of nitrite to nitrogen dioxide (eq. 38 and 39) and, thus, promote the deleterious protein nitration[747,779,780] observed, *e.g.*, during myocardial ischaemia-reperfusion injury[781-785]. (The presence of nitrated proteins in circulating erythrocytes is controversial.[747,786]). Moreover, the acidic conditions (characteristic of ischaemia) favour the nitration reactions, at the same time as decrease the rate of nitrogen dioxide scavenging by thiols, therefore increasing, effectively, the efficiency of protein nitration.[780]

In this context, it should be mentioned that an alternative nitration mechanism was proposed, which involves the previous nitrite binding to the haem iron, followed by the reaction of hydrogen peroxide with the bound nitrite to form an iron-peroxynitrite species that is, then, responsible for the nitration reactions.[749,787] However, the occurrence of this mechanism is limited to the presence of high, non-physiological, nitrite concentrations.[749]

Since the nitrite can remove (reduce) the ferryl and radical ferryl species[788,789], it was suggested that reactions 38 and 39 could function (i) as "sinks" of those oxidising species (thus hampering their oxidation and peroxidation reactions[772,790-796]) and (ii) as regenerators of Mb (to scavenge more hydrogen peroxide) **-***i.e.*, nitrite oxidation as a mechanism of cytoprotection[797], and not as a source of deleterious nitrogen dioxide radical[289]. (Note that the high concentration of thiols and other antioxidants present in cells should scavenge the radical until a relatively high concentration is formed.) Overall, it is clear that the reactions 38 and 39 "have two faces" and, *in vivo*, it is likely that a delicate balance takes place between the protective and deleterious role of nitrite/hydrogen peroxide/Mb[289]. The antioxidant capacity and the extent of the oxidative injury (namely inflammatory (where nitrite accumulates) and ischaemic injuries) could dictate whether the Hb/Mb peroxidase-like activity should be protective or deleterious.

$$(Mb)Fe^{2+} + H_2O_2 \longrightarrow (Mb)Fe^{4+}{=}O + H_2O \qquad (46)$$

space (=blank line)

In what concerns the hexa-coordinated Nb (see section 3.1.1.2. for the description of this hexa/penta-coordinated HG), as expected, its oxidising and nitrating activities are dependent on the conditions that favours the penta-coordination, with disulfide-containing Nb exhibiting a higher affinity for nitrite than the reduced protein.[438] Because, in contrast with Mb and Hb, the met-Nb does not react with hydrogen peroxide to form ferryl-haem species[438,452], its nitrating activity has been attributed to the nitrite binding to the haem iron, followed by reaction with hydrogen peroxide, to yield an iron-peroxynitrite nitrating species[438]. In this context, it is worth noting the apparent inability of Nb to form cytotoxic ferryl-haem species, what could be related and relevant to its observed role in cellular survival.[431,435]

3.1.2.2. Haem-dependent nitrite oxidation - peroxidases

It is remarkable that reactions 37 to 39, described for the autocatalytic nitrite oxidation by Hb, represent precisely a typical mechanism of a peroxidase-catalysed reaction[798-802]: in general, a native ferric peroxidase reacts with hydrogen peroxide to form compound I (equivalent to reaction 37); the enzyme is then regenerated by (i) two one-electron reduction steps, yielding compound II (equivalent to eq. 38) and subsequently the ferric protein (equivalent to eq. 39), or by (ii) one two-electron reaction, oxidising halides to the respective hypohalous acids (as, *e.g.*, in myeloperoxidase), or oxidising a second molecule of hydrogen peroxide to dioxygen (as in catalase). Hence, taking as model the mechanism above described for oxy-Hb, is not difficult to envisage how a peroxidase enzyme can account for the nitrite oxidation (eq. 47), as long as nitrite can bind to the active site and the reaction is thermodynamically feasible.

37+38+39 global reaction:

$$H_2O_2 + 2NO_2^- + 2H^+ \longrightarrow 2^{\bullet}NO_2 + 2H_2O \qquad (47)$$

For instance, nitrite is readily oxidised by myeloperoxidase at acidic pH (<6), in the presence of hydrogen peroxide, with a rate that is limited by the reaction with compound II (equivalent to reaction 39).[803-808] In fact, nitrite is a good substrate for myeloperoxidase compound I (equivalent to reaction 38; $k \approx 2x10^6 M^{-1}s^{-1}$ (pH 7) or $10x10^6 M^{-1}s^{-1}$ (pH 5)), but it reacts more slowly with myeloperoxidase compound II (equivalent to reaction 39; $k \approx 6x10^2 M^{-1}s^{-1}$ (pH 7) or $900x10^2 M^{-1}s^{-1}$ (pH 5))[807,808]. Therefore, if a "fast substrate" is available to reduce compound II (and regenerate the enzyme), the myeloperoxidase can rapidly oxidise nitrite to nitrogen dioxide.[808,809] The subsequent nitrogen dioxide-mediated nitration reactions could constitute a feasible protective response against (the onset of) inflammatory injury[752,753] (as mentioned in the introduction of this section, 3.1.2.) or a defensive mechanism against pathological microorganisms (*e.g.*, *P. aeruginosa*[810]). However, under chronic inflammatory conditions, when nitrite accumulates[814-816] (as a consequence of the induction of inducible NOS), myeloperoxidase could catalyse a burst of nitrogen dioxide, being the probable responsible for the biomolecules nitration observed in a wide range of inflammatory diseases involving activated neutrophils and macrophages.[809] A parallel situation is thought to occur in asthma and other allergic inflammatory disorders (characterized by activation of eosinophils)[809,817-820], with nitrite being sequentially oxidised by eosinophil peroxidase compound I and compound II to yield nitrogen dioxide[803,804,809,817,820-823]. The eosinophils, however, are more efficient (at least 4-fold) at promoting nitration than the neutrophils, because the nitrite oxidation by compound II is faster ($k \approx 6x10^3 M^{-1}s^{-1}$ at pH 7.4) than in myeloperoxidase[817,823].

With lactoperoxidase a different situation occurs, since nitrite rapidly reduces compound I ($k \approx 2x10^7 M^{-1}s^{-1}$ (pH 7.2)[824]) by two electrons, directly to the ferric state, with no observable nitrogen dioxide formation[803,804,809,817,820-822,824,825], as catalase does[756,821,826-827]. Nevertheless, the one-electron reduction of compound I can be carried out by a small molecular mass phenolic substrate (Ph-OH; eq. 48), followed by nitrite reduction of compound II to yield the nitrogen dioxide (eq. 49; equivalent to eq. 39) and subsequent phenol nitration (eq. 50).[828] In addition, at least *in vitro*, in the presence of a high nitrite concentration, lactoperoxidase is able to promote the nitration via an iron-peroxynitrite species (formed by reaction of hydrogen peroxide with nitrite-bound haem), as was suggested for Mb (section 3.1.2.1.).[828]

(peroxidase)compound I + Ph-OH ⟶ (peroxidase)compound II + Ph-O$^{\bullet}$ + H^+ (48)

(peroxidase)compound II + NO_2^- + $2H^+$ ⟶ (peroxidase)Fe^{3+} + $^{\bullet}NO_2$ + H_2O (49)

Ph-O$^{\bullet}$ + $^{\bullet}NO_2$ ⟶ HO-Ph-NO_2 (50)

In summary, it is presently widely accepted that the peroxidases-mediated oxidation of nitrite contributes to the observed deleterious nitration in several pathologies, namely in inflammatory conditions.[809]

3.1.2.3. Haem-dependent nitrite oxidation - cytochrome *c*

The "peroxidase-like mechanism" of nitrite oxidation can be further generalised, anticipating that other haemic proteins with peroxidase-like activity[829] can also promote the nitrogen dioxide radical formation, contributing to cellular oxidative damage[831-833]. In this respect, the alleged hexa-coordinated, "multi-task" Cc stands out.

As described above, besides its "respiratory" role (electron carrier/hexa-coordinated haem), Cc displays also peroxidase-like activity (catalyst/penta-coordinated haem). (See section 3.1.1.2.(b) for the description of this hexa/penta-coordinated haemic protein, its several proposed roles and suggested "function switching" mechanisms.) Presently, several studies have shown that, in the presence of hydrogen peroxide, Cc catalyses the oxidation of various reducers (including endogenous antioxidants, such as glutathione or ascorbate) through the formation of a compound I-like intermediate, in which one oxidising equivalent is present as an oxoferryl-haem species and the other as a protein tyrosyl radical (equivalent to reactions 37 plus 38).[831,834-839] The nitrite binding to the oxoferryl-haem and its subsequent oxidation yields nitrogen dioxide radical, in a reaction similar to the peroxidases one.[840] The nitrogen dioxide would, then, nitrate Cc itself (described to increase the fraction of penta-coordinated Cc) and proximal molecules[840],

being responsible for possible beneficial and deleterious effects, in a delicate balance, as was above discussed for Hb/Mb (section 3.1.2.1.).

As expected from the previous description of the Cc "activity switching" mechanisms, the presence of cardiolipin[517,529,830,841], oxidation by peroxynitrite[523] or reactive halogen species[520], nitration[522,523] and also partial proteolysis[840,842-845] greatly increase the peroxidase and nitrating activities of Cc.

In this context, it is of note that nitrated proteins and cytoplasmatic Cc are both characteristics of cells undergoing apoptosis; moreover, mitochondria depleted *versus* replete with Cc display significant differences in nitration yields[840]. This "coincidence" could be due (at least in part) to the nitrite oxidase activity of Cc, which would constitute a plausible mechanism for the cell to control the molecular and spatial specificity of the nitration reactions.[840]

space (=blank line)

Before concluding this section, it has to be here emphasised that the Cc peroxidase-like activity can constitute an important antioxidant defence against hydrogen the peroxide-dependent oxidative damage in mitochondria and cytoplasm.[846] Besides phagocytes, mitochondria are a relevant source of hydrogen peroxide, either under normal (when it oxidises up to 1-2% of the oxygen consumed to hydrogen peroxide and superoxide radical[847]) and under "not normal" conditions (*e.g.*, during ischaemia-reperfusion injury or in the presence of redox cycling drugs and electron transport inhibitor drugs[848-851]). Hence, Cc could be an antioxidant molecule that scavenges the hydrogen peroxide formed (besides the scavenging of the superoxide radical already mentioned). In this scenario, the presence of nitrite could contribute to "regenerate" the Cc (equivalent to reactions 38 and 39), as was discussed for Hb/Mb (section 3.1.2.1.), exerting a beneficial (antioxidant) effect. Nevertheless, several deleterious effects have been associated with the Cc peroxidase-like activity. E.*g.*, (i) contribute to the peroxidation of mitochondrial cardiolipin, promoting the Cc release[518,519] (section 3.1.1.2.), or (ii) "support" the nitrite oxidase activity, contributing, in this way, to the formation of nitrogen dioxide radical, a powerful oxidising and nitrating compound.

3.1.2.4. Nitrite oxidation - summary

One of the mechanisms that prevents the *in vivo* accumulation of nitrite is its oxidation. While the formation of the relatively "inert" nitrate is usually associated with the nitrite reaction with oxygenated Hb and Mb, it should not be forgotten that the same reaction produces, besides hydrogen peroxide, the reactive nitrogen dioxide radical. On the other hand, the formation of that oxidising and nitrating radical has been linked with the nitrite reaction with proteins with peroxidatic activity **-**again haemic proteins.

In summary, to oxidise nitrite, either by one or two electrons, mammals are (once more) doing "substrate adaptations" to the haem redox chemistry of haemic proteins already present in cells to accomplish other functions. However, contrary to its reduction, the nitrite oxidation seems to have two quickly identifiable "faces": (i) the beneficial, controlled formation of the signalling/defensive nitrogen dioxide and (ii) the deleterious, pathological, nitrogen dioxide overproduction. The "ugly face" of this nitrite handling arises from the accumulation of nitrite and hydrogen peroxide, *in vivo*, under conditions of oxidative and nitrosative stress, when both can be responsible for biomolecules modifications (oxidation and nitration) and, consequently, for pathologies.

Following the identification of nitrated proteins, *in vivo*, under a variety of pathological conditions, the mechanisms of nitrotyrosine and nitrotryptophan formation became the focus of interest by the medical scientific community. For long, the observation of nitrotyrosine residues was considered as an unequivocal fingerprint of the peroxynitrite formation. However, the occurrence of nitrated proteins in cells would reflect, not only the peroxynitrite formation or the NO trapping by tyrosyl radicals, but also the nitrite oxidation by "inflammatory" oxidants[745,803,852] or by haemic proteins such as the ones here described. The existence of these different nitrating pathways supports the relevance of this nitrite-dependent posttranslational protein modification for normal cellular signalling, but also in injury[840].

Regardless of its physiological and pathological significance, the mechanisms of formation of nitrogen dioxide radical are only beginning to be studied at a molecular level of detail. On the nitrite oxidation "chapter", the future goals include, not only the mechanistic aspects, but, mainly, the understanding of its *in vivo* interplay and relative relevance.

3.1.3. Mammalian nitrite handling - concluding remarks

To conclude this discussion on the mammalian nitrite handling, two last points must be addressed.

First: In a review of the mammalian nitrite roles, it must be mentioned that nitrite itself has been suggested to be a signalling molecule[200,376] **-**with no need to be converted into the "active" molecule. Although the nitrite stability and *in vivo* abundance would make it an interesting signalling molecule, it is difficult to proof that it is nitrite itself, and not NO or nitrogen dioxide, the responsible for the observed effects[853]. If confirmed, this mechanism may overcome the impasse of the rapid NO scavenging.

Second: If nitrite is a key molecule for cellular homeostasis, how is its concentration controlled to respond to daily changes in dietary intake and in NO metabolism (NOS activity/NO consumption)? In humans, nitrite is obtained from diet sources such as vegetables (*e.g.*, spinach, lettuce or beetroot), curried meat[6,723,854-856] or drinking water[857], either directly[856] or indirectly from the nitrate reduction by commensal bacteria in the mouth and gastrointestinal tract[703,716,717,722,724] (see the introduction of section 3.1.1.4.). Most of this exogenous nitrite (which it is not converted in the stomach) diffuses to the systemic circulation, where it is transported to resistance vessels and tissues.[716] The second main source of nitrite is the NO oxidation itself[149,858,859], with 70% of plasma nitrite being probably derived from endogenously produced NO[315,860]. To this pool of nitrite contributes the oxidation of NO through (i) reaction 26, (ii) via ceruloplasmin[174] and (iii) reaction with dioxygen (eq. 34→44→45; however, under the low physiological NO concentrations, the reaction 34 is believed to be too slow ($k \approx 10^{-6}$-$10^{-7} M^{-2} s^{-1}$) to significantly contribute to the nitrite formation). All the nitrite sources are, thus, potentially subjected to great daily variation. Hence, how does a cell control the nitrite concentration to cope with its oxidation and reduction reactions? Presently, the mechanisms of nitrite transport and intracellular accumulation/export are poorly understood and mainly restricted to the erythrocyte (where they possibly involve the anion exchanger AE1 of erythrocyte band 3 (see[352]))[861-865]. But it would be very interesting if the nitrite transport across cell membranes was modulated by the oxygen concentration, allowing the nitrite transport to be

directed to cells that need it to produce NO.[864,865] Future work will dictate if this is a reasonable hypothesis.

space (=blank line)

Undoubtedly, the knowledge of nitrite physiological roles in mammals has evolved considerably over the last two decades, giving a novel *relevance* to the formerly "*irrelevant*" nitrite. This knowledge offers/will create new therapeutic approaches for the management of several pathological situations[866-871], including, among others, ischaemia injury, cardiovascular dysfunctions, myocardial infarction, stroke, pulmonary hypertension[180,181,188,192,194,225,236,237,274,467,872-885] (all based on nitrite reduction) or infection (as a bactericide/fungicide in stomach and airways; based on nitrite oxidation)[714,812,813,886-889] (see also[810]). Thus, the mammalian nitrite metabolism research will certainly reserve many surprises for the future.

3.2. NITRITE ON SIGNALLING (AND OTHER) PATHWAYS IN PLANTS

As all other organisms, plants must perceive and respond to a plethora of external stimulus and internal signals. The NO is one of the signalling molecules used by plants to respond to abiotic and biotic challenges, as well as for their survival: NO has been shown to be involved, *e.g.*, in response to temperature, salt or drought stresses, in disease resistance pathways, germination, flowering, root development, leaf senescence or stomatal closure[890-930]). However, the plant NO formation and signalling pathways are, by far, less well characterised than the mammalian counterparts.

Plants have several potential NO-generating proteins, localised in different subcellular compartments, but only few of them have been thoroughly studied. Presently, it is accepted that NO can be formed through oxidative and reductive pathways.

The oxidative pathways are believed to produce NO, aerobically, through the oxidation of organic compounds such as polyamines[931,932], hydroxylamine[933] and arginine[934-938]. Surprisingly, although arginine-dependent NO formation (inhibited by mammal NOS inhibitors) can be measured and localised in plant tissues and organelles, no homologous NOS (gene or protein) was yet found in

higher plants.[890,918,937,939-941] In fact, the two previous NOS candidates for higher plants (a variant of the P protein of the glycine decarboxylase complex[942] and AtNOS1 [939,943]) were found not to be NOS enzymes.[944,945] Nevertheless, the green algae *Ostreococcus tauri* was shown to hold a NOS (45% similar to human NOS[946,947]) and *Symbiodinium bermudense*[948,949] and *Chattonella marina*[950] were shown to have NOS-like activity **-**raising the intriguing question of why higher plants do not have/need a NOS, contrary to animals, algae or prokaryotes.

In the reductive pathways, apparently the predominant ones[914,933,951,952], NO is formed through the (by now familiar) nitrite reduction and is favoured (once more) by low dioxygen concentrations and acidic conditions (see references throughout the following sections). However, in plants, the nitrite reduction occurs in a different "scenario": nitrate and nitrite, both precursors and end-products of signalling NO, are also normal substrates of the plant nitrogen assimilation pathway (see section 2.1.; Fig. 1, orange arrows). When nitrate is the main nitrogen source available, to survive, plants must assimilate it under conditions that range from normoxia to anoxia; nitrate and nitrite can, thus, accumulate to very high (millimolar) concentrations, in particular under hypoxia/anoxia (further discussed below) **-**a situation clearly different from the modest (nano- to micro-molar) nitrite concentrations found in mammalian tissues, where nitrite is not involved in any *primary* biosynthetic pathway. As a consequence, when plants use nitrite to synthesise signalling NO, they must do it in a controlled and parallel way to the nitrogen assimilation **-**is this the reason why plant nitrate reductase is one of the possible nitrite reductases/NO synthases? These circumstances make the "signalling" nitrite/NO metabolism more complex in plants, but also allow roles for nitrite not possible in mammals, like the maintenance of the cellular redox status under hypoxia (as will be discussed in section 3.2.1.4.).

The nitrite reduction/NO formation in plants has been ascribed to proteins such as the molybdenum-containing cytoplasmatic nitrate reductase (C-NaR) or the haem-containing non-symbiotic HG, Cc, CcO or cytochrome bc_1 (Complex III) (see references in the following sub-sections). Unexpectedly, the assimilatory CSNiR (responsible for the assimilatory nitrite reduction to ammonium) does not reduce nitrite to NO (see sections 2.1. and 4.1.2. for details about CSNiR; Fig. 1, orange arrows).

In order to restrict the scope of information presented to a manageable size, only a few proteins will

be here discussed: some were chosen due to their parallelism with the mammals and other because of their novelty. Therefore, only C-NaR, XO/XD, AO (molybdoenzymes), non-symbiotic HG (haemic proteins) and the plasma membrane-bound nitrite reductase and nitrate reductase (the novel "players") will be discussed (in sections 3.2.1.1.-3.2.1.4.), together with the protein-independent nitrite reduction (section 3.2.1.5.). In addition, also the nitrite oxidation by plants will be briefly described (section 3.2.2.).

3.2.1. Nitrite reduction in plants

3.2.1.1. Molybdenum-dependent nitrite reduction - cytoplasmatic nitrate reductase

The nitrite-dependent NO formation in plants, either *in vivo* or *in vitro*, has been ascribed mainly to the C-NaR. This enzyme is involved in the first and rate-limiting step of plant nitrate assimilation pathway (see section 2.1.; Fig. 1, orange arrows), where it catalyses the nitrate reduction to nitrite, with the simultaneous oxidation of NADH (eq. 51).[590,953-959] The C-NaR is a homodimeric molybdoenzyme, belonging to the sulfite oxidase family (see section 4.; Fig. 6-c); it holds (per monomer), besides the molybdenum centre, where the nitrate reduction takes place, one b_5 haem and one FAD centre that is involved in the NADH binding and oxidation. In accordance with its key role on the nitrogen metabolism, C-NaR is highly regulated by complex transcriptional, translational and posttranslational mechanisms (that respond to nitrogen, carbon dioxide and dioxygen availabilities, pH, temperature and light), being rapidly degraded (half-life of six hours in darkness).[953,955-957,959,960] Besides this well established role on the reduction of nitrate, C-NaR from different species were shown to also catalyse the subsequent nitrite reduction to NO (eq. 52), not only *in vitro*[914,961-963], but also *in vivo*, through studies with (i) transgenic plants expressing a permanently active[964] C-NaR[914,970-972], (ii) C-NaR knock-out mutants (*nia*1 and *nia*2 genes)[909,974-981], (iii) inactive C-NaR (*e.g.*, plants with tungstate supply instead of molybdate)[975,976,982-987], (iv) among others[907,929,930,988-998]. The nitrite reduction by C-NaR was also studied *in silico* and it was

found that both nitrate and nitrite are easily reduced (to nitrite and NO, respectively), although, as expected, nitrate is the preferred substrate.[999]

These two C-NaR activities **-**nitrate and nitrite reduction**-** seem to be controlled by the nitrate, nitrite and dioxygen concentration. (a) Under normoxic conditions, the cytoplasmatic nitrate availability (in millimolar range[1000]) "auto-controls" the nitrite reduction by C-NaR, because (i) nitrate competitively inhibits (K_i of 50μM [914,1001]) the nitrite reduction/NO formation and (ii) the nitrite concentration is 1-2 orders of magnitude lower than the respective K_m value ($\approx$100μM [914]; note that nitrite is promptly transported to and reduced by CSNIR on the leaves chloroplasts or roots plastids)[914]. (b) Under hypoxic and acidic conditions[1002], the C-NaR concentration and activity are increased[914,963,1017-1026], at the same time as the nitrite reduction by CSNiR is decreased, especially in hypoxic roots (due to the decreased pentose phosphate pathway-dependent NAD(P)H generation[914,1001,1017,1019-1021,1025, 1027]). As a consequence, when nitrate is the main nitrogen source, nitrite accumulates in hypoxic tissues[1017,1019,1020,1028,1029] and its reduction by C-NaR is progressively increased, leading to the NO formation[914,952,1017]. The same C-NaR "activity switch", from mere nitrate reductase to NO synthase activity, is observed upon nitrite accumulation[1030] triggered by inhibition of the photosynthetic activity[983,1032-1034], or by the expression of an antissense CSNiR (transgenic plants with very low CSNiR activity that, consequently, accumulate nitrite)[974,975,1035].

$$NO_3^- + NADH + H^+ \longrightarrow NO_2^- + NAD^+ + H_2O \quad (51)$$

$$NO_2^- + 1/2NADH + 3/2H^+ \longrightarrow {}^{\bullet}NO + 1/2NAD^+ + H_2O \quad (52)$$

In summary, when C-NaR activity is increased (to an extent that nitrite formation exceeds its rate of consumption by CSNiR) and/or nitrite accumulates to an extent that CSNiR could not cope with it, the nitrite reductase activity of C-NaR would become significant and the formation of NO is catalysed **-**C-NaR "activity switching".[914] The concept of doing "substrate adaptations" is, in plants, obvious and opportune: if plants are using C-NaR to abstract one oxygen atom from nitrate, why not to use the same redox system to continue the reaction and abstract the second oxygen atom?

Clearly, the amount of NO produced would be very low, predicted to be much less than 1% of the

nitrate reducing activity (due to competitive inhibition by nitrate).[914,975] However, as was discussed for mammals, the NO concentration should be kept very low, within the characteristics of a local signalling molecule. Obviously, and again as in mammals, the plant NO formation should be tightly controlled and it can be argued that the well known complex C-NaR regulation serves, not only to control the nitrogen assimilation, but also to regulate the formation of the signalling NO.

The C-NaR/nitrite-dependent NO formation has been suggested to be involved in (i) stomatal closure[909,919,976,1036,1037], (ii) immune defence mechanisms (similar to the mammalian inducible NOS, since pathogen signals induce the C-NaR and increase the NO formation[977,979,981,986,987,1038]), (iii) onset of germination[951] or (iv) in the phenylpropanoid metabolism[1039]. This enzyme is also suited to play a role as a cytoplasmatic nitrite sensor, to "signalise" the presence of toxic nitrite concentrations.[973] However, (once more) as suggested in mammals, nitrite in plants may be acting, not only as a NO source, but also as an oxygen sensor: it is intriguing that an enzyme that is rapidly degraded in darkness[973] is increased during hypoxia (that also leads to nitrite accumulation) **-**is this a coincidence or a strategy?

space (=blank line)

Before finishing this section, another C-NaR feature must be discussed: the dioxygen reduction to superoxide radical by C-NaR.[963,1040,1041] This reaction not only consumes the electrons needed to reduce both nitrate and nitrite, but also produces superoxide that scavenges the NO (hindering its physiological function) and forms the strong oxidising peroxynitrite (as was discussed for mammalian XO/AO). However, the NO formation by purified C-NaR was described to be rather insensitive to the presence of air[975], suggesting that the reduction of dioxygen would not compete with the nitrite reduction. In addition, the dioxygen reduction would also compromise the *in vivo* nitrate reduction under normoxia, what was not yet reported to occur. Surely, the relative extension of the nitrate reduction *versus* nitrite reduction *versus* dioxygen reduction will depend on the respective *in vivo* concentrations and kinetic specificity constants **-**which remain to be determined. If the oxygen reductase activity is significant *in vivo*, then oxygen would control the C-NaR at two time scales: short-term (determining the nature of the reaction products) and long-term (increasing/decreasing C-NaR activity and concentration).

3.2.1.2. Molybdenum-dependent nitrite reduction - xanthine dehydrogenase/oxidase and aldehyde oxidase

The molybdoenzyme XD is an important candidate to reduce nitrite to NO in plants. As the mammalian enzyme (see sections 3.1.1.3. and 4.2.3.), plant XD is involved in the purine catabolism and in the generation of reactive oxygen species[1042-1044], in the cytoplasm[1045] and in peroxisomes[1046-1048] (although its presence in peroxisomes is still controversial[1049]). However, the conversion of XD into a XO form is not common to all plants: while the enzyme from *Arabidopsis thaliana* does not have the two corresponding cysteine residues[1042] involved in the conversion mechanism of the mammalian enzyme (see section 4.2.3.), the pea leaf peroxisomal enzyme was described to exist mainly (70%) as a XO form[1046-1048]. (In fact, the conversion is also not common to all animals, as, *e.g.*, pigeon XD can not be converted into XO.) Besides the purine catabolism, plant XD has been suggested to be involved in a variety of challenging environmental conditions, where increased XD activities and reactive oxygen species production were observed, namely upon plant-pathogen interactions[1050-1052], hypersensitive response[1053], drought stress[1043] and natural senescence[1042,1054].

The fact that mammalian XD/XO is able to reduce nitrite to NO (section 3.1.1.3. and 4.2.3.) anticipates a similar role for the homologous plant enzyme. In accordance, *in vivo* inhibition studies (with allopurinol) have revealed a probable role for the enzyme in the formation of NO upon phosphate deficiency, in white lupin roots.[1055] In this context, other studies, planned to assess the *in vivo* C-NaR-dependent NO formation by inhibiting C-NaR with tungstate, or using cyanide, should be revaluated. Both tungstate and cyanide should inhibit XD/XO, by replacing the molybdenum atom and removing the sulfo group of the catalytic centre, respectively (see section 4.2.3.), besides interfering severely with the metabolism[1056]. Thus, the use of these and other non-specific inhibitors should be done with care: the observed decreased NO formation, attributed to C-NaR, might be due (also) to the inhibition of XD/XO. Nevertheless, the definitive establishment of plant XD/XO as a NO source must wait for the characterisation of the nitrite reductase activity of purified XD/XO **-**what was not yet

accomplished[1057].

Another relevant candidate to contribute to the plant NO formation is AO. Like the mammalian enzyme, the plant AO is a "strict" oxidase that catalyses only the reduction of dioxygen (not NAD^+)[1043,1058]. Initially, it was described to catalyse the formation of only hydrogen peroxide[1043], but a recent work demonstrated the expected superoxide radical formation[1059]. The plant isoenzymes (*Arabidopsis thaliana*, *e.g.*, contains four AO[1060-1063]) are, at least, (i) responsible for the oxidation of the abscisic aldehyde into the absicisic acid[1062,1064] (one plant hormone involved in development processes and in a variety of abiotic and biotic stress responses[1065-1067]) and (ii) were implicated in the biosynthesis of indole-3-acetic acid (an auxin phytohormone) during early stages of plant development[1060]. Its involvement in plant NO formation is still speculative **-**although promising.

3.2.1.3. Haem-dependent nitrite reduction - globins

HG[1068] are a large family of ancient haemic proteins with the globin fold (typically eight α-helices[1069]) that are widely distributed in all kingdoms of life. Besides the well known dioxygen transporters Hb, Mb and leghaemoglobin[1070], that use a pentacoordinated *b* haem to reversibly bind and transport dioxygen, the HG family also comprises **-**among many others**-** several hexacoordinated haemic proteins, including the recently discovered mammalian Nb and Cb (described in section 3.1.1.2.(a)), the cyanobacterium *Synechocystis* HG (section 3.3.) and the plant non-symbiotic HG[1077] (NS-HG), whose physiological functions remain a matter of debate. Herein, only class 1 NS-HG will be addressed (since it is the most studied one), although, most of the discussion could be extended to, at least, class 2 NS-HG.

Following the discussion on the mammalian hexa-coordinated HG, it would be expected that plant NS-HG would be able to reduce nitrite to NO upon conversion to a penta-coordinated state. Furthermore, as was described for mammalian Nb and Cb, also plant NS-HG have been found to be induced by low dioxygen concentrations, suggesting a cellular protective role during hypoxia[1079,1081,1088,1093, 1097,1111-1116]. However, and surprisingly, this nitrite reductase activity only

recently began to be explored, with the discovery that rice deoxygenated NS-HG (deoxy-NS-HG) reduces nitrite to NO (equivalent to eq. 13) with a remarkable rate constant of $83 M^{-1}s^{-1}$, at pH 7, under anaerobic conditions.[1117] The pH effect was not yet thoroughly explored, but it is expected that the rate further increases as the pH value decreases. Given the (i) low affinity constant for coordination by the distal histidine ($K \approx 1\text{-}2$ [1090,1091]), that dictates the formation of a high fraction of "open" penta-coordinated molecules, and because plants (ii) can be often subjected to extreme hypoxia/anoxia (see[1002]), (iii) during which nitrite accumulates, (iv) the pH values decrease, (v) NS-HG are induced and (vi) can become deoxygenated, the nitrite reductase/NO synthase activity of plant NS-HG can be of physiological relevance.

Nevertheless, and as expected, the NO formed can be rapidly trapped by deoxy-NS-HG (equivalent to eq. 15)[1117] or be oxidised by oxygenated NS-HG (oxy-NS-HG; equivalent to eq. 16). In fact, in the literature, plant NS-HG are mentioned, mainly, as NO scavengers responsible for the NO oxidation to nitrate[993,1075,1087,1099,1101,1103,1111,1114-1116,1118-1130]. The rate constants towards dioxygen ($k_{on} \approx 60\text{-}70 \mu M^{-1}s^{-1}$ and $k_{off} \approx 0.04\text{-}0.2 s^{-1}$ [1088,1092-1094] that results in a global oxygen affinity (2-3nM) two orders of magnitude higher than the CcO one) suggest that these NS-HG remain oxygenated even at extremely low oxygen concentrations[1092,1094], thus supporting their role as NO scavengers (and not as deoxy-NS-HG-dependent nitrite reductases). Also the observation of reduced NO formation in plants overexpressing NS-HG supports this role.[1114,1121] In this respect, it is interesting that NS-HG are also induced by conditions where the NO formation might be increased, namely by nitrate, nitrite or by NO itself[1124,1125,1129,1131]. This up-regulation is more in line with a scavenger role, *i.e.*, defence against the deleterious excessive NO formation (that generates reactive nitrogen and oxygen species and also interferes with the "normal" NO signalling) than with a NO synthase role. Noteworthy, also the hypoxia-mediated induction of NS-HG could be related with the NO accumulation that is known to occur under low dioxygen stress. Accordingly with these evidences, NS-HG would be key players of NO homeostasis, acting in a similar way to the one described for mammalian oxy-Hb and oxy-Mb (section 3.1.1.1.), thus, preventing unwanted NO effects.

But, as reasoned above, the nitrite reductase/NO synthase activity of NS-HG can also be of physiological relevance. Hence, it is plausible that, *in vivo*, a delicate balance takes place between NO scavenging and NO formation, as was exemplified for Mb, that was suggested to "translate" the

mismatch between the oxygen supply and consumption into an increased NO flux, when the dioxygen concentration decreases (end of the section 3.1.1.1.). However, in the plant NS-HG case, the reaction should be shifted towards lower dioxygen concentrations (because NS-HG become deoxygenated at lower oxygen concentrations) and the fluxes of NO should be higher (as the rate constant and available nitrite concentrations are higher).

In addition to (i) control the (physiological) NO homeostasis, (ii) prevent the (pathological) deleterious effects of excessive NO formation and (iii) generate NO under extreme hypoxia/anoxia, the plant NS-HG can (iv) contribute to decrease (consume) the nitrite that can accumulate to dangerous concentrations during hypoxia/anoxia, not only through its reduction, but also via its hypothetical oxidation (equivalent to eq. 14), and (v) maintain the cellular redox status under hypoxia and anoxia (the last point will be addressed in section 3.2.1.4.).

space (=blank line)

Crucial for all the above (catalytic) roles of plant NS-HG is the protein conversion to an "open" penta-coordinate state, as was discussed for the mammalian hexa-coordinated Nb and Cb (and also Cc). In accordance, it is of major importance to characterise potential mechanisms that control the conversion of hexa- into penta-coordinated states. Those mechanisms would dictate if NS-HG are posttranslationally redox/allosteric-regulated nitrite reductases, as the mammalian hexa-coordinated haemic proteins are believed to be. Also decisive for the *in vivo* significance of the NS-HG reactions (as is for all the others HG) is the existence of regenerating systems that efficiently re-reduce the oxidised proteins. In plant roots, one of the most plausible systems comprises the enzyme monodehydroascorbate reductase and NADH (at least in barley, with K_m (NS-HG)=0.3μM).[1101,1126] Nevertheless, this system is believed to be slow to account for a "catalytic" re-reduction of NS-HG[1117] and the identification of more efficient systems are one of the challenges for future research. It is also essential to study (and compare) the nitrite reductase activity of the other plant HG. Not only of class 2 and 3 NS-HG, but also of leghaemoglobins, that are penta-coordinated, present in higher (millimolar) concentrations and could display a behaviour more in line with the Mb one (section 3.1.1.1.). To conclude, also the question of how the nitrite-dependent NO avoids the *dogmatic* haem scavenging, to be able to fulfil is signalling role, has to be answered in the plant kingdom.

space (=blank line)

In the context of the plant haem-dependent nitrite reduction, it is noteworthy that Cc should also be a physiologically relevant nitrite reductase in plants.

3.2.1.4. *New* pathways - plasma membrane-bound nitrite reductase

Plants are also able to produce nitrite-dependent NO in root apoplast, through the recently identified root specific, plasma membrane-bound (apoplast-faced) nitrite reductase (RPM-NiR). This novel enzyme catalyses the formation of NO from nitrite using a not yet identified physiological electron donor[1132,1133]; its activity is maximal under the acidic (pH 6) conditions characteristic of hypoxia and is reversibly inhibited by dioxygen[1132,1134]. Most important, it can account (and exceed (500nmol/g FW/h [1116])) for the NO formation rates observed under hypoxic conditions (10-50nmol/g FW/h [914,1114]). The RPM-NiR was suggested to act in concert and in tight association with the root specific, plasma membrane-bound, succinate-dependent, nitrate reductase (RPM-NaR)[1023,1132-1138], which (due to its localisation on the apoplastic side of the plasma membrane) is assumed to catalyse the *in vivo* reduction of apoplastic nitrate to nitrite.

The root apoplastic nitrite-dependent NO formation might, thus, be one of the primary signals that report the presence of nitrate in roots.[1133] For the same reasoning, the toxicity cause by high nitrate concentrations might be the result of increased NO formation. In addition, it may also act as an oxygen sensor (since RPM-NiR-dependent NO formation is reversibly inhibited by dioxygen).[1132,1134] Furthermore, a role in root developmental[1139] or in regulation of mycorrhizal inoculation[1140] were also envisaged.

Nonetheless, the root apoplastic nitrite might have another function. The root, the organ that provides nutrients and water to the whole organism, is commonly subjected to hypoxia (see[1002]). To ensure short-term cell viability under hypoxic conditions, plants promote the glycolysis and fermentation pathways, to synthesise ATP and regenerate NAD^+. However, presently there is evidence that an additional pathway, nitrate/nitrite-mediated, may be important for plant survival under hypoxic

conditions.[993,1114,1115,1120] In fact, it has long been known that the presence of nitrate increases the tolerance to flooding[1141-1144], allegedly (and still controversial) because nitrate assimilation (reduction to ammonium; see section 2.1.; Fig. 1, orange arrows) consumes four moles of NAD(P)H (eight electrons) and six protons, thus being more efficient at regenerating NAD^+ and consuming protons than any of the fermentation reactions[1009,1018,1025,1116]. But the NO formation by the sequential activity of RPM-NaR and RPM-NiR puts forward the hypothesis that the known beneficial nitrate role might be due to the operation of a new cycle, involving nitrate/nitrite/NO, that actively recycles NADH (Fig. 4).[1116] The C-NaR is also suggested to contribute to this cycle, although its activity in the roots is believed to be lower than the RPM-NaR one.[1023,1116] The cycle is proposed to be closed by a hypoxia-induced oxygenated class 1 NS-HG, which, due to its high oxygen affinity (2-3nM), remains oxygenated even at extremely low oxygen concentrations (see section 3.2.1.3. for details about these proteins). The NS-HG would oxidise the NO to nitrate (equivalent to eq. 16) and would be, subsequently, recycled by a reductase (Fig. 4).[1116,1123] Because the RPM-NiR activity is controlled by the dioxygen concentration, as well as the induction of the NS-HG, the metabolic flux through the cycle would be limited to hypoxic conditions, not compromising the nitrogen assimilation (nitrate reduction to ammonium) under normoxia. This cyclic pathway is suggested to oxidise 2.5 NADH molecules per nitrate molecule recycled, helping, in this way, to maintain the redox status of the cell along with the fermentation pathways[1116,1123]. Hence, in plants, nitrite may play a *new* role: contribute to the oxidation of NAD(P)H under hypoxia and, ultimately, maintain the cellular redox and energy status.

In this context, it is noteworthy that nitrite might also be involve in another alternative pathway that would enable plants to maintain the ammonium production and/or ATP generation even under anoxia (< 1nM).[1145] In accordance to a recently suggestion[1145], under anoxia, plants would be able to reduce nitrite to ammonium through three individual steps (eq. 53) that would replace the normoxic one-step CSNiR-catalysed reaction (see section 4.1.2. for details about this reaction). The last of these three steps was suggested to be catalysed by deoxy-NS-HG that were shown to reduce, not only nitrite to NO (section 3.2.1.3.), but also the hydroxylamine to ammonium ($25 mM^{-1}s^{-1}$).[1145] The reasonability of an haem to catalyse the hydroxylamine reduction to ammonium is supported by the known reactivity of the *c* haem of CcNiR, sirohaem of CSNiR (sections 4.1.1. and 4.1.2.) and *b* haem of Hb and

Mb.[1145-1047] Nevertheless, this alternative pathway raises two important questions: how the NS-HG transfers the two electrons needed to reduce the hydroxylamine molecule and how is the three-electrons reduction of NO carried out. In summary, this alternative nitrite-mediated pathway would remove toxic nitrogen metabolites, at the same time as it would act as an electron sink (as the cyclic pathway above mentioned), by coupling the reducing power produced during anaerobic glycolysis to the ammonium production.[1145] Globally, both proposals suggest that under hypoxia, nitrogen oxides and oxo-anions are essential metabolites to reduce the NAD(P)H concentrations and maintain the ATP/ADP ratio sufficiently high to enable plants to survive.

$$NO_2^- \xrightarrow{(1e^-)} {}^{\bullet}NO \xrightarrow{(3e^-)} NH_2OH \xrightarrow{(2e^-)} NH_4^+ \qquad (53)$$

space (=blank line)

The increasing number of evidences that root apoplastic nitrite/NO may play important roles *in vivo* demand for a deeper knowledge of the two root specific RPM-NaR and RPM-NiR enzymes. Unfortunately, the present data is still limited (and raises more questions than answers). The RPM-NiR is believed to be different from the CSNiR and to share with RPM-NaR (i) the electrons source (succinate, probably via the quinone pool of the plasma membrane) and (ii) the regulatory mechanisms (activity dependent on nitrate supply and inhibition by dioxygen).[1134] The RPM-NaR has also been described to be different from the known NaR of higher plants.[1138] Instead, there are evidences that it might be similar to the prokaryotic "respiratory" membrane-bound nitrate reductases NaRGHI (see section 4.3.1. for details about these enzymes).[1148] Recently, the successful RPM-NaR extraction (from *Hordeum vulgare*) indicated that this protein is remarkably similar in sequence to the prokaryotic NaRH subunit. Interestingly, NaRH is not the subunit responsible for nitrate reduction (instead, it arbores four Fe/S centres responsible for electron transfer). Furthermore, a data base study suggested that *Populus trichocarpa* might hold homologous NaRH (75% identity) and NaRG (68% identity) peptides, although the existence of NarI-related peptide in plants remains questionable.[1148] This alleged similar structure and localisation of RPM-NaR puts forward the hypothesis of a similar function, that is, the hypothesis that higher plants are able to

carry out part of the denitrification pathway (Fig. 1, blue arrows; section 2.2.).[1148] This polemic hypothesis raises the possibility that nitrate/RPM-NaR/nitrite/RPM-NiR might be able to contribute to the ATP synthesis under hypoxic/anoxic conditions **-**one unprecedented role for nitrite in higher organisms, as a "respiratory" substrate! Such hypothesis, however, must wait for the future evaluation of the hypothetical ability of nitrate/nitrite reduction to create a proton motive force at root plasma membranes.[1148]

3.2.1.5. Other nitrite reduction reactions

In addition to the nitrite reduction reactions so far discussed, plant could also rely on a protein-independent NO source. As previously discussed (section 3.1.1.4.), the non-proteic NO formation relies on the nitrite decomposition to dinitrogen trioxide (eq. 31→32), which can, then, dismutate to NO and nitrogen dioxide radical (eq. 23) or be converted to a RSNO (eq. 24), in an overall reaction that is highly pH and reducer-dependent. The plant protein-independent nitrite reduction was suggested to contribute to the NO formation on the roots and seeds apoplast[908,1149,1150]. The acidic pH[1151-1153], the availability of nitrite in soil and the presence of reducing antioxidants (like ascorbate and phenolic compounds[1150,1154]), suggested that the apoplast could be involved on the NO generation in those tissues. In addition, this protein-independent NO formation could occur at microlocalised acidic environments, as in the chloroplast.[1154]

space (=blank line)

Besides the apoplast, plasma membrane, cytoplasm, also mitochondria, peroxisomes and chloroplasts have been found to generate nitrite-dependent NO although the proteins responsible for the catalysis were are not yet identified.[952,996,1057,1155-1158]

3.2.2. Nitrite oxidation in plants

Plants also handle nitrite to oxidise it to nitrate and nitrogen dioxide radical using haemic proteins with peroxidase-like activity **-**once more a "substrate adaptation" to the haem redox chemistry of haemic proteins already present in cells to accomplish other functions. Following the discussion on the mammalian nitrite oxidation, it would be expected that, at least, plant peroxidases, HG and Cc would behave as "non-dedicated" nitrite oxidases, although this oxidative chemistry has been (comparatively) poorly explored (see section 3.1.2. for details about these oxidative reactions that will not be here repeated). The horseradish peroxidase-mediated nitrite oxidation reaction is one exception. Nitrite was found to be readily oxidised by this plant peroxidase][804,806,821,822,1159-1162], in the presence of hydrogen peroxide (equivalent to eq. 37-38), with a rate constant similar to the myeloperoxidase one ($k \approx 7 \times 10^5 M^{-1}s^{-1}$ (pH 6.9)[1159]), but limited by the slow reaction with the peroxidase compound II (equivalent to reaction 39; $k \approx 7 M^{-1}s^{-1}$ [1160]). Hence, only in the presence of a "fast substrate" (*e.g.*, a phenolic compound; $k \approx 10^3$-$10^6 M^{-1}s^{-1}$ [1160]) to reduce compound II (and regenerate the enzyme), is the nitrite oxidation catalytically efficient. In addition, also a number of plant HG with peroxidase-like activity are shown to be able to oxidise nitrite and mediate protein nitration, at least *in vitro* .[1163]

3.2.3. Plant nitrite handling - concluding remarks

In summary, the nitrite reduction to NO in plants could be accomplished by diverse metalloproteins, with different cellular roles and tecidular and subcellular localisations, suggesting that nitrite-dependent NO performs specific functions in the various subcellular compartments that, perhaps, need to be independently orchestrated. In spite of the diversity, all the pathways reviewed depend on hypoxic/anoxic conditions to operate and, as was discussed for mammals, it is possible that *each* individual pathway is activated when the dioxygen concentration decreases below *its own threshold* of oxygen-dependent activity; in this way, all pathways would act in a concerted and self-regulated manner, with each pathway being relevant under different conditions

and in different tissues. Thus, in plants, like in mammals, nitrite could act not only as a source of the signalling NO, but also as an oxygen sensor. Nevertheless, due to the nitrite specific function in plants, as a ordinary metabolite of nitrogen assimilation, the control of its CSNiR-independent reduction has to be more complex (or at least different) than the mammalian one.

To reduce nitrite to NO, higher plants and mammals share some strategies: both do "substrate adaptations" to previously existent redox systems, depending on proteins that are not "normally" committed to synthesise NO. The C-NaR is a remarkable example of such "activity switching" to obtain a "new" NO synthase from an "old" (previously existent) protein. For this reason, it would be very interesting to understand if plant XD/XO and AO are also able to generate NO. The NS-HG (although their physiological functions remain a matter of debate) seem to represent another example. But plants use also different, unique, strategies to handle nitrite: the RPM-NaR and RPM-NiR enzymes constitute a new and promising pathway, not only for NO synthesis, but also for nitrite-dependent NADH "recycling" under hypoxia/anoxia.

The major challenge in plants is, undoubtedly, to attain the same level of knowledge we now have about mammalian NO formation (and signalling) pathways, focusing on (i) the characterisation of the pathways so far identified and on potential new ones (inspired by the mammalian ones) and (ii) on how those different pathways could act in a concerted manner to produce the signalling network observed in plants.

Regarding the first point, the study of NO formation by plant proteins (both nitrite-dependent and independent) is still a new field: most of the enzymatic reactions were not characterised, neither kinetically nor at molecular level, and the great majority of studies relied on the use of inhibitors (most not specific at all) and on genetic manipulation (that, besides silencing the target enzyme, also extensively alters the metabolism, as is observed with C-NaR knock-out mutants). The "bottle-neck" here is, certainly, the capacity to obtain the purified proteins in sufficient quantities and the development of new, more specific, *in vivo* assays. In this point, the most imperative questions are, perhaps, related with the molecular composition and chemistry behind the RPM-NaR and RPM-NiR reactions and the expected ability of XD/XO and AO to form NO. Also the characterisation of the reaction mechanism, at a molecular level of detail, of C-NaR (a sulfite oxidase family member) is crucial to our understanding of the molybdenum-dependent reactivity towards

nitrite. In addition, although outside of the scope of this paper, it is clear that the search for a higher plant NOS will continue. Presently, it seems that higher plants have lost the specific NOS in the course of evolution. Is its activity carried out using a different chemistry and, consequently, a protein not related to NOS?

Concerning the second point, the questions are more numerous and exciting. How plants regulate the nitrite-dependent NO signalling in the presence of a variable nitrate (the nitrite source) supply? Why to have two NO forming nitrate reductases, one in the cytoplasm and another facing the apoplasm? Are plants really doing denitrification?

space (=blank line)

Nitrite/NO metabolism in humans is becoming increasingly important, offering innovative therapeutic approaches. The plant nitrite/NO metabolism is also promising. The future world will be characterised by increasing concentrations of carbon dioxide, global warming and the occurrence of extreme meteorological phenomena. In such scenario, the knowledge of plant nitrite/NO metabolism would, certainly, be essential for understanding and managing crop productivity.

Since the Industrial Revolution, the carbon dioxide concentration in the atmosphere has increased *ca* 40% and predictions are that it may double by the end of the XXI century.[1164] Plants could mitigate this increase through its photosynthetic assimilation, but it was shown that the elevated carbon dioxide inhibits the assimilation of nitrate into organic compounds[1164-1169] **-**explaining the inability of many plants to sustain rapid growth under elevated carbon dioxide. Nitrate is the most abundant form of inorganic nitrogen in agricultural (temperate well aerated) soils[1170] and, if plants are not able to assimilate it, crops will become depleted of organic nitrogen compounds and, thus, compromised.[1167] Therefore, the relative availability of soil nitrate and ammonium will be crucial to determine the crops productivity and the food quality in the near future. However, if the nitrate assimilation decline is already being studied in several plants[1164,1169], the effects on the generation of NO were overlooked and, plausibly, NO is a key molecule on the response to increased carbon dioxide. Furthermore, to understand how plants respond to the soil available nitrogen form, the NO metabolism has, certainly, to be considered. Also under extensive flooding, the NO should be involved in crop productivity, since the oxygen availability influences nitrite-dependent NO formation, which, in turn, controls "respiration" in plant mitochondria.[1171-1173]

3.3. NITRITE ON SIGNALLING (AND OTHER) PATHWAYS IN BACTERIA

In prokaryotes, the NO formation had for long been thought to occur only in denitrification (Fig. 1, blue arrows; section 2.2.), where the anaerobic reduction of nitrogen compounds is used to derive energy. More recently, NO was found to take part in other dissimilatory pathways that "copy" the first steps of denitrification (Fig. 1, gray and violet arrows; section 2.3.). Therefore, on the organisms that carry out those pathways, NO is an ordinary intermediate metabolite, *i.e.*, a reaction product and substrate of a "respiratory" pathway. In addition, NO is also as a signalling molecule that activates the genes required for its own anabolism/catabolism (a common regulatory strategy in Biology) through, *e.g.*, regulators of the FNR family (in *Pseudomonas*, *Paracoccus* and *Rhodobacter*).

However, presently, it is clear that the prokaryotic NO is also involved in "non-respiratory" pathways, *e.g.*: (i) in the biosyntheses of secondary metabolites (nitration of tryptophane in *Deinococcus radiodurans*[1174] and of the tryptophanyl moiety of thaxtomins (plant toxins) in *Streptomyces turgidiscabies*[1175-1177]), (ii) regulation of recovery from radiation damage (of the radiation-resistant *Deinococcus radiodurans*)[1178], as well as in (iii) cytoprotection against oxidative stress (in *Escherichia coli*, *Bacillus subtilis*, *Bacillus anthracis*, *Staphyloccuos aureus*, through different mechanism, such as activation of catalase, inhibition of the Fenton chemistry by S-nitrosation of reduced thiols and through transcription factors like OxyR, SoxR, Fur or FNR[1179-1184]). In this context, it is intriguing that NO is important for the pathogen survival[1184,1185] and, simultaneously, for the host defence (phagocytes produce reactive oxygen and nitrogen species to damage pathogens and protect themselves from infection) **-**clearly, there is still much to be learned about host/pathogen interactions.

In prokaryotes, the aerobic NO formation is catalysed by enzymes homologous to the oxygenase domain of the mammalian NOS (see section 3.1.1.), but lacking the reductase domain.[1175,1186-1196] These prokaryotic enzymes successfully reduce arginine to NO, using cellular reducing equivalents

that are not normally committed to the NO production.[1191,1196,1197] The *Sorangium cellulosum* NOS is an exception, since the enzyme holds a "fused" reductase domain (even though with a different domain organisation and holding an Fe/S).[1198,1199] Interestingly, the prokaryotic NOS is found only in a subset of bacteria, mostly gram-positive (*Exiguobacterium*, *Staphylococcus*, *Geobacillus*, *Bacillus*, *Rhodococcus*, *Streptomyces* and *Deinococcusus*), but also gram-negative (*Sorangium cellulosum* [1198]), and in an archeon (*Natronomonas*)[1177,1178,1184,1190,1191,1194,1196,1200,1201] -rising the questions of why are not prokaryotic NOS widespread and what is the evolutionary relationship between the animal and prokaryotic proteins? (Note that plants do not seem to have a NOS enzyme.)

Nevertheless, bacteria are able to synthesise NO in a NOS-independent manner, through (once more) the nitrite reduction, therefore expanding the formation of NO to other prokaryotic organisms. *Escherichia coli* and *Salmonella enterica* are two (long known) examples of bacteria that, not having a NOS enzyme, are able to produce NO when grown under nitrate "respiring" (anaerobic) conditions.[1202-1207] Also *Microcystis aerugrinosa* (a cyanobacterium that usually cause cyanobacterial blooms)[1208] and *Bacillus vireti* (whose genome indicates that it carries out DNRA; Fig. 1, green arrows) are able to generate nitrite-dependent NO. In addition, also known NOS holder organisms may rely on the nitrite reduction to produce NO (*e.g.*, in the NOS mutant of *Streptomyces*, a small amount of thaxtomin is still produced[1175-1177]). However (and as was discussed for plants), this prokaryotic nitrite-dependent, "non-respiratory", NO formation should be carried out in a tightly controlled and parallel way to the nitrogen assimilation and/or dissimilation.

Until recently, the nitrite-dependent NO formation was assumed to arise from the activity of CSNiR and CcNiR (see sections 2.1-2.2. and 4.1.1.-4.1.2.), because studies with mutants suggested that both enzymes would be largely responsible for the NO production.[1207,1209] However, no NO generation could be observed with purified enzymes (as will be discussed in sections 4.1.1. and 4.1.2., both enzymes catalyse the nitrite reduction to ammonium without releasing any intermediates). Instead, both enzymes were proposed to catalyse the NO reduction, as a detoxification process[1210-1212.] (see also section 4.1.1.2.).

Concurrently, other studies suggested that the nitrite-dependent NO formation is due to the nitrite reduction by NaR under anaerobic, nitrate-rich conditions, upon nitrite accumulation.[1202-1206,1208,1213-1218] (Prokaryotes use nitrate for dissimilatory and assimilatory processes (section 2.1.-2.2.) and, for

those purposes, hold three types of NaR enzymes, "respiratory" membrane-bound NaR, periplasmatic NaR and assimilatory cytoplasmatic NaR, all of which will be described in section 4.3.1..) Presently, there are an increasing number of studies showing that most of the NO is formed by the "respiratory" membrane-bound NaR[1218-1220], with the periplasmatic NaR contributing very little to the bacterial NO (less than 3%)[1219,1220]; the hypothetical contribution of the assimilatory cytoplasmatic NaR was not yet investigated. Furthermore, an *in silico* study supported the feasibility of "respiratory" NaR to catalyse the nitrite reduction to NO (with an energy barrier of ≈15kcal/mol).[999]

The NO formation by the "respiratory" NaR would depend on a combination of anaerobic, nitrate-sufficiency and nitrite accumulating conditions, which would, not only promote the reaction, but also induce the enzyme expression.[47,1218,1220] Remarkably, these conditions are similar to the ones described for the plant C-NaR-dependent NO generation (in the previous section). As in C-NaR, nitrate competitively inhibits the "respiratory" NaR-catalysed nitrite reduction (*e.g.*, *S. enterica* specificity constant for nitrite is ≈150 times lower than for nitrate[1220]) and the nitrite reduction is promoted only when the nitrate concentration decreases and nitrite builds up (nitrite K_m value in the millimolar range[1220]). In accordance, under nitrate-limited growth conditions, when both nitrate and nitrite are present at low micromolar concentrations and the expression of "respiratory" NaR is repressed, the NO formation is very low.[1220] Nevertheless, the extension of the prokaryotic NO synthesis seems to be dependent on the organism (and, probably, on the NO role), with the *E. coli* NO generation estimated to be less than 1% [1203,1219], while the *S. enterica* NO formation can account for up to 20% [1221] of the nitrate reduced.[1020]

The similarities in the nitrite-dependent NO formation by plant C-NaR and bacterial "respiratory" NaR are noteworthy. Furthermore, also the fungus *Aspergillus* NaR was shown to be able to reduce to nitrite to NO.[991] This similar activity suggests that the nitrite reduction/NO formation could be a general feature of all types of NaR enzymes. In this respect, it is intriguing why the bacterial periplasmatic and assimilatory NaR would not be able to catalyse the nitrite reduction. It can be argued that the sulfur-rich coordination of molybdenum centre of periplasmatic NaR (see sections 4.3.1. and 4.; Fig. 6-c) hampers the nitrite reduction. However, definitive conclusions must wait for kinetic and spectroscopic characterisation of the purified enzymes (the studies cited above were carried out with NaR mutants; the hypothetical NO formation by purified periplasmatic and

cytoplasmtic NaR was not yet kinetically characterised neither studied *in silico*).

space (=blank line)

Although the "respiratory" NaR is presently believed to be the major source of nitrite-dependent signalling NO, other sources remain to be identified. Due to the parallelism with mammals, two NO sources will be described below, an HG and a XO family enzyme member.

The deoxygenated hexa-coordinated HG are believed to be relevant NO sources (see sections 3.2.1.3. and 3.1.1.2.(a) for details about this protein family) and the protein from the cyanobacterium *Synechocystis*[1222,1226] was shown to reduce nitrite to NO with a remarkable rate constant of $68 M^{-1}s^{-1}$, at pH 7, under anaerobic conditions[1117] **-**together with NS-HG plant, these are the fastest HG-dependent nitrite reductases described so far. The significant rate of nitrite reduction, combined with a high (millimolar) available nitrite concentration (due to the nitrogen metabolism of this photosynthetic autotroph), makes this hexa-coordinated HG a potentially relevant NO synthase under hypoxic/anoxic conditions. Nonetheless, as other HG (those here described and, *e.g.*, the well documented bacterial flavohaemoglobin[1227]), this bacterial protein is also a potential NO scavenger[1117], whose physiological relevance is related, among other possible roles, with the organism defence against the oxidative and nitrosative bursts from the immune systems of their hosts[496,1228,1229]. The discussion of the bacterial HG reactivity towards nitrite, NO and dioxygen, as well as of its physiological relevance, follows the same lines as the other hexa-coordinated HG (sections 3.1.1.2. and 3.2.1.3.), in particular those of the plants (also autotrophs) and will not be here mention.

The XO family member aldehyde oxidoreductase (AOR) can also contribute to the bacterial NO formation. AOR was first described by Moura *et al.*[1230] and is believed to be an aldehyde scavenger, acting in a complex chain of electron transfer proteins that links the oxidation of aldehydes to the reduction of protons.[1231] AOR is a molybdoenzyme belonging to the XO family (Fig. 6-c), structurally similar to the mammalian XO and AO, even though lacking the FAD domain and holding a slightly different molybdenum centre (see section 4.2.3. for details). As the mammalian enzymes, AOR was recently shown to catalyse the nitrite reduction to NO.[623] Once again, the amount of NO produced would be dependent on the accumulation of nitrite (K_m value in the millimolar range[623]) and is estimated to be low ($k^{app} \approx 13 M^{-1}s^{-1}$ [623]). In addition, the physiological relevance of this bacterial NO formation pathway would depend on the competition between nitrite and the physiological

oxidising substrate, flavodoxin.[1232] Overall, the AOR-dependent NO formation would be controlled by the cellular redox status (that determines the flavodoxin redox state, as well as of other proteins involved in the "respiratory" pathways) and by the availability of nitrite. Accordingly, the following cytoprotective pathway against oxidative stress could be suggested: under normal conditions, the electron transporters involved in the "respiratory" pathways would be reduced and the AOR aldehyde oxidising activity would be coupled with the reduction of protons; in a situation of oxidative stress, as the proteins began to become oxidised and the "respiratory" pathways began to be disrupted, the nitrite would accumulate and AOR could link the aldehyde oxidation to the nitrite reduction. The NO, thus, formed could, subsequently, participate in potential signalling cascades "designed" to protect the organism from the oxidative stress damage. In this way, this hypothetical mechanism would allow the bacteria to "translate" a situation of oxidative stress (a change in the cellular redox status) into a differentiated NO flux that would be, subsequently, "translated" into a biological defensive response.

The nitrite reduction by bacterial NaR and AOR, fungus NaR, plant C-NaR and mammalian XD/XO and AO suggest that all forms of life can use a molybdoenzyme when they need to produce NO for other purposes than "respiration". This *consensus* emphasises the relevance of the molybdenum chemistry to catalyse the oxygen atom abstraction from nitrite and suggests that it is worth investigating the possible role of bacterial XD in nitrite reduction.

4. BIOLOGICAL MECHANISTIC STRATEGIES TO HANDLE NITRITE

Nitrite participates in several different metabolic pathways, with remarkably different biological purposes (sections 2. and 3.). From a mechanistic point of view, these pathways can be better thought as a series of nitrogen compounds, with oxidations states ranging from 5+ (nitrate) to 3- (ammonium), that are interconverted **-**oxidised and reduced**-** by several metalloenzymes, as is represented in figure 5. The nitrite oxidation involves the abstraction of one electron (to form $^{\bullet}NO_2$) or the addition of one oxygen atom (to form NO_3^-), while the reduction can be a "simple" abstraction of one oxygen atom (to yield NO), or a "complex" abstraction of both oxygen atoms with the addition of four protons (to NH_4^+). To carry out these distinct reactions, Biology developed several strategies, exploring different nitrite binding modes (Fig. 7) and using, in the great majority of cases, haemic iron and molybdenum, but also copper (Fig. 6).

Haem is one of the most important and most employed cofactor groups in Biology, participating in electron transfer, catalysis, sensing and transport of small molecules.[9,1233,1234] One haem consists of an iron atom coordinated by four equatorial nitrogen atoms of a porphyrin ring (Fig. 6-a) and by one or two axial ligands. The number of the axial ligands is dictated by the haem function. For electron transfer (*e.g.*, "respiratory" Cc), the haem has two axial ligands from the protein, typically histidine and/or methionine residues, leaving no vacant coordinating position ("closed" haem).[1235,1236] For small molecules transport, the haem is coordinated by only one amino acid residue (*e.g.*, by a histidine residue in Hb or Mb), leaving one "open site" for binding (in the example, of dioxygen).[9,1233] The same situation is (usually) found in catalysis, where a water/substrate molecule occupies the sixth coordination position (water is a weak ligand that can be easily replaced by the substrate) **-**the "ready" enzymes, accordingly to the nomenclature of Moura *et al.*.[1236] However, there are some enzymatic active sites where the haems are hexa-coordinated (*e.g.*, bacterial cytochrome *c* peroxidase[1237-1239] or Cd_1NiR (see section 4.2.1.)) and need an activation step to loose an axial ligand to become penta-coordinated **-** the "unready" enzymes.[1236] The same situation is found on the Nb or Cc (sections 3.1.1.2.). As was and will be discussed, the existence of "unready" proteins can be explored by Biology as a regulatory strategy to control the cellular metabolism. The nature of the fifth protein-derived haem axial ligand seems to be dependent on the activity type of the haemic enzyme: cytochromes P_{450} [1240] and NOS[1241] have a cysteine,

whereas peroxidases have a histidine[1242] and haemic catalases have a tyrosine residue[1243]. But there are also some enigmas to solve, like the unusual lysine coordination present on CcNiR (see section 4.1.1.). The haem great versatility is also conferred by the structure of the porphyrinic ring (Fig. 6-a), as is well exemplified by the reactivities with nitrite of CcNiR (*c* haem: forms and releases NH_4^+), Cd_1NiR (d_1 haem: forms and releases NO) and Mb (*b* haem: forms and bounds NO).

Copper is not so widely used, but it also plays crucial roles for the organisms survival (*e.g.*, in oxidative phosphorylation (CcO) or antioxidant defence (superoxide dismutase)) and is employed in one of the nitrite reductase enzymes **-** the CuNiR (section 4.2.2.). This metal participates in redox reactions, either in "simple" electron transfers (*e.g.*, plastocyanin or pseudoazurin) or in catalysis (*e.g.*, activation and reduction of dioxygen, superoxide radical, nitrous oxide and (of course) nitrite). A copper centre consists of a copper atom coordinated by nitrogen, oxygen or sulfur atoms from different amino acid residues. According to their geometry and electronic structure, the copper centres have been divided into several groups: (i) type 1 (T1; Fig. 6-b), where the copper is coordinated by two histidines, one cysteine and a variable axial ligand (*e.g.*, a sulfur atom of a methionine residue, as will be described for the CuNiR T1 centres); (ii) type 2 (T2), with no sulfur atom coordination (*e.g.*, the three histidines and water/nitrite molecule of the CuNiR T2 centres); (iii) type 3, with coupled binuclear copper centres[1244-1247]; (iv) trinuclear copper clusters (containing type 2 and 3 centres)[1248-1250]; (v) mixed-valence binuclear Cu_A centre[1251-1255]; (vi) heteronuclear Cu_B-$haem_A$ center (CcO)[1256]; (vii) and binuclear Cu_Z center (nitrous oxide reductase)[1257-1260].

Molybdenum is essential to most organisms **-**including humans**-** catalysing important redox reactions of the metabolism of carbon, nitrogen, and sulfur (many of which constitute critical steps in the global biogeochemical cycles of those elements).[586,1261-1266] With the exception of the iron/molybdenum cofactor of nitrogenase (involved in the dinitrogen fixation (section 2.1.; (Fig. 1, yelow arrow)) and a few other heteronuclear centres (whose physiological function is not yet fully understood)[1267-1269], molybdenum is found in a mononuclear form **-**hereafter designated as molybdenum centre. In these centres, molybdenum is coordinated by the *cis*-dithiolene group of one or two pyranopterin cofactor molecules (Fig. 6-c(i)) and by oxygen, sulfur or selenium atoms in a diversity of

arrangements that determinates the classification of the molybdoenzymes into three families[586]: XO, sulfite oxidase and dimethylsulfoxide reductase families (Fig. 6-c(ii)). (In addition, a fourth family might be created to hold a molybdenum-containing enzyme recently described in mammals, the mitochondrial amidoxime-reducing component (mARC)[1270-1072], whose classification is presently unknown.)

space (=blank line)

The mechanistic strategies of biological utilisation of nitrite will be discussed in the following sections (4.1.-4.3.). The starting point will be the protein structure (*"Enzymatic machinery"*). A description of the reaction mechanism will follow (*"Promiscuity"* [1273] and *"Mechanism"*), where we will confine our considerations only to the active sites and the structure-activity relationships will be (as much as possible) systematically explored to discuss the mechanistic strategies that Biology developed to handle nitrite.

4.1. NITRITE REDUCTION TO AMMONIUM

The great majority of biological redox reactions involves one or two-electron reduction/oxidation steps. When higher reductions/oxidations are needed, Biology divides the required reaction in several individual reactions of one or two-electron reduction/oxidation, each catalysed by a specific enzyme. The denitrification pathway (Fig. 1, blue arrows) is a clear example where nitrate is reduced by five electrons to dinitrogen in four individual steps (eq. 4). The uncommon multi-electron reactions, where several electrons are transferred, through a single enzyme, without the release of any intermediate, are devoted to key steps of the metabolism. Oxidative phosphorylation (dioxygen reduction by four electrons to water, catalysed by CcO), "respiration" and assimilation of sulfur (sulfite reduction by six electrons to sulfide, catalysed by sulfite reductases), assimilation of molecular nitrogen (dinitrogen reduction by six electrons to ammonium, catalysed by nitrogenases) are some of the few examples found on Biology.

The assimilation and "respiration" of nitrite constitute two remarkable examples of what was stated

above (Fig. 1, orange and green arrows, respectively). In both pathways, nitrite is reduced by six electrons to ammonium, in a reaction catalysed by a single enzyme, without the release of any intermediate. The metal chosen for this catalysis is iron, probably a reminiscence of the prebiotic ammonium formation on the early Earth, where nitrite is believed to had been readily reduced to ammonium by reduced iron.[1274]

However, assimilation and "respiration" serve different cellular purposes and evolved to be two distinct metabolic pathways, located in different cellular compartments (cytoplasm/chloroplasts strom and periplasm/cytoplasm membrane, respectively), catalysed by structurally different enzymes, containing different redox centres and amino acid sequences (here discussed separately in section 4.1.1. and 4.1.2.).

4.1.1. Dissimilatory nitrite reduction to ammonium

The dissimilatory nitrite reduction to ammonium (eq. 54) is achieved within the *c* haem of the multi-*c*-haems-containing nitrite reductase (CcNiR) enzymes (ammonium:cytochrome oxidoreductase, EC 1.7.2.2).

$$NO_2^- + 6e^- + 8H^+ \xrightarrow{\textit{c}\text{ haem}\ [Fe]} NH_4^+ + 2H_2O \qquad (54)$$

$$E^{o\prime}\ (NO_2^-/NH_4^+) = +0.75\ V$$

4.1.1.1. Enzymatic *machinery*

CcNiR (also known as NrfA[1275]) are part of larger complexes. In the periplasm of *Wolinella succinogenes*[68,1276,1277], *Desulfovibrio desulfuricans*[1278] or *D. vulgaris*[1279,1280], CcNiR forms a stable membrane-associated complex with NrfH, most likely $NrfA_4NrfH_2$. NrfH is a small (≈20kDa), membrane-anchored, tetra-haemic (*c* type) quinol oxidase, that provides electrons to CcNiR directly from the membrane quinone pool, in a fast and efficient way.[1276,1277,1281-1283] *Escherichia coli* CcNiR, on

the contrary, uses a "soluble" periplasmatic penta-haem cytochrome, NrfB, as a direct redox partner ($NrfA_2NrfB_2$; which, in its turn, acts in conjunction with a ferredoxin (NrfC) and a putative membrane quinol oxidase (NrfD)).[1283-1287]

All CcNiR structurally characterised to date host penta-haemic or octa-haemic subunits. The penta-haem CcNiR are homodimers (≈120kDa), with each monomer folded as a single domain (*Wolinella succinogenes* (Fig. 8)[1276], *Escherichia coli*[1285], *Sulfurospirillum deleyianum*[1288], *Desulfovibrio desulfuricans*[1278,1289,1290], *Desulfovibrio vulgaris*[1279,1291,1292], *Shewanella oneidensis*.[1293] These CcNiR contain five covalently bound *c* haems (Fig. 6-a) per monomer, arranged in near-parallel and near-perpendicular haem pairs. Four of the haems (#2 to #5 (Fig. 8-b)) are bis-histidinyl-coordinated (CysXXCysHis binding motif) and, together, form a "wire" that facilitates the fast and effective electron transfer from the physiological partner (likely through haem #2 [1285]) to the active site.[1294] The fifth haem (#1 (Fig. 8-b)) constitutes the enzyme active site and exhibits an unusual coordination by a lysine residue in the proximal position (CysXXCysLys_{134} motif, *W. succinogenes* numbering) and by water/hydroxyl group in the distal position (Fig. 8-c).[1288,1295,1296] The active site also comprises conserved histidine (His_{277}) and arginine (Arg_{114}) residues, which undergo hydrogen-bonding to nitrite and seem to play a key role in the reduction process (as will be discribed). The active site pocket is completed with one tyrosine (Tyr_{218}) and one glutamine (Gln_{276}) residues that form a conserved calcium binding site (at ≈10Å from the iron atom). The presence of calcium is essential for the enzyme activity[1297], possibly due to a structural and/or catalytic roles (through, respectively, keeping the distal histidine away from the iron atom/conformational stabilisation through electrostatic interactions[1290,1297,1298] and facilitating the proton transfer steps[1299]). In the *D. desulfuricans* enzyme, a second calcium ion was identified coordinated to the propionates of the non-catalytic haems #3 and #4, whose main role is believed to be structural.[1290] Further more, two channels allow the nitrite entrance and the ammonium release to the protein surface (the last one on the opposite site of the substrate entry).[1276,1288] The first is a funnel-like entrance, with a significantly positive electrostatic surface potential (that stabilises the negatively charged substrate and presumably supplies the necessary protons (eq. 54)[1290]), whereas the second has a predominantly negative electrostatic surface potential to assist the efflux of the cationic product.

The octa-haem CcNiR (*Thialkalivibrio nitratireducens* [1300-1302] and *Thioalkalivibrio paradoxus* [1303])

is an homo-hexameric enzyme containing eight haems per monomer. The monomer of the octa-haem CcNiR consists of two domains: one N-terminal domain, with three haems in a unique fold, and one catalytic C-terminal domain, with five haems in an arrangement similar to the one found in the penta-haem CcNiR. Despite the low (20%) sequence homology with known penta-haem CcNiR, the catalytic haem of octa-haem CcNiR comprises the lysine residue (CysXXCysLys motif) at the proximal position and the histidine, arginine and tyrosine residues at the distal side, as well as channels for the substrate and product transport and the two conserved calcium binding sites. However, octa-haem CcNiR has special structural features, such as an unusual topography of the product channels that open into the void interior space of the protein hexamer, and a covalent bond between the conserved tyrosine and the adjacent cysteine.

4.1.1.2. *Promiscuity*

In addition to nitrite, CcNiR also catalyses the reduction of NO and of hydroxylamine to ammonium (although with lower specific activities).[1295,1297,1304-1308] Surprisingly, CcNiR is also able to catalyse the reduction of NO to nitrous oxide (eq. 8)[1305,1309,1310], a reaction typical of denitrifiers (Fig. 1, blue arrows). Although the NO reductase activity of CcNiR has been known for long, only recently it begun to be re-evaluated, when it was realized that CcNiR is a key player of the oxidative and nitrosative stress defence network of *W. succinogenes* and *E. coli*, mediating resistance, not only to hydroxylamine and NO, but also to hydrogen peroxide-induced stress[1311].[1310,1312-1316] Moreover, CcNiR catalyses the six-electron reduction of sulfite to sulfide (and with a specific activity higher[1317-1320] than the reported for the true dissimilatory sulfite reductase), connecting, in this way, the sulfur and nitrogen cycles.[1297,1318-1322] CcNiR is, thus, one more "multi-task" protein, involved in, at least, two distinct functions, anaerobic "respiration" and stress defence.

4.1.1.3. Mechanism

CcNiR catalyzes the reduction of nitrite (NO_2^-) to ammonium (NH_4^+). In order to catalyse this reaction, CcNiR has to remove two oxygen atoms from nitrite (cleavage of two N-O bonds), at the same time as it has to add six electrons and eight protons (eq. 54), and do all of these without releasing any intermediates (with no doubt, a remarkable reaction).

To achieve this purpose, the properties of the catalytic haem are certainly crucial, starting from the back-bonding interaction between the iron and nitrite, that, as will be described below, is quite important for the substrate binding mode and strength and for the N-O bond activation. Also the unusual lysine coordination present on CcNiR should have a decisive functional role, although theoretical calculations have not yet shown any striking electronic reasoning for lysine choice over the more conventional histidine ligand. Nevertheless, this odd coordination should had represented an evolutionary advantage, since it requires the additional biological effort of producing a specialised haem lyase (co-evolution).[1323-1326] In addition to the direct iron coordination, the second sphere coordination should play an essential role to define the catalytic properties of the haem. In CcNiR, the active site arginine and histidine residues seem to be important in directing the nitrite binding mode, which, in turn, would condition the reaction product: since CcNiR abstracts both oxygen atoms from the nitrogen, without realising any intermediate, it can be anticipated that the "nitro" mode is the productive one (Fig. 7-a, 7-b(i)). This proposal is supported by theoretical studies that point to the "nitro" coordination as the energetically more favourable one[1327,1328] and by the crystallographic structures showing the "nitro" binding mode in nitrite and hydroxylamine complexes of oxidised CcNiR.[1301,1327]

space (=blank line)

The catalytic cycle is believed to begin from a Fe^{2+}-OH_2 complex (Fig. 9-b), since the reduced enzyme binds nitrite much more strongly than the oxidised form.[1327-1333] In spite of the less favourable electrostatic interaction (Fe^{3+}...NO_2^- *versus* Fe^{2+}...NO_2^-), the back-bonding interaction with reduced iron (an effect described below) makes nitrite affinity for Fe^{2+} higher (stronger Fe-N bond) than for Fe^{3+}.

After the reduction of Fe^{3+}-OH_2 (the enzyme resting state (Fig. 9-a)), the Fe-O bond is weakened,

as a consequence of the increase in the Fe-O distance, and the axial water molecule is readily displaced by nitrite, resulting in a low spin Fe^{2+}-NO_2^- complex (Fig. 9-c).[1327,1328,1332] When nitrite is approaching the iron, the positively charged Arg_{114} and protonated His_{277} would establish strong hydrogen bonds with the negative nitrite oxygen atoms, "anchoring" the nitrite in the "nitro" mode (Fig. 9-c).[1328] These hydrogen bonds would cause an asymmetric charge distribution in the nitrite, with the oxygen atoms gaining additional negative charge and, thus, becoming more susceptible to electrophilic attack. Simultaneously, the N-O bonds became longer and more single bond-like (1.25 instead of 1.5 bond order) and, thus, weaker, which should facilitate the cleavage.[1328]

In addition to these interactions with the positively charged residues, a back-bonding interaction of the nitrogen atom with the iron is of key importance for the strength of the Fe-N bond and for the initial activation of the N-O bond[1327,1328,1330,1331]: on the nitrite-iron complex, the nitrite LUMO, which has π-anti-bonding character, interacts with the iron HOMO, d_{xz} orbital, receiving electron density from the iron. Moreover, theoretical calculations[1328] suggest that the nitrite is positioned with its plane perpendicular to the iron d_{xz} orbital (nitrite plane nearly bisects the N(porphyrin)–Fe–N(porphyrin) angle) in a way that the π-acceptor capability of the nitrite nitrogen atom is maximised.[1328,1334,1335] This back-bonding interaction makes two effects: (i) an Fe-N bond stronger (responsible for the strong binding of the substrate to the reduced active site) and (ii) a N-O bond weaker (*i.e.*, longer and with a lower order, because of the electron density transfer into an orbital that is anti-bonding with respect to the N-O bond).

Upon nitrite binding with the concomitant N-O bond activation, the reaction proceeds with the abstraction of the first oxygen atom, through heterolytic cleavage (Fig. 9-c→f). The first oxygen atom to be abstract is the one closest to the His_{277}, as suggested by the crystal structure of oxidised CcNiR complexed with the putative reaction intermediate hydroxylamine.[1327] To do this abstraction, this oxygen has to be converted into a good leaving group, which is achieved with the addition of two protons (Fig. 9-c→f). And, in fact, the protonated His_{277}, positioned at a short distance from the nitrite oxygen atom (1.56Å [1328]) and with a "fine-tuned" acidity (the pK_a value can be readily "tuned" by the protein environment [1336]), is well suited for this role.[1328,1337] Remarkably, His_{277} is located to facilitate the donation of protons and at the same time to "anchor" the nitrite in the correct position to maximize the back-bonding interaction with the iron atom.[1328] At high pH values (>8, where His_{277} is

most likely deprotonated), is the Arg_{114} (with a pK_a>>7) that probably acts as a proton donor.[1328] After the protonation steps, the {FeNO}^{6} intermediate[1338] (Fig. 9-f) is formed and the first water molecule is released.

The reaction is proposed to proceed towards the formation of a {Fe-NH(O)}^{8} complex (Fig. 9-g). To accomplish it, the enzyme active site must be "recharged" with the necessary protons and electrons, what was suggested to be achieved through two consecutive proton-coupled electron transfers.[1299] In addition, the enzyme must either overcome or avoid the formation of a {FeNO}^{7} complex, which is thought to impair the progression of the reaction. (In fact, due to its high stability (deep potential energy minimum[1299]), such a {FeNO}^{7} complex can be prepared *in vitro* (reacting reduced CcNiR with NO) and it has been well characterised spectroscopically, kinetically[1327,1340-1343] and at theoretical level[1299].) Thermodynamic and kinetic theoretical analysis suggested that the active site "recharging" mechanism through the two proton-coupled electron transfer steps is a probable pathway to overcome the formation of the {FeNO}^{7} complex and lead to the formation of the {Fe-NH(O)}^{8} complex[1299] (Fig. 9-f→g): the first step proceeds rapidly and is highly exothermic (≈-46kcal/mol), with the formation of the {Fe-NH(O)}^{8} complex in a subsequent rate-limiting equilibrium reaction (with an activation barrier of 6.5kcal/mol). In addition, the theoretical calculations revealed that, if the first electron can be added either via simple reduction or coupled with proton transfer, the second one can only be supplied upon the addition of a proton.[1299] The comprehensive theoretical study carried out suggested also that the conserved calcium biding site present at close proximity of the iron atom (see the "*Enzymatic Machinery*") can effectively modulate the proton supply process[1299]: a proton localised on the calcium site would be more favourably transferred, because its energy is higher (≈6kcal/mol) than the one of a proton in a pure water solution. As a result, the calcium site can facilitate the proton transfer steps.

Concerning the electrons, the two electrons transferred to the active site (Fig. 9-f→g) should be readily taken up by one of the NO π-anti-bonding orbitals; the double occupation of the π-anti-bonding orbital leads to the splitting of one of the N-O bonds and to the formation of two separate lone electron pairs on the NO nitrogen and oxygen atoms[1299], what, in its turn, should make the NO more easily protonable.[1299,1327] However, at this reaction point, it is not the oxygen atom to be abstract that is thought to be protonated, but the nitrogen atom[1299,1327], resulting in the formation of

the {Fe-NH(O)}^{8} complex (Fig. 9-g).

In a few words, the proton-coupled electron transfer mechanism efficiently "recharges" the enzyme active site: the electron transfer haems donate the electrons; the change in the reduction state is immediately accompanied by proton transfer from a well-organised network of proton donors in the inlet channel, part of which is the calcium site.[1299] The "recharged" enzyme, then, promptly reduces the {FeNO}^{6} to the reactive {Fe-NH(O)}^{8} intermediate (Fig. 9-f→g). In this context, it can be speculated that the reasoning for the presence of four non-catalytic haems in CcNiR is the requirement for the timely electron transfer. This speculation, however, is questioned by CSNiR that, in spite of catalysing the same reaction, has only one Fe/S centre to transfer electrons to the active site (see section 4.1.2.).

At this stage (Fig. 9-g), the enzyme still has one oxygen atom to remove and several protons and electrons to add (eq. 54). Unfortunately, the mechanism of this last part of the reaction is not yet so well characterised. The reaction should continue to form an iron-hydroxylamine complex (Fig. 9-h). The formation of such intermediate is suggested by the known hydroxylamine reductase activity of CcNiR (that reduces this compound with half of the specific activity observed with nitrite (see section 4.1.1.2.)). The oxidised form of this complex (Fe^{3+}-hydroxylamine) was, in fact, observed in crystals of oxidised CcNiR, where the oxygen was found hydrogen-bonded (3.0Å distance) to the N_{ε} atom of the Arg_{114}.[1306,1327] In order to form this complex, the reaction is suggested to proceed with the alternating transfer of two electrons and two protons, possibly as two proton-coupled electron transfers (with no critical energetic barriers, regardless the sequence of transfers[1327]) to yield the Fe-NH(H)(OH) complex (Fig. 9-h).

Now, the reaction is almost completed. The addition of one more proton converts the hydroxyl moiety in a water molecule (the second one), that leaves the enzyme (Fig. 9-h→i). After the addition of one electron, the oxidation of Fe^{2+} coupled to a proton transfer, would yield the Fe^{3+}-ammonium complex (Fig. 9-i). Theoretical calculations indicate that the crucial role of the active site Tyr_{218} (revealed by the almost complete inactivation of the enzyme when this residue is mutated to a phenylalanine[1320]), is probably carried out at this final stage, possibly in a radical step involving hydrogen atom transfer.[1328]

The Fe^{3+}-ammonium complex can be readily dissociated, due to the lack of significant π-interactions

between the amine nitrogen and the iron (Fe^{3+}/Fe^{2+}-NH_3 are standard σ-donor bonds) (Fig. 9-i→a)[1276,1327]. Alternatively (energetically similar[1327,1333]), the Fe^{3+}-ammonium complex is first reduced to Fe^{2+}-ammonium (Fig. 9-j), after which the ammonium is displaced by a new nitrite molecule (Fig. 9-j→c), closing the catalytic cycle. Note that, until this point, all the reaction steps were proposed to take place with reduced iron, which is made possible due to the efficient electron transfer to the active site. Ammonium is, subsequently, electrostatically guided to the protein surface through the product channel.

space (=blank line)

In summary, the CcNiR-catalysed reaction is presently suggested to start from a ferrous-nitrite complex. The nitrite reduction proceeds with the initial cleavage of the first N-O bond, to form an $\{FeNO\}^6$ complex (Fig. 9-c→f). This complex is subsequently reduced and protonated to eventually form an iron-hydroxylamine complex (Fig. 9-f→h). Each reduction step increases the proton affinity of the previous intermediate, and each protonation increases its electrophilicity. Once the hydroxylamine intermediate is formed, further reduction and protonation lead to the lysis of the last N-O bond and the product is released (Fig. 9-h→c).

The success of the first N-O bond cleavage (Fig. 9-c→f) is due (at least) to the back-bonding with iron and to the hydrogen bonds with second sphere amino acid residues. The back-bonding interaction (i) strengths the Fe-N bond and (ii) weakens the N–O bond. The hydrogen bonds (i) direct the nitrite to the correct position to allow the back-bonding interaction; (ii) enhance this back-bonding (stabilising the nitrite π-anti-bonding orbital, bringing it energetically even closer to the iron *d* orbitals); (iii) cause an asymmetric charge distribution that activates the N–O bond (make it more single bond-like) for reductive cleavage, and (iv) supply the necessary protons.

The success of the second N-O bond cleavage is ascribed to the NO π-anti-bonding orbital that can be readily occupied by two electrons; this "localised" reduction makes the NO nucleophilic and easily protonable to yield, first the $\{Fe\text{-}NH(O)\}^8$ complex (Fig. 9-g), and, ultimately, the hydroxylamine intermediate (Fig. 9-h).

4.1.2. Assimilatory nitrite reduction to ammonium

The assimilatory nitrite reduction to ammonium (eq. 55) is achieved within the sirohaem of the sirohaem-containing nitrite reductase (CSNiR) enzymes (either ferredoxin- or NAD(P)H-dependent enzymes, ammonia:ferredoxin oxidoreductase, EC 1.7.7.1 or ammonia:NAD(P)$^+$ oxidoreductase, EC 1.7.1.4, respectively[1344]).

$$NO_2^- + 6e^- + 8H^+ \xrightarrow{\text{sirohaem (Fe)}} NH_4^+ + 2H_2O \qquad (55)$$

$$E^{o'}(NO_2^-/NH_4^+) = +0.75\ V$$

4.1.2.1. Enzymatic *machinery*

CSNiR have been studied from a number of higher plants, algae, and cyanobacteria, with the spinach chloroplast enzyme being the most extensively characterised CSNiR (also the first 3D structure known[1345]), whereas much less is known about the enzymes from cyanobacteria[1346] or from algae[1347-1349]. Recently, the tobacco enzyme became the focus of an increasing interest.[1350-1352]

CSNiR (product of *nasB*, *nirB* and *Nii* genes, in, *e.g.*, *Paracoccus denitrificans* [1353], *E. coli* [1354] and tobacco[1355], respectively) are monomers (≈65kDa), folded into three domains, in a fairly compact structure, containing one [4Fe-4S] centre and one sirohaem buried within the monomer (Fig. 10)[1345,1359,1352,1356]. The [4Fe-4S] centre is coordinated by four cysteine sulfur atoms, with the characteristic $Cys_{441}XXXXXCys_{447}X_{(n)}Cys_{482}XXXCys_{486}$ motif (spinach enzyme numbering), and transfers the electrons from the reduced ferredoxin (the physiological electron donor) to the sirohaem (Fig. 10-c)[1357,1358]. Sirohaem (Fig. 6-a) is found in nitrite and sulfite reductases, where it constitutes the enzymes active site.[1359,1360] The sirohaem is connected to the Fe/S through the Cys_{486} residue that also coordinates the haem iron (Fig. 10-c). Despite of being strongly coupled by the bridging sulfur, the Fe/S and sirohaem act as independent one-electron carriers in catalysis[1360] and in titrations[1345,1357]. The haem sixth coordination position is occupied by a water molecule.[1361] The active site also comprises conserved Arg_{109}, Arg_{179}, and Lys_{224} residues, which are thought to facilitate the binding and subsequent reduction (hydrogen-bonding/proton donation) of the negatively charged

nitrite.[1345,1350,1360] In addition, several other potentially positively charged residues seem to be essential to stabilise the sirohaem eight carboxylate groups (some of which are conserved in sulfite reductase[1362-1367]) and are, probably, crucial to determine the functional properties of the sirohaem[1345]. CSNiR has a wide (> 8Å) tapered channel that leads to the surface of the protein, where the Fe/S and sirohaem are located, and through which nitrite, ammonium and solvent have a clear path between the protein surface and the haem distal side. It is also at the surface of this channel that the reduced ferredoxin is proposed to bind, to deliver one electron at a time to the CSNiR Fe/S, "closing" the channel from the solvent in the binding process.[1345,1356,1368,1369]

4.1.2.2. *Promiscuity*

Like CcNiR, CSNiR also catalyses the reduction of hydroxylamine to ammonium[1348,1370-1372] and of sulfite to sulfide, but with lower affinity comparatively to nitrite[1373,1374]. Remarkably, the mutation of the active site residue asparagine 226 (neighbouring of the Lys_{224}) to a lysine, in tobacco enzyme, results in an increase of the sulfite reductase activity of one order of magnitude.[1351]

4.1.2.3. Mechanism

The CSNiR-catalysed nitrite reduction to ammonium also takes place without the release of detectable intermediates and several biochemical, spectroscopic and crystallographic studies support that the reaction mechanism is similar to the CcNiR one and to the dissimilatory sulfite reductase one[1375] on its key aspects. (i) The reaction is believed to begin from a low spin (sirohaem)Fe^{2+}-nitrite complex[1357,1361,1372,1373,1376-1381], with the nitrite molecule bound through the "nitro" mode[1361,1379-1381], stabilised through hydrogen bonds with positively charged Lys_{244}, Arg_{179} and Arg_{109} residues[1345,1350]. (ii) The first oxygen atom to be abstracted is the one closest to the Lys_{224} (also in a similar way to

what was proposed for the dissimilatory sulfite reductase[1375]), where Lys_{224} and Arg_{179} are the probable proton donors to form the first water molecule and the Fe^{2+}-NO intermediate[1350,1361,1381-1383]. (iii) The Fe^{2+}-NH(H)OH intermediate[1348,1360,1381] was proposed to be formed with the protons derived from the "recharged" Arg_{179} and Arg_{109} residues and from a water molecule hydrogen-bonded to the "recharged" Lys_{224} [1350].

space (=blank line)

In spite of catalysing the same reaction, with a mechanism that it is suggested to be similar, there are some remarkable differences. The first obvious distinctive features are the haem type, *c* haem or sirohaem, and its axial coordination, lysine or cysteine. Thus, contrary to what may be expected, CSNiR is not structurally related to CcNiR; instead, it is related to the other enzyme known to have a sirohaem, the assimilatory sulfite reductase (which converts sulfite into sulfide, for incorporation into sulfur-containing biomolecules).[1362,1374] Furthermore, it must be bear in mind that the utilisation of different haem types requires a higher biological effort to synthesise all molecules.[1384-1389] The rationalisation for these two choices **-***c versus* sirohaem**-** must wait for theoretical studies on the different haem types and coordinations.

Another distinctive feature is the CSNiR ability to "store" electrons. Unlike CcNiR, with its four *c* haems, CSNiR has only one Fe/S to hold one electron. It has also only a single ferredoxin binding site[1345,1360,1390,1391] to receive one electron at a time, from the ferredoxin [2Fe-2S] centre (*i.e.*, the physiological partner is a one-electron donor). Those facts mean that the CSNiR must receive the necessary six electrons in one-electron steps and make the elucidation of the electron transfer particularly challenging.

Remarkably, these two characteristics (catalytic haem and electron transfer centres) are not important to determine nature of the reaction product. The two active site structures represent two "solutions" to solve the same "problem", but within distinct biological purposes ("respiration" *versus* assimilation). A similar phenomenon will be discussed for the "respiratory" nitrite reduction to NO, where two *truly* different "solutions" were found for the same reaction (iron- and copper-dependent enzymes).

4.1.3. Nitrite reduction to ammonium by other enzymes

Tetrathionate reductase (TTR) and hydroxylamine oxidoreductase (HAOR) are two multi-haemic enzymes that were shown to also catalyse the reduction of nitrite to ammonium.[1392-1397] TTR catalyses the reduction of tetrathionate ($S_4O_6^{2-}$) to thiosulfate ($S_2O_3^{2-}$) (eq. 56). The *Shewanella oneidensis* enzyme is a perisplamatic protein (≈55kDa) that holds eight *c* haems, seven of which are bis-histidinyl-coordinated (with CysXXCysHis binding motifs) and one, the catalytic site, has the unusual lysine coordination characteristic of CcNiR (note that the enzyme holds an equivalent CysXXCysHis motif, with the coordinating lysine being located elsewhere in the primary structure).[1393,1394] HAOR catalyses the oxidation of hydroxylamine to nitrite (eq. 57), in the second step of nitrification (section 2.2.; Fig. 1, black arrows).[1392,1395-1400] The enzyme from *Nitrosomonas europaea* is a homotrimer (≈210kDa) of octa-haemic monomers. Seven of the haems are typical bis-histidinyl-coordinated *c* haems, whose role is only electron transfer, while the active site is constituted by a histidinyl-coordinated P_{460} haem. The P_{460} haem (Fig. 6-a) found in HAOR and in periplasmatic cytochrome P_{460} [1401], is the only haem known to withdraw electrons from an iron-coordinated substrate, a property adequate to catalyse an oxidation reaction (eq. 57).

$$S_4O_6^{2-} + 2e^- \xrightarrow{\text{c haem}} \boxed{Fe} \longrightarrow 2S_2O_3^{2-} \qquad (56)$$

$$NH_2OH + H_2O \xrightarrow{P_{460}\text{ haem}} \boxed{Fe} \longrightarrow NO_2^- + 4e^- + 5H^+ \qquad (57)$$

The structures of these two enzymes show significant similarities in the haem "architecture" with CcNiR. Five of the haems of TTR are almost overlapped with the CcNiR haems, including the substrate binding site and the position of the coordinating lysine at the active site. And four of the haems of HAOR are superposable with the non-catalytic haems of CcNiR, with the catalytic haems positioned flipped in respect to each other. In addition, both TTR[1393,1394] and HAOR[1395-1397] are able to catalyse the reduction of nitrite and hydroxylamine to ammonium as CcNiR. In this context, it is surprising how

HAOR, an enzyme that is "tuned" to catalyse the oxidation of hydroxylamine, is able to catalyse those reduction reactions. However, *in vitro*, in the presence of sufficient reducing power (reduced methyl viologen), HAOR and CcNiR reactions probably proceed through comparable intermediates (eq. 58) (although the HAOR presents lower (two orders of magnitude) specificity constants[1395,1396]).

$$Fe\text{-}NH_4^+ \longleftarrow Fe\text{-}N(H_2)OH \rightleftharpoons Fe\text{-}N(H)O \rightleftharpoons Fe\text{-}NO \rightleftharpoons Fe\text{-}NO_2^- \qquad (58)$$

The occurrence of those reductase activities under more physiological conditions (where the P_{460} haem should be mostly oxidised[1397]) is, nevertheless, controversial. However, the TTR catalytic specificities towards nitrite and hydroxylamine (comparable with the ones of CcNiR)[1394], suggest that this enzyme may have a role in the biological nitrogen cycle. It was hypothesised that the multi-haemic enzymes of the "oxidative branches" of the nitrogen cycle (HAOR (nitrification) and hydrazine oxidoreductase (AnAmmOx); see section 2.2.) had evolved from the more ancient penta-haemic nitrite reductase enzymes, with an octa-haemic nitrite reductase as a possible intermediate in the process, under a variety of environmental pressures, that triggered the function changing from reduction to oxidation[1321,1402]

4.2. NITRITE REDUCTION TO NITRIC OXIDE

NO is a remarkable biomolecule. Its formation constitutes the first committed step in denitrification and is an essential step in the primitive AnAmmOx and other "respiratory" pathways (Fig. 1, blue, gray and violet arrows, respectively), where nitrogen compounds are used to derive energy (sections 2.2.-2.3.). The NO is also a vital mammalian signalling molecule and, for its synthesis, Biology developed a "dedicated" enzyme **-**NOS**-** and several nitrite "recycling" pathways to ensure the NO formation when the NOS activity is impaired (due to decreased dioxygen availability; see sections 3.1.1.). Remarkably, also plants and bacteria are using similar "rescue" nitrite-dependent pathways to

produce NO (sections 3.2.1. and 3.3.). So, the biological nitrite reduction to NO is an ubiquitous, universal reaction that was "invented" for the anaerobic world and has been "reinvented" and employed ever since.

To carry out this "old" reaction, Biology developed different "solutions", using three metals to do the reduction: iron, copper and molybdenum. For the "respiratory" function, two distinct classes of enzymes were developed, the haem-dependent (Cd_1NiR; section 4.2.1) and copper-dependent (CuNiR; section 4.2.2) nitrite reductases. Remarkably, the "solution" first found (pre-oxic era), the iron-dependent enzyme, was so successful that was used in different strategies of survival (denitrification, AnAmmOx, "denitrification/intra-aerobic methane oxidation" and possibly others), further emphasising the *in vivo* importance of NO as the alleged first deep electron sink on Earth (before the emergence of dioxygen)[148,1403]. For signalling pathways, Biology "reuses" iron and molybdenum-based redox systems, present in cells to accomplish other functions, to carry out the nitrite reduction to NO (as was discussed in sections 3.).

4.2.1. Dissimilatory nitrite reduction to nitric oxide by an iron-dependent enzyme

The dissimilatory iron-dependent nitrite reduction to NO (eq. 59) is achieved with the d_1 haem of the *c* and d_1 haems-containing nitrite reductase (Cd_1NiR) enzymes (nitric oxide:cytochrome oxidoreductase, EC 1.7.2.1).

$$NO_2^- + 1e^- + 2H^+ \xrightarrow{d_1\ \text{haem}} [Fe] \longrightarrow {}^{\bullet}NO + H_2O \qquad (59)$$

$$E^{o\prime}\ (NO_2^-/{}^{\bullet}NO) = +0.34\ V$$

4.2.1.1. Enzymatic *machinery*

Cd_1NiR (product of *nirS* genes) are periplasmatic homodimers (≈120kDa), with each monomer folded into two domains: (i) one typical bacterial α-helical cytochrome *c* N-terminal domain

(electron transfer domain), containing one *c* haem, and (ii) one eight-bladed β-propeller C-terminal domain (catalytic domain), comprising one d_1 haem (*Pseudomonas aeruginosa* (Fig. 11)[1404-1407] and *Paracoccus pantotrophus*[1408-1412]). The *c* haem is responsible for the electron transfer from different electron carriers (see section 2.2.) to the d_1 haem (Fig. 6-a), which constitutes the enzyme active site.[1405,1411,1413-1418] The active site also comprises two conserved histidine residues (His_{327} and His_{369}, in *P. aeruginosa*, and His_{345} and His_{388}, in *P. pantotrophus*), which are essential for catalysis (Fig. 11-c) as will be discussed.[1404-1412,1419,1420]

P. aeruginosa Cd_1NiR has the *c* haem coordinated by Met_{88} and His_{51}, while the d_1 haem is coordinated by His_{182}, in the proximal position, and by a hydroxyl group, in the distal position (Fig. 11-c)[1406]. When the d_1 haem is oxidised (regardless of the *c* haem state[1421]), this hydroxyl group is hydrogen-bonded to the Tyr_{10} of the *partner* monomer (the 7-29 loop is "swapped" over the two monomers)[1404] **-***i.e.*, the haem of the active site is an a "closed" hexa-coordinated state. After d_1 haem reduction, the N-domain 56-62 loop is displaced (6Å) and the Tyr_{10} is (4.2Å) shifted away from the d_1 haem iron[1405,1421]. This concerted movement in the N-domain of one monomer "opens" the active site haem present in the C-domain of the *partner* monomer, allowing nitrite to bind to the haem iron. (For the following mechanism discussion, it should be here emphasised that it is the electron transfer to d_1 haem, and not the *c* haem reduction, that is responsible of these conformational changes.[1421])

In *P. pantotrophus* Cd_1NiR, the structural changes are more complex. In spite of the similar overall structure, in *P. pantotrophus*, the N-terminal segment is not domain-"swapped", but "wrapped" around the same monomer at the interface between the two domains.[1408]. In addition, the as-isolated, oxidised, enzyme has the *c* haem His_{17}-His_{69}-coordinated and the d_1 haem His_{200}-Tyr_{25}-coordinated (proximal and distal positions, respectively).[1408,1410,1411] This haems coordination is surprising. First, because neither His_{17} nor Tyr_{25} are conserved in other Cd_1NiR, apart from the very closely related *P. denitrificans* enzyme (note that Tyr_{25} is not equivalent to the Tyr_{10} of the *P. aeruginosa* structure, since it belongs to the same monomer). Second, because Tyr_{25} is coordinating the iron directly. The presence of a His_{200}-Tyr_{25}-coordinated active site haem, with no vacant position for substrate binding, indicates that the *P. pantotrophus* Cd_1NiR is isolated in an inactive form **-**an "unready" enzyme (as denominated in section 4.). However, in a situation parallel to that of

P. aeruginosa Cd_1NiR, reduction of the *P. pantotrophus* enzyme triggers a concerted movement in a N-domain loop that changes the coordination sphere of both haems: the *c* haem becomes Met_{106}–His_{69}-coordinated and the tyrosine residue of the d_1 haem is displaced to yield an "open" penta-coordinated active site **-**now, a "ready" enzyme.[1404,1411,1422,1423] Accompanying the structural changes, the pre-reduction of *P. pantotrophus* Cd_1NiR leads to a catalytically more active enzyme, which exhibits higher k_{cat} values[1424] than the as-isolated enzyme[1417,1428].

The conformational changes **-**that culminate with the haem ligand "switching"**-** needed to activate the Cd_1NiR can be relevant to the *in vivo* regulation of the enzyme (and not be only an experimental problem that has to be overcame). Actually, the *P. pantotrophus* Tyr_{25}Ser mutant, which has a "locked" penta-coordinated d_1 haem iron, is an active enzyme without needing the reductive activation step.[1429] Similarly, the *P. aeruginosa* Tyr_{10}Phe mutant is functionally and spectroscopically identical to the wild-type.[1430,1431] Therefore, the presence of the tyrosine residue, which is not essential for catalysis, could be an intentional biological regulatory strategy: to have an enzyme whose reactivity can be controlled through a mechanism of the type "unready" *versus* "ready". An enzyme with such "tuneable" reactivity would allow the organism to quickly respond towards different cellular/environmental conditions/stimuli[1423,1429] (as was previously discussed for Nb and Cc).

space (=blank line)

The Cd_1NiR structural changes, however, are not driven only by reduction, but also by the protonation state of the residues at the active site. In fact, it is remarkable how comparable are the structures of the oxidised *P. aeruginosa* His_{369}Ala and His_{327}Ala mutants (pH 5.5-6.5)[1406,1419,1423] and of the reduced wild-type *P. pantotrophus* Cd_1NiR crystallized under anaerobic conditions at pH 9.0 (at this pH, the conserved histidine residues are likely deprotonated and, thus, uncharged and electrostatically similar to the His-Ala mutants)[1423]: these three structures (i) preserve the individual *c* and d_1 coordination and folding characteristic on reduced enzymes and (ii) show the *c* domain ≈60º rotated (≈20Å displaced) relative to the d_1 domain. Although the mutants conformational alterations were initially interpreted as suggesting that the two conserved histidines are crucial for the enzyme structural organization[1406], the parallel structure of the wild-type *P. pantotrophus* enzyme indicates that it is the protonation state of those histidines that is determinant for the conformation adopted by Cd_1NiR. Therefore, it is probable that, not only the localised iron charge (oxidised *versus* reduced),

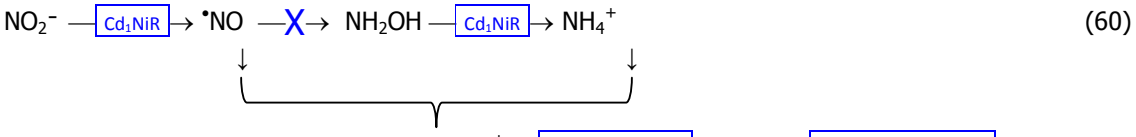
but also the overall charge in the active site controls the enzyme conformation.

These remarkable Cd_1NiR structural changes, driven by oxidation/reduction and also protonation/deprotonation, further support that the conformational changes are a deliberate mechanistic strategy of Cd_1NiR -the fourth protein type (here described) with allosteric/posttranslational control of the catalysis.

4.2.1.2. *Promiscuity*

Besides the nitrite reduction to NO, Cd_1NiR catalyses the two-electron reduction of hydroxylamine to ammonium and the four-electron reduction of dioxygen to water, in the presence of any of its electron donors.[1417,1428,1432] In fact, the enzyme was long thought to be an oxidase and classified as a cytochrome oxidase (EC 1.9.3.2).

Contrary to CcNiR, the Cd_1NiR does not catalyse the reduction of NO to hydroxylamine (eq. 60). This is a very interestingly observation, because that reaction would prevent the primitive "recycling" of nitrogen through the AnAmmOx (eq. 61 and Fig. 1, gray arrows), suggested in section 2.2..



(60)

(61)

4.2.1.3. Mechanism

Comparatively to the nitrite reduction to ammonium, the formation of NO is considerably simpler. As will be discussed, the Cd_1NiR-catalysed reaction involves the nitrite binding to the reduced d_1 haem iron, followed by the abstraction of one oxygen atom and the addition of one electron. In this way, the Cd_1NiR reaction can be considered as a "copy" of the first part of the CcNiR reaction illustrated in figure 9-b→f. However, the well known very high affinity of NO to haem iron raises an intriguing question: how is the NO released from the active site haem of the Cd_1NiR?

space (=blank line)

The Cd_1NiR catalytic cycle begins after the reductive activation of the resting (inactive) enzyme, with the concomitant tyrosine displacement from the d_1 reduced iron (Fig. 12-a→b). For NO formation, the "nitrito" binding mode would be *a priori* expected (Fig. 7-a), although all the binding modes are feasible (Fig. 7-b(ii)-(v)). However, the crystal structures showed that nitrite binds to Cd_1NiR in the "nitro" mode (Fig. 12-c), with the nitrite oxygen atoms forming hydrogen bonds with the two conserved histidine residues (His_{327} and His_{369}, in *P. aeruginosa*, and His_{345} and His_{388}, in *P. pantotrophus*).[1404,1405,1408,1411,1419,1433,1434] This Cd_1NiR-nitrite interaction mode is clearly a "copy" of the CcNiR one and the initial nitrite binding and activation is believed to be ruled by the same principles that were proposed for CcNiR: back-bonding and hydrogen bond. However, it should be here emphasised that it is plausible that the d_1 haem unique features (two electron withdrawing carbonyl groups (Fig. 6-a) and inversion of the iron *d* orbitals energy levels, both discussed below) may made the d_1 haem less effective at such back-bonding than the *c* haem of CcNiR[1435]. In addition, in the Cd_1NiR, the enzyme reduction not only provides the reduced iron, for which the nitrite affinity is higher[1406,1412], but, in addition, "opens" the active site haem.

Subsequent to the nitrite binding, and again as in CcNiR, the oxygen atom to be abstracted is protonated, the N-O bond is heterolytically cleaved and a water molecule is released (Fig. 12-c→e).[1406,1420,1433,1434] The two conserved histidine residues are well positioned to act as proton donors[1404-1412,1419,1420] and their key role on this step is supported by the abolishment of the nitrite reductase activity on the His_{327}Ala and His_{369}Ala mutants[1406] and by theoretical calculations[1433]. Nonetheless, the two histidine residues are not equivalent, and the His_{369} (located at a shorter distance from both nitrite oxygen atoms) seems to be essential to control the stability of the enzyme-

substrate complex (possibly via the formation of two hydrogen-bonds).[1406,1407,1433] These steps (Fig. 12-c→e) culminate with the formation of a ferrous {FeNO}^{6} complex, as was described for CcNiR (Fig. 9-f).

From this point on, the similitudes end and the CcNiR and Cd_1NiR reactions follow separate strategies. While the CcNiR "objective" is to retain the NO bound to be further reduced, the Cd_1NiR should promote the rapid NO release (the purpose of an NO synthase enzyme).

In the {FeNO}^{6} complex, the NO is already formed and it is "waiting" to be released from the active site. Initially, due to the known very slow dissociation of ferrous-NO complexes of other haemic proteins (see, *e.g.*, the discussion about the haemic proteins in sections 3., and also the references[243-257,1420]), the Cd_1NiR ferrous {FeNO}^{6} complex (Fig. 12-e) was considered to be a "dead-end" product. For that reason, the NO was suggested to be released from the ferric {FeNO}^{6} complex (Fig. 12-f).[1405,1406,1413,1340,1438] Such ferric complex is formed by intramolecular iron oxidation (equivalent to eq. 25), with the transfer of one electron from the Fe^{2+} to the bound NO (Fig. 12-e→f, "valence isomerisation"); the NO π-anti-bonding orbital would readily accommodate this electron.

Although reasonable, this initial proposal was never proven. In fact, several studies failed to show kinetically competent NO dissociation from the ferric complex.[1425,1438] In addition, several experimental evidences suggested that the last step of NO formation should be different: (i) nitrite reduction is not inhibited after pre-incubation of reduced enzyme with a large excess of added NO, showing that the fully reduced enzyme-NO complex is not a "dead-end" species[1420,1439,1440]; (ii) the NO release is triggered by the intramolecular *c* to d_1 haem electron transfer[1439,1441]; (iii) the presence of the electron donor (that transfers electrons to the *c* haem) must contribute to effecting NO release[1417,1438,1442,1443]; (iv) the Cd_1NiR mutant (*P. pantotrophus* Met_{106}His mutant) with the *c* haem "locked" in the oxidised state (with a reduction potential of -60mV [1425]) is able to reduce nitrite to NO, at the reduced d_1 haem, but unable to release the NO from the ferric complex thus formed[1425]. Together, these data indicate that the iron-NO complex has to be first reduced, with one electron transferred from the *c* haem (Fig. 12-f→g), and that the NO is released from the ferrous {FeNO}^{7} complex thus formed. However, this new via of NO release is, once more, contrary to the well known properties of the ferrous-NO complexes (see above).

After much debate about the iron-NO complexes dissociation, this impasse was recently overcome with the demonstration that the NO can be rapidly released from the ferrous d_1 haem: k_{off} of 200s^{-1} and 65s^{-1}, from *P. pantotrophus* Cd_1NiR[1440], and of 35 and 6 s^{-1}, from *P. aeruginosa* Cd_1NiR[1420,1437] (biphasic dissociations in both cases), values that compare with k_{off}≈10^{-5}-$10^{-3}s^{-1}$ from Mb and Hb or guanylate cyclase[244,250,252]. Moreover and most important, these (d_1)Fe^{2+}-NO dissociation rates are sufficiently high to be catalytically relevant, with the NO dissociation being the probable rate-limiting step in *P. pantotrophus* Cd_1NiR (k_{off}≈k_{cat}=72s^{-1} [1417]) and, possibly, in *P. aeruginosa* Cd_1NiR (k_{off}≈k_{cat}≈ intramolecular electron transfer from *c* to d_1 ≈3-6s^{-1}).[1406,1413,1417,1420,1441,1444] The agreement between the catalytic kinetic parameters and the rates of (d_1)Fe^{2+}-NO dissociation[1445] strongly supports the NO release from the ferrous $\{FeNO\}^7$ complex during the catalytic cycle.[1420,1434,1440] The present data, however, does not allow to discriminate if the NO is released from the "mixed-valence" protein, (d_1)Fe^{2+} - (*c*)Fe^{3+} (Fig. 12-g), or from the fully reduced enzyme, (d_1)Fe^{2+} - (*c*)Fe^{2+} (Fig. 12-h).[1420,1440]

Once the ferrous $\{FeNO\}^7$ complex is formed, a new nitrite molecule is able to react with the enzyme at a rate limited by the NO dissociation, showing that the high affinity of ferrous d_1 haem for nitrite actively contributes to the NO dissociation during the catalytic cycle.[1437] Thus, after nitrite displacement of the bound NO, the reduced d_1 haem can immediately start a new catalytic cycle (Fig. 12-g→h→c or 12-g→i→c), not being inhibited in between.

Interestingly, the release of NO from a ferrous $\{FeNO\}^7$ complex can rationalise why an enzyme that catalyses a one-electron reduction has two one-electron redox centres: nitrite reduction oxidises the (initially reduced) d_1 haem and the *c* haem is needed to rapidly re-reduce the d_1 haem in order to the NO release be triggered.

space (=blank line)

The surprising rapid NO dissociation from the (d_1)Fe^{2+}-NO complex is largely controlled by the unique features of the d_1 haem structure. An elegant and definitive demonstration of the relevance of haem was the observation that apomyoglobin (that has a different binding pocket), when reconstituted with d_1 haem, releases NO at a rate four orders of magnitude greater then the native *b* haem-containing Mb (2s^{-1} [1440] *versus* 1,2x$10^{-4}s^{-1}$ [244,245,248,249]). The mechanism by which the d_1 haem achieves this fast NO release is, presently, not well understood, but two of the d_1 features

should be important. First, the peculiar ordering of the *d* orbitals energy levels of the d_1 haem iron: $(d_{xz},d_{yz})^4$ $(d_{xy})^1$, instead of the common $(d_{xy})^2$ $(d_{xz},d_{yz})^3$.[1410,1446] The fact that the d_1 iron HOMO lies in the haem plane (instead of lying above and below the haem plane (d_{xz},d_{yz})) would not favour its interaction with the NO SOMO/LUMO (π-anti-bonding) and, thus, the NO would be less strongly bound to the d_1 then to a *b* type haem.[1410] Second, the presence of two electron withdrawing carbonyl groups in the d_1 structure (Fig. 6-a) would weaken the electron donation of the iron orbitals to the NO nitrogen atom, contributing also to weaken the Fe-N bond.[1440]. Future spectroscopic and theoretical studies will undoubtedly contribute to a better understanding of the properties of this unique haem ring.

In addition to the unique properties given by the singular d_1 haem structure, the Cd_1NiR catalysis seems to be also dependent on deliberate conformational changes **-** allosteric control of the catalysis. The remarkable Cd_1NiR conformational changes, driven by oxidation/reduction and/or protonation/deprotonation (described on the *"Enzymatic machinery"*), can, in fact, represent an additional mechanism to control the catalysis and they were recently evoked to explain the negative cooperativity in the intramolecular electron transfer[1444]: this decrease in the intramolecular electron transfer rate as the level of enzyme reduction increases may, in principle, arise from a decrease in the electronic coupling between the *c* and d_1 haems, which, in turn, may be caused by conformational changes. Accordingly, the modifications of the domain-domain interfaces, within and between monomers (relocation of Tyr_{10}, displacement of the 56-62 loop and the large rotation/sliding of the *c* domain), while disrupting the hydrogen bonds network, would decrease the electronic coupling between the *c* and d_1 haems.[1444] In the same way, the decreased intramolecular electron transfer rate as the pH is increased towards basic values (5.8 to 8.0)[1447] can be attributed to conformational changes.

But the allosteric control may have other implications. It is tempting to think that the *c* and d_1 domains are acting as "rigid bodies", linked by flexible loops that propagate the changes (oxidation/reduction and protonation/deprotonation) occurred in one haem pocket to the other one. In this "modular" structure, it can be speculated that the *c* haem oxidation/d_1 haem reduction (Fig. 12-f→g) and/or the deprotonation of the conserved histidines (Fig. 12-c→e) would initiate a conformational change that modifies the position of the residues involved on the stabilization of the

(d_1)Fe-NO complex and/or affects the d_1 affinity for ligands, facilitating, in this way, the NO release. After the NO release, the enzyme would have to undergo another conformational change, to return to the "initial" conformation that would favour the nitrite binding. This step could be triggered by the reduction of the *c* haem (by the external electron donor) or by the re-protonation of the histidine residues. Obviously, future work will be needed to evaluate the possibility of these conformational changes being part of the catalytic mechanism.

To finish this discussion, it should be mentioned that a dynamic and cooperative network of bonds (not controlled by the conformational changes) was also purposed to be responsible for the strong nitrite binding and fast NO release.[1434] A combination of high field electron-nuclear double resonance techniques (to detect the hydrogen bonds) and density function theory calculations (to correlate the experimental results with the structure)[1434] puts forward the following hypothesis: (i) in the beginning of the catalytic cycle, the positively charged histidine residues "attract" the nitrite, hold it in place and donate the necessary protons to the oxygen atom abstraction; (ii) at the same time, the Tyr_{10} establishes a hydrogen bond with the nitrite nitrogen atom; (iii) after the iron-NO complex formation, the now deprotonated His_{369} forms a hydrogen bond with the Tyr_{10}, removing it from the proximity of the still bound NO, *i.e.*, preventing it to form an hydrogen bond with the NO and, in this way, facilitating the NO release. Alternatively, if the His_{369} is rapidly reprotonated and forms an hydrogen bond with the NO oxygen atom (stabilizing the Fe-NO complex), it was purposed that the *simultaneous* hydrogen bond between the tyrosine and the NO nitrogen atom ((Tyr)O-H $\cdots$ N(O)-Fe) would populate the (partial sp^2) nitrogen non-bonding orbital, weakening the Fe-N bond, and, therefore, facilitating the NO release.[1434] With this proposal, the nitrite reduction is facilitated by a dynamic and cooperative network of hydrogen bonds[1448] that, first, keeps the nitrite in the haem and then, after the oxygen abstraction step, changes and no longer holds the NO in the active site. The role ascribed to His_{369} has been difficult to prove. The fast NO dissociation displayed by the His_{369}Ala mutant (only two times slower than the wild-type[1420]) seems to indicate that this residue is not involved on the NO release. However, in the mutant, the Tyr_{10} is displaced such that it cannot form an hydrogen bond with the NO nitrogen atom.[1434] Also the role for the tyrosine residue has been criticised, because the Tyr_{10}Phe mutant is catalytically active.[1430,1431] Nevertheless, it can be argued that, in the absence of Tyr_{10}, its role can be played by His_{327}.[1434] A more definite assignment of

the tyrosine role must wait for measurement of the rate of NO release from the $Tyr_{10}Phe$ mutant (or from *Pseudomonas stutzer*i Cd_1NiR, which does not have an equivalent tyrosine).

space (=blank line)

In summary, as presently suggested, the catalytic cycle of Cd_1NiR can be considered as a "copy" of the first part of the CcNiR reaction, until the $\{FeNO\}^6$ complex is formed (Fig. 12-b→e *versus* Fig. 9-b→f). Once this complex is formed, the CcNiR keeps the NO bound to be further reduced by two electrons, yielding an $\{FeNO\}^8$ complex. The Cd_1NiR, on the other hand, reduces that complex by only one electron (to $\{FeNO\}^7$) and, after that, the rapid NO dissociation is promoted (Fig. 12-f→g→h). The dissociation mechanism has to be fast, in order to compete with the hypothetical reduction by a second electron, and efficient, to effectively promote the NO release. The nature of this mechanism is (to us) the most interesting aspect of the Cd_1NiR-catalysed reaction: what happens after the electron transfer from *c* to d_1 haem that triggers the fast NO release? Undoubtedly, the unique d_1 haem has a leading role in controlling the Cd_1NiR reactivity with NO. Its exceptionally high NO dissociation rate, in conjunction with the allosteric control of catalysis and the probable dynamic network of hydrogen bonds, act synergistically to efficiently synthesise and release NO.

space (=blank line)

The Cd_1NiR uses the same basic *machinery* **-**an haem**-** to deal with the same substrate **-**nitrite**-** as CcNiR and CSNiR, but "tuned" by a different ring structure to yield a different product, the NO. The d_1 haem evolved specifically to form and release NO: (i) it is present only in the Cd_1NiR enzyme[1449], where it is "tailored" to meet the challenging mechanistic requirements of NO formation and release, and (ii) this "tailored" structure required the development of an huge and specific *machinery* to be synthesised (d_1 synthesis requires a substantial biological effort, met by the *nirECFD-LGHJN* genes products[1450]). The evolutionary success of the d_1 haem structure is put in evidence by the presence of the Cd_1NiR enzyme in so many organisms still today.

In spite of all the successful efforts to create the d_1 haem, and due to the importance of the nitrite reduction/NO formation, Biology made other "experiments" that culminate with the development of a different, *de novo*, invention of the oxic era: the CuNiR.

4.2.2. Dissimilatory nitrite reduction to nitric oxide by an copper-dependent enzyme

The dissimilatory copper-dependent nitrite reduction to NO (eq. 66) is achieved with the copper centre of the copper-containing nitrite reductase (CuNiR) enzymes (nitric oxide:cytochrome oxidoreductase, EC 1.7.2.1).

$$NO_2^- + 1e^- + 2H^+ \xrightarrow{\text{Cu centre}} {}^{\bullet}NO + H_2O \qquad (66)$$

$$E^{o\prime}\,(NO_2^-/{}^{\bullet}NO) = +0.34\ V$$

4.2.2.1. Enzymatic *machinery*

CuNiR (product of *nirK* genes) are periplasmatic homotrimers (≈110kDa), with each monomer folded into two eight-stranded β-barrel domains, called I and II (*Alcaligenes faecalis* [1451-1454], *Achromobacter cycloclastes* (Fig. 13)[1455-1457], *Alcaligenes xylosoxidans* [1458-1464], *Rhodobacter sphaeroides* [1465]). The three monomers are tightly associated around a central channel of 5-6Å, with domains I positioned at the corners of the trimer and domains II forming the core of the molecule. An extensive network of hydrogen-bonds (within and between monomers) maintains the rigidity of these complex structure, where ≈1/3 of the monomer surface has to be used on the trimer formation.[1456]

The CuNiR enzymes contain two different copper centres per monomer, one T1 and one T2 (see section 4.; Fig. 6-b): the T1 centre is responsible for the electron transfer from the physiological partners to the T2 centre, which constitutes the enzyme active site. Each T1 centre is located within domain I of each monomer, while the T2 is found bound in a cleft formed by apposition of domain II of one monomer and domain I of the adjacent monomer (Fig. 13-a,b). The T1 centre copper is coordinated by two histidine (His_{95} and His_{145}) and one cysteine (Cys_{136}) residues, in a distorted trigonal planar geometry, and by one methionine residue (Met_{150}) forming a weaker interaction in an axial position (*A. cycloclastes* numbering (Fig. 13-c)). The T2 centre copper (the active site) is coordinated by three histidines (His_{100}, His_{135} and His_{306}) and by a water molecule, in a distorted

tetrahedral geometry (Fig. 13-c). The two centres are connected through the (T1)Cys_{136} - (T2)His_{135} bond, which enables a path for rapid electron transfer across the ≈12.5Å that separates the copper atoms.[1451,1463,1466,1467] The CuNiR active site also comprises conserved aspartate, histidine and isoleucine residues (Asp_{98}, His_{255} and Ile_{257}, the last two provided by the domain II of the adjacent monomer, Fig. 13-c) that are essential for catalysis .[1454,1462,1463,1468,1469]

space (=blank line)

Historically, CuNiR were classified into two sub-groups, depending on their colour being blue (*e.g.*, *A. xylosoxidans* CuNiR) or green (*e.g.*, *A. faecalis*, *A. cycloclastes* or the recently described *Sinorhizobium meliloti* [1470] CuNiR) in the as-isolated oxidized state. The colour of the enzyme is given by the T1 centre: while the methioine sulfur ligand of the *A. xylosoxidans* blue enzyme deviates only slightly from the axial position, the one of the *A. faecalis* green enzyme is in a considerably tilted position (relatively to the His-His-Cys plane), resulting in a distorted tetrahedral and flattened tetrahedral geometries, respectively.[1459] The perturbed geometry of the green enzymes modifies the copper-ligands interactions, resulting in the redistribution of absorption intensity in the charge transfer and ligand field transitions[1471]: the absorption at ≈600nm decreases, the intensity of the absorption envelope at ≈450nm increases, and the EPR signal change from axial to rhombic symmetry. Besides the small differences in the geometry of the T1 centre, green and blue CuNiR show a marked difference in the overall surface charge distribution. This is probably responsible for the different specificity towards the redox partner, pseudoazurin for green CuNiR and azurin for blue CuNiR (supporting the electron transfer via protein-protein complex formation).[1456,1459,1472,1473]

More recently, the resolution of the structure of a CuNiR isolated from the *Hyphomicrobium denitrificans* [1474] lead to the definition of a novel family of CuNiR, characterised by an homohexameric structure containing an additional T1 copper centre per monomer[1475]. The *H. denitrificans* CuNiR monomer is folded in three domains: one N-terminal β-barrel domain (≈15kDa), with one T1 copper centre, and two C-terminal domains (≈35kDa) containing the T1 and T2 centres in the characteristic organisation of "classic" trimeric CuNiR. The N-terminal and C-terminal T1 copper centres are blue and green, respectively, given the enzyme its greenish-blue colour in the oxidized form.[1475-1478] A genome analysis points toward the wide occurrence of this "new" N-terminal extended CuNiR, which probably exists also into two distinct subclasses as the "classic" family, *i.e.*, blue and green trimeric

CuNiR.[1479] The *H. denitrificans* CuNiR molecule is a dimer of trimers (a trigonal prism-shaped hexameric structure) containing twelve T1 and six T2 copper centres, in a total of 18 copper atoms per molecule.[1474] However, and surprisingly, the additional copper centre was found to be too far away (>24Å) from the catalytic core to effectively participate in electron transfer.[1474,1480] Nevertheless, that distance can be shortened to an appropriate value for electron transfer by a simple rotation of the "extra" domain (a movement that could take place, hypothetically, when the redox partner protein binds).[1480,1481]

In addition to the N-terminal extended CuNiR, genome analysis identified the occurrence of another type of CuNiR with a C-terminal extension containing a *c* type haem.[1479,1482] In accordance, the *Ralstonia pickettii* enzyme was shown to be a trimeric protein (≈50kDa tri-domain monomer) with each monomer holding, besides the two copper centres, a single *c* haem.[1480,1483] The enzyme folds as the "classic" (only copper-containing) CuNiR, with the additional haem domain of one monomer localised in close proximity to the T1 centre of the adjacent monomer, with a copper-to-haem edge distance of 10.1Å (an electron transfer compatible distance).[1480] The observed folding clearly defines an electron transfer path between the haem domain and the catalytic core, supporting the functional importance of the haem during turnover.[1480] In this context, the "new" *c* haem-copper-containing enzyme can be thought as "fused version" of the electron transfer complex of a "classic" CuNiR with cytochrome c_{551}, whose structure was recently elucidated.[67] In this "fused version", the enzyme may have captured the partner gene to construct an effective self-sufficient electron transfer system, where the additional domain acts as a physiological electron donor: electron donor and acceptor proteins fused together by genomic acquisition for functional advantage.[1480]

The enzyme from *Pseudoalteromonas haloplanktis* constitutes a different example of a "fused" *c* haem-copper-containing nitrite reductase. This protein folds as a unique trimeric "domain-swapped" structure, with the haem domain localised at the surface of the T1 centre from the adjacent monomer at a haem-to-copper distance of 10.6Å.[1481] The conformation of the *P. haloplanktis* enzyme, thus, shows a self-sufficient electron transfer system, but the structural aspects of the domain-domain interface and the electron transfer kinetics indicate that the haem-copper domain interaction should be highly transient (similar to the interaction of two non-covalently bound proteins in an electron

transfer complex).[1481] In addition, and as mentioned above for the additional copper centre of the *H. denitrificans* enzyme, the haem does not seem to be involved in the electron transfer from the cognate redox partner protein to the catalytic core.[1481] Clearly, there is still much to be learned about the biological function of the "extra redox domains" of the extended CuNiR enzymes. The occurrence of several types of "extensions" support that they are of biological importance; otherwise, why were not the additional domains evolutionally split or lost?[1481]

Another example of how the "classic" CuNiR structure can be transformed is provided by the *Neisseria gonorrhoeae* enzyme: its structure revealed the deletion of specific surface loops and the inclusion of a lipid modification site in a N-terminal extension (this enzyme is the major bacterium anaerobically induced outer membrane lipoprotein), as well as a C-terminal extension that is glycosylated.[1484,1485]

The "classic" and "new" N- and C-terminal extended CuNiR enzymes constitute, thus, fascinating examples of how Biology, once had found a "solution", reuses it recurrently, introducing only minor adjustments to respond to specific cellular demands.

4.2.2.2. *Promiscuity*

In addition to the nitrite reductase, CuNiR has significant superoxide dismutase activity (≈56% of the bovine enzyme activity)[1459,1461] and is also able to catalyse the reduction of dioxygen to hydrogen peroxide (being inactivated in the process)[1486].

4.2.2.3. Mechanism

Although it was initially thought that the CuNiR reaction mechanism would be a "copy" of the Cd_1NiR one[1340], the most recent results point towards a different strategy. One of the key differences between CuNiR and Cd_1NiR is the nitrite binding mode: nitrite binds to the T2 centre, either oxidised or

reduced, in an asymmetric "bidentate nitrito" mode (Fig. 7-a). Several crystal structures and theoretical calculations[1453,1456,1457,1469,1487-1493] show that nitrite is bound through its two oxygen atoms, in a distorted square pyramidal geometry (with the two oxygen atoms of nitrite plus the two nitrogen atoms of the histidine residues in the distorted base), where the longest Cu-O bond is with the oxygen atom closest to the conserved aspartate residue (Fig. 14-c). This surprising binding mode (unexpected because copper model complexes bind nitrite, generally, through the nitrogen atom[1453,1494-1497] (exceptions in[1498-1500])) is thought to be directed by the conserved active site amino acid residues, namely the aspartate and histidine residues, but also by the hydrophobic isoleucine residue present on top of the nitrite binding site.[1453,1454,1457,1469,1493,1501,1502]

The mechanism of nitrite reduction to NO by CuNiR is less well characterised than the Cd_1NiR one, with several details being not known yet.[1503] To begin with, there is a debate about which specie binds the nitrite - the oxidised or reduced T2 centre - with both hypotheses being supported by several crystallographic and kinetic studies.[1340,1453,1454,1456,1461,1492,1493,1504-1517] In solution, CuNiR follows a random sequential steady-state mechanism, with two alternative routes: (i) T2 centre reduction followed by nitrite binding or (ii) nitrite binding to the oxidised T2 followed by T2 reduction.[1507,1510,1515,1517] The prevailing route is determined by the nitrite concentration and pH (*e.g.*, the second route prevails at high nitrite concentration or high pH, when the T1 to T2 electron transfer is the rate-limiting step). Moreover, the kinetic studies suggest that the reduced T2 centre can exist in two interconvertible forms, where only one form is catalytically active.[1461,1507,1509] Although the inconsistent results may be ascribed to different assay conditions and/or to the presence of CuNiR molecules with inactive reduced T2 centres, a definitive consensus is far from being established and both routes are here considered **-**random sequential mechanism. Accordingly, the beginning of the catalytic cycle is depicted with the T2 centre reduction, followed by nitrite binding (Fig. 14-a→b→c) and with the nitrite binding to the oxidised T2 centre, after which an electron is transferred from the T1 centre (Fig. 14-a→d→c).

Several elegant combinations of experimental and theoretical studies confirmed that the T2 centre reduction is triggered by a protonation step (Fig. 14-a→b or d→c).[1458,1467,1492,1506,1514,1518-1522] Theoretical calculations[1490,1492] suggested that it is protonation of the conserved aspartate residue that increases (electrostatically) the reduction potential of the T2 centre and, thus, drives the

experimentally observed electron transfer from T1 to T2 (with T1 oxidation/T2 reduction), followed by nitrite reduction and NO release. In addition, nitrite binding has also been suggested to induce a favourable shift in the reduction potential of the T2 centre.[1493] Therefore, the nitrite-bound reduced T2 centre, with a neighbouring protonated aspartate residue (Fig. 14-c), is suggested to be the key complex that initiates the nitrite reduction[1492], regardless of the order of the events that leads to its formation.

Once the square pyramidal (T2)Cu^{+}-O(N)O complex (Fig. 14-c) is formed, the reaction is believed to proceed with the protonation of the oxygen atom to be abstracted. Steady-state kinetic studies shown the existence of two protonation equilibriums that were attributed to the only two ionisable conserved residues present in the T2 centre, the aspartate (to which is attributed a pK_a of ≈5) and histidine residues (pK_a ≈7).[1454,1457,1462,1467,1487,1501,1505,1506,1523,1524] Both residues were found to be essential for the CuNiR activity[1454,1462,1468,1487,1501,1506,1520], but theoretical calculations suggested that the proton is transferred from the protonated aspartate residue[1492] (Fig. 14-c→e). The subsequent N-OH bond cleavage is thought to be facilitated by an emergent back-bonding interaction between the copper and the nitrite oxygen atoms[1492]**:** (i) on the initial nitrite-copper complex (Fig. 14-e) there is no back-bonding[1525]; (ii) along the reaction coordinate, as the N-OH bond is elongated, its σ-anti-bonding orbital is lowered in energy and becomes mixed with the copper HOMO, the d_{x2-y2}, receiving electron density from the copper. This back-bonding is made possible by the "bidentate nitrito" binding mode of nitrite, which allows for an efficient interaction between the copper and the nitrite oxygen atoms.[1492] The back-bonding interaction would strengthen the Cu-OH bond and weaken the N-OH bond, leading to the N-O bond homolysis and, eventually, to the NO dissociation from the copper complex (Fig. 14-e→a).[1492]

Unfortunately, the details of the NO release from the copper complex are still elusive. The catalytic cycle would continue with the addition of a second proton, probably transferred from the histidine residue[1454,1462,1468,1487,1501,1506,1520], to yield a bound water molecule (Fig. 14-a), but the order of the protonation / NO release was not yet definitively established. Nevertheless, the side-on Cu^{+}-NO complex observed in some crystal structures[1457,1489,1491] does not seem to be part of the catalytic cycle[1509,1526].

space (=blank line)

In summary, the key steps for the CuNiR-catalysed NO formation are the proton transfer from the aspartate residue to the nitrite molecule, followed by the electron transfer from the reduced copper to the now protonated nitrite **-**proton transfer triggering electron transfer.[1492] According to the suggested mechanism, the success of the N-O bond lysis by the CuNiR is due to the unusual nitrite binding mode and to the presence of a proton donor amino acid residue[1492]: (i) the "bidentate nitrito" binding mode of nitrite enables an effective back-bonding interaction that lowers the activation barrier for the N-OH bond cleavage and simultaneously stabilises the Cu-OH complex to be formed; (ii) the presence of a proton donor residue (the protonated aspartate) is essential for the stabilisation of the copper complex that will be formed after the N-O bond cleavage (Cu-OH). It should be noted that, if the N-O bond was cleaved without the proton being transferred, it would result in the formation of a Cu^{2+}-O^{-} complex, where the charge density is not stabilised (energetically very unfavourable, >50kcal/mol *versus* <16kcal/mol [1492]). In this point, the choice of an aspartate, a residue with a low pK_a value that is not usually associated with the proton donation, is intriguing (in the CcNiR, CSNiR and Cd_1NiR-catalysed reactions, the proton donors were found to be the "expected" positively charged residues, namely histidine, arginine and lysine residues). The strategies to reduce nitrite to NO followed by Cd_1NiR (an *old* enzyme) and CuNiR (a *de novo invention* of the oxic era) are, thus, remarkably different.

4.2.3. Signalling nitrite reduction to nitric oxide by a molybdenum-dependent enzyme

The mammalian signalling molybdenum-dependent nitrite reduction to NO (eq. 63) can be achieved with the molybdenum centre of the xanthine oxidase (XO) enzyme (xanthine:dioxygen oxidoreductase, EC 1.17.3.2).

$$NO_2^- + 1e^- + 2H^+ \xrightarrow{\text{pyranopterin}\ [Mo]} {}^{\bullet}NO + H_2O \qquad (63)$$

$$E^{o\prime}(NO_2^-/{}^{\bullet}NO) = +0.34\ V$$

As described under *"Nitrite on signalling pathways"*, besides mammalian XO, also mammalian AO (section 3.1.1.3.) and bacterial AOR (section 3.3.) **-**all enzymes of the XO family (Fig. 6-c; section 4.)**-** catalyse the nitrite reduction to NO. Mammalian XO and AO are structurally very similar, both comprising one identical molybdenum centre (described below), two Fe/S and one FAD centre. Bacterial AOR holds a slightly different molybdenum centre (apparently with a terminal oxo group, instead of the sulfo group of XO and AO[1527], and with the pyranopterin cofactor esterificated with cytidine monophosphate (Fig. 6-c(i)) and only two Fe/S (no FAD centre). *Desulfovibrio gigas* AOR was the first (in 1995) XO family member for which the crystal structure was determined[1528-1530], with the first mammalian XO and AO structures being reported only in 2000 [1531] and in 2012 [1532,1533], respectively. In order to restrict the information presented to a manageable size, for the sake of simplicity, this review will focus only the mammalian XO, the *bench mark* of this family of molybdoenzymes.

Physiologically, mammalian XO is a key enzyme in purine catabolism, where it catalyses the hydroxylation of both hypoxanthine and xanthine to the terminal metabolite urate, with the simultaneous reduction of dioxygen to superoxide anion radical and hydrogen peroxide.[585-591] In this sense, the XO is quite different from the previously described enzymes: XO has two "classic" substrates **-**hypoxanthine/xanthine and dioxygen**-** and two active sites to react with each substrate **-**molybdenum and FAD centres, respectively.

4.2.3.1. Enzymatic *machinery*

Mammalian XO is a cytoplasmatic (see details in section 3.1.1.3) homodimer (≈290kDa), with each monomer folded into three domains[1531,1534]: (i) one small (≈20kDa) N-terminal domain, constituted by two subdomains (characterised by α-helical and β-sheet structures), each with one [2Fe-2S] centre (Fe/S I and II, respectively); (ii) a second (≈40kDa) domain, holding one FAD; and (iii) one large (≈85kDa) C-terminal domain, also constituted by two subdomains that bind the molybdenum centre at their interface (Fig. 15-a,b). Although the two monomers contact each other through the

molybdenum domain, their molybdenum centres are ≈50Å apart, suggesting that the two monomers act independently.

The molybdenum centre, solvent-accessible at the bottom of a 14,5Å hydrophobic channel, is the XO active site responsible for the hydroxylation half-reaction.[1531,1534] It holds the molybdenum atom coordinated in a distorted square pyramidal geometry, with an apical oxo (=O) group and with the four equatorial positions occupied by one essential sulfo (=S) group, one labile hydroxo (-OH) group and two sulfur atoms of the *cis*-dithiolene (–S–C=C–S–) group of the pyranopterin cofactor molecule (Fig. 6-c(i); Fig. 15-c).[1531,1534,1535]. This active site also comprises four conserved glutamate (Glu_{802}, Glu_{1261}), glutamine (Gln_{767}) and arginine residues (Arg_{880}, bovine enzyme numbering), essential for the hydroxylation reaction (Fig. 15-c). In addition, two phenylalanine residues (Phe_{914}, Phe_{1009}) seem to be important to position the substrate (hypoxanthine/xanthine) in front of the equatorial labile Mo-OH group, in a plane parallel to the apical Mo=O group of the molybdenum center (Fig. 15-c). Closest to the molybdenum centre, is the Fe/S I, coordinated by the $Cys_{113}XXCys_{116}X_{(n)}Cys_{148}XCys_{150}$ motif. Further away, in the proximity of the FAD centre, the Fe/S II is coordinated by $Cys_{43}XXXXCys_{48}XXCys_{51}X_{(n)}Cys_{73}$, in a folding that resembles that of plant [2Fe-2S] ferredoxins.[1536,1537] At last, FAD, the active site responsible for the reduction half-reaction, is bound in an extended conformation in a deep cleft of the FAD domain, with the *si*-side of the isoalloxazine ring accessible to the solvent. On the whole, the four redox centres (molybdenum, Fe/S I, Fe/S II and FAD centres) are aligned in an almost linear fashion (Fig. 15-b), defining an intramolecular electron transfer pathway that rapidly delivers the electrons from the molybdenum centre (where the hydroxylation takes place) to the FAD (to reduce dioxygen).

space (=blank line)

In the context of the XO enzymatic *machinery*, it is pertinent to mention here the structural changes responsible for the interconversion of XD into XO.

The mammalian XO enzymes are synthesised as a NAD^+-dependent dehydrogenase form **-**XD**-** and, under normal physiological conditions, exist mostly as such in the cell.[585-591] However, the XD form can be readily converted into a "strict" oxidase form **-**the XO[1538] The distinction between XD and XO is made based only on the electron acceptor used by each form: while XD transfers the electrons preferentially to NAD^+ (although it can also use dioxygen), XO fails to react with NAD^+ and uses

exclusively dioxygen (as the oxidase classification indicates).[1550-1554,1560,1561] At this point, it should be mentioned that both dioxygen and NAD^+ react at the FAD centre (*i.e.*, the electrons introduced at the molybdenum centre, during xanthine hydroxylation, are transferred to the FAD in both enzyme forms; once in the FAD centre, the electrons react with dioxygen or NAD^+, depending on the affinity of each enzyme form).

The dehydrogenase into oxidase conversion, either *in vivo* or *in vitro*, can be reversible, through oxidation of the Cys_{535} and Cys_{992}, or irreversible, by proteolysis after Lys_{551} or Lys_{569}.[589,1531,1549-1556,1558,1560,1562-1566] The substrate switching (dioxygen *versus* NAD^+) triggered by the conversion is achieved by the following mechanism[589,1531,1566]: the disulfide bond formation or the proteolysis drive a movement of the 423-433 loop, on the *si*-face of the FAD isoalloxazine ring, that shifts the Asp_{429} away and bring the Arg_{426} guanidinium group closer. In this way, the loop displacement changes the electrostatic potential (from negatively to positively charged) in the vicinity of the FAD, increasing the FAD midpoint potential. In addition, the novel loop position obstructs the access to the FAD binding site, blocking the NAD^+ binding to FAD. In concert, these conformational changes, that occur only in the FAD centre, are responsible for the different substrate specificity of XD and XO. The fact that the global folds of XD and XO show no significant changes at the Fe/S and molybdenum centres[589,1531,1566] is consistent with the kinetic studies that demonstrate that the two enzyme forms are virtually identical in respect to the binding and catalysis of substrates at the molybdenum center[585-591]. This is also the case of the nitrite reduction reaction (that, as will be described, occurs at the molybdenum centre).

space (=blank line)

The concerted conformational changes responsible for the dehydrogenase into oxidase conversion suggest that the conversion could play a role *in vivo*, in a situation similar to the one discussed for Nb, Cc or Cd_1NiR. Indeed, the dehydrogenase into oxidase conversion could be the basis of several cellular regulatory strategies: (i) proteolysis, triggered by an hypoxic event, would contribute to the formation of an enzyme form **-**XO**-** that favours the nitrite reduction/NO formation (instead of the NAD^+ reduction by XD; as discussed in section 3.1.1.3.); (ii) as oxidative stress conditions develop (after reperfusion or in an event not related to ischaemia), the concentration of cellular reduced thiols decreases and the population of disulfide-containing XO molecules increases, would be the reactive

oxygen species ($O_2^{\bullet-}$ and H_2O_2) formed by XO that would be responsible for signalling cascades (see, *e.g.*,[1567,1568]). The same mechanism would contribute to some reactive oxygen species-mediated diseases[627-635] (like ischaemia-reperfusion injury[636-641,1569-1572] or ethanol toxicity[570,642-647]), in situations where the cellular antioxidants could not cope with the overproduction of those reactive species by XO, accounting, in this way, for the well-accepted XO pathological role. Therefore, XD/XO could be another protein type with allosteric/posttranslational control of the catalysis.

4.2.3.2. *Promiscuity*

To no other of the enzymes here described, the term *promiscuity* makes more sense: XO has a "broad specificity" for both reducing and oxidising substrates. Besides the well-known hydroxylation of hypoxanthine and xanthine, XO catalyses the oxidation of a wide variety of aldehydes and substituted pyridines, purines, pteridines and related compounds.[567-573,585-591,609,610,1573,1574] XO can also catalyse the reduction of several compounds apart from dioxygen (or NAD^+ by XD), including several sulfoxides, N-oxides and nitrate and nitrite.[611-623] This *promiscuity* has suggested the XO involvement in the xenobiotic metabolism, where it can oxidise the more-polar nitrogen-containing heterocycles. Therefore, XO, in conjunction with AO and the cytochrome P_{450}-containing system (that efficiently oxidises lipophilic aromatic compounds), appear to be responsible for the detoxification of aromatic compounds, activation/deactivation of several pro-drugs (*e.g.*, anti-neoplasic and anti-hypertensive drugs), natural products and "toxic" activation of drugs and azo dyes (used as colorants in food, drink, and cosmetics) in mammals.[605,607,623-626,1575-1582]

4.2.3.3. Mechanism - xanthine hydroxylation

Before discussing the nitrite reduction mechanism, it is useful to briefly review the xanthine hydroxylation reaction mechanism.

In general, the molybdoenzymes (of all families) catalyse the transfer of an oxygen atom from water to the product or from the substrate to water, in reactions that imply a net exchange of two electrons and in which the molybdenum cycles between Mo^{6+} and Mo^{4+}.[585-591] It is based on this catalytic feature that these enzymes are commonly referred to as oxo-transferases. Most of the enzymes of the XO family, and XO in particular, catalyse the cleavage of a C-H bond, with the subsequent oxygen atom insertion in a novel C-O bond, in reactions of oxidative hydroxylation. But there are exceptions, namely, the hydroxybenzoyl-CoA reductase, that catalyses the irreversible dehydroxylation (an oxygen abstraction reaction) of the phenol ring, and the CO dehydrogenase, since the CO_2 formation from CO does not involve the hydrolysis of a C–H bond.

In its reaction with xanthine, the XO molybdenum centre catalyses the insertion of its equatorial oxygen atom into the xanthine moiety to produce urate (eq. 64; Fig. 16-a→b).[585-591] The two electrons, thus, introduced into the molybdenum ($Mo^{6+}\rightarrow Mo^{4+}$) are, then, rapidly distributed throughout the Fe/S and FAD centres, according to their reduction potentials. At the FAD centre, the electrons are finally transferred to dioxygen (or NAD^+, in XD), to give superoxide anion radical and hydrogen peroxide (or, in XD, NADH). The intramolecular electron transfer (Mo→Fe/S I→Fe/S II→FAD) is, therefore, an integral aspect of the XO catalysis. The XO-catalysed hydroxylation reaction is, in this way, quite different from the monooxygenases reaction, as XO generates (rather than consumes) reducing equivalents and uses dioxygen as an oxidant and not as the source of oxygen atoms (which in the XO case is, ultimately, water).

$$\text{xanthine} + H_2O \xrightarrow{\text{pyranopterin}\ \boxed{Mo}} \text{urate} + 2e^- + 2H^+ \qquad (64)$$

Before resume to the nitrite reduction mechanism itself, two remarks should be made: (i) the nitrite reduction is virtually identical in both XO and XD[623]; (ii) for nitrite to be reduced, the electrons driven

from the xanthine oxidation (or other XO reducing substrate) can not be deviated to dioxygen, NAD^+ or other electron acceptor **-**reason why, the mammalian nitrite reduction/NO formation is thought to occur only in hypoxic-anoxic conditions (as described in 3.1.1.3.).

4.2.3.4. Mechanism - nitrite reduction

To catalyse the nitrite reduction to NO, XO, like Cd_1NiR and CuNiR, has to bind nitrite, transfer one electron, cleave a N-O bond and release the NO thus formed. However, the mechanism by which the XO molybdenum centre carries out this reaction still has several lacunae to fill. Nevertheless, an outline of the mechanism can easily be drawn (Fig. 16) taking in account the following points.

First, nitrite is believed to be bound through one of its oxygen atoms ("nitrito" binding mode (Fig. 7-a)). The $Mo^{6+,5+,4+}$ chemistry is dominated by the formation of oxides and sulfides, but the strong tendency of molybdenum to bind oxo groups is balanced by its ability to easily lose a single oxygen atom[1583]; this chemistry makes the molybdenum cores excellent "oxygen atom exchangers", as long as the thermodynamics of the reactions is favourable[1584]. In accordance, and as far as we know, all substrates or products of XO family enzymes interact with the molybdenum atom through an oxygen atom.[585-591] Second, nitrite should bind to the reduced molybdenum centre. Kinetic and spectroscopic (EPR) studies showed that the NO formation only occurs after molybdenum reduction and suggested a role for nitrite in the displacement of urate from the active site.[623] Third, the reduced molybdenum transfers one electron to nitrite, being oxidised in this process. Spectroscopic (EPR) assays demonstrated unequivocally that the reduced molybdenum centre (enzyme reduced with compounds that interact at the molybdenum, Fe/S or the FAD centres) is oxidised in the presence of nitrite.[623] Moreover, the simultaneously NO formation (see the fourth point below) showed that nitrite was concomitantly oxidised. Fourth, the molybdenum centre catalyses the N-O bond cleavage and releases the NO thus formed. That the molybdenum is, in fact, the centre responsible for the NO formation was definitively demonstrated with a combination of spectroscopic (EPR to follow the molybdenum oxidation) and electrochemical (to measure the NO formation) methods, using the

molybdenum specific inhibitor allopurinol.[623] In addition, the nature of the product of the nitrite reduction (*i.e.*, NO) was independently confirmed by several other methodologies.[618,619,623] In this context, it should be noted that one XO family enzyme catalysing a reduction, oxygen abstraction, reaction is not a novelty, since the 4-hydroxybenzoyl-CoA reductase catalyses the irreversible dehydroxylation of the phenol ring (to yield benzoyl-CoA).[1585-1587] Fifth, the reaction is thought to be triggered by a protonation event. The nitrite reduction/NO formation is greatly accelerated at acid conditions (pH<7) and involves two protonation equilibriums with pK_a values of 5.9 and 6.8.[1588] The residues responsible for the protonation equilibriums, however, were not yet identified. This pH dependency is also observed on Cd_1NiR[1420,1589] and CuNiR enzymes[1454,1457,1462,1467,1487,1501,1505,15061523,1525] and must be due to the protons necessary to carry out the reaction (eq. 63). Sixth, the NO formation and release is expected to involve only one bond cleavage. Bearing in mind that nitrite is believed to be bound via the "nitrito" binding mode, the simplest way to release NO would be through one bond cleavage (MoO-NO (Fig. 7-b(ii))). Although it is possible that NO release requires two bond cleavages (MoON-O and then Mo-ON (Fig. 7-b(iii))), it is improbable: besides involving an extra "unnecessary" step, that step is not consistent with the known molybdenum chemistry, since it would leave it without any oxo group (not consistent with the known molybdenum chemistry mentioned in the first point).

In view of these considerations, it is suggested that the XO-catalysed nitrite reduction follows the mechanism depicted in figure 16.[623,1590] After the molybdenum reduction by xanthine (Fig. 16-a→b), nitrite binds to the reduced molybdenum, via the "nitrito" binding mode, displacing the urate (Fig. 16-b→c).[623] Once the Mo^{4+}-O-N-O complex is formed, the reaction is suggested to proceed with the protonation of the oxygen atom bound to the molybdenum (Fig. 16-c→d).[1590] The conserved Glu_{1261} residue is well positioned to act as the proton donor, but this key role must wait for theoretical studies to be confirmed.[1590] This protonation step could trigger the electron transfer from the reduced molybdenum to the now protonated nitrite, as was described for CuNiR (section 4.2.2.), causing the N-OH bond homolysis and subsequent NO release (Fig. 16-d→e).[1590] The mechanism by which the N-OH bond cleavage is undertaken is presently not known. However, it is tempting to think that the strategy followed by XO would be analogous to the CuNiR one, since both metals share the same square pyramidal geometry and have a redox active HOMO on the xy plane (d_{xy} and d_{x2-y2}, for

molybdenum[1591-1593] and copper, respectively).[1590] Furthermore, also in XO, the protonation of nitrite leads to the formation of a more stable "future" metal complex: in this case, the Mo^{5+}-OH (Fig. 16-e) instead of Mo^{5+}-O^- (similar to the CuNiR Cu-OH *versus* Cu-O^-). The pK_a of the molybdenum coordinated ligands change dramatically with the oxidation state, with the lower oxidation states possessing highly protonated ligands.[1594,1595] For this reason, on the Mo^{5+} complex (Fig. 16-e), both the oxygen and the sulfur atoms should end up protonated, either the protonation event occurs before (as suggested) or after the NO release. Finally, also the choice of the proton donor **-**if it is confirmed**-** seems to be similar: one aspartate in CuNiR and the Glu_{1261} residue in XO.[1590]

In this point, it is instructive to compare the nitrite reduction to the enzymatic reductive dehydroxylation of 4-hydroxybenzoyl-CoA to yield benzoyl-CoA (which requires the cleavage of a C-OH bond and the formation of a new C-H bond). This reaction is proposed to follow a Birch-like reduction mechanism, involving single electron and proton transfer steps to the phenol ring, with formation of radicalar intermediates.[1586] Moreover, for the reaction to proceed, it is considered essential to stabilise the ketyl radical intermediate formed, what is accomplished by the presence of the carbonyl moiety on the phenol ring. On the suggested nitrite reduction mechanism, the reduction of nitrite also involves single electron and proton transfer steps and the formation of radicalar "intermediates". But, in the nitrite case, the radicals formed **-**NO and the Mo^{5+} core**-** are stable enough to have independent existence.

At this stage (Fig. 16-e), one molecule of NO is already formed and released. However, because xanthine oxidation is a two electron process ($Mo^{6+}\rightarrow Mo^{4+}$; eq. 64), the molybdenum centre still has one electron (Mo^{5+}) to reduce another nitrite molecule. Thus, the reaction is proposed to proceed with the binding of a second nitrite molecule.[623] To generate a good leaving group, water (Mo^{5+}–OH_2), the consumption of one proton is suggested (Fig. 16-e→f). Subsequently, nitrite displaces the water molecule (Fig. 16-f→g) and, after a second cycle of nitrite reduction/molybdenum oxidation, a second NO molecule is released (Fig. 16-g→h→a). The molybdenum is now in a 6+ oxidation state (Fig. 16-a), which would favour the deprotonation of its ligands[1594,1595], and ready to start another catalytic cycle.

space (=blank line)

In summary, although the mechanism of XO-catalysed nitrite reduction is not yet fully understood,

it seems probable that a strategy similar to the CuNiR one is employed. Therefore, in XO, nitrite is suggested to bind to the reduced molybdenum centre, in a "nitrito" binding mode, and, after a protonation event, the N-OH bond is believed to be homolytically cleavage and the NO "automatically" released.

space (=blank line)

The molybdenum unique chemistry makes the molybdenum centres excellent '"oxygen atom exchangers", precisely what is needed to catalyse the oxygen atom transfer from nitrite to water (or urate), to form NO (eq. 63). In this context, it is surprising that no "dedicated" molybdenum-containing nitrite reductase is known to exist: the molybdenum centres are widely used for oxo-transfer reactions (both abstractions and insertions) of the carbon, sulfur and nitrogen metabolism (including in NaR and MoNiOR)[585-591,1261-1266], but Biology did not develop any "dedicated" molybdoenzyme to catalyse the nitrite reduction. These considerations made us think that, probably, the living organisms are using molybdenum to synthesise NO, but employing enzymes that *we attribute* to other functions. Mammalian XO and AO, plant C-NaR and bacterial AOR and NaR may become the first examples of such utilisation to be described.

4.3. NITRITE OXIDATION TO NITRATE

The nitrite oxidation has received far less attention than its reduction. On the "prokaryotic world", where it constitutes the last step of nitrification (Fig. 1, black arrows; section 2.2.), the nitrite oxidation has been less studied, because ammonium oxidation has long been thought to be the limiting step[101]; whereas on the "eukaryotic world", until the last decade, both nitrite and nitrate were considered "useless" end-products of NO metabolism.

Bacterial nitrite oxidation is carried out by chemolithoautotrophs, that derive energy from the oxidation of nitrite to nitrate, in a strictly aerobic process, where all the carbon needs can be satisfied with carbon dioxide assimilation (through the Calvin cycle)[96-99,102,1596-1598]. Nevertheless, the nitrite oxidation is thermodynamically unfavourable and the lithotrophic growth is slow and inefficient.[1597,1599]

The nitrite-oxidising bacteria are, thus, hard to maintain and not many groups are trained to keep them in culture[102], reason why they **-** and their key enzyme nitrite oxidoreductase **-** have been poorly studied.

Interestingly, the anaerobic AnAmmOx bacteria "*Candidatus* Kuenenia stuttgartiensis" (see section 2.2.) also seem to oxidise nitrite to nitrate. It was recently suggested that the gene product initially annotated as a nitrate reductase (nitrite forming) may be involved on the nitrite oxidation (nitrite consuming).[110,1600] This oxidation would produce electrons that could be transferred to the quinone pool (through a membrane-bound subunit), or be used in different reduction reactions (via putative blue copper- and haem-containing proteins).

Eukaryotic nitrite oxidation began to be studied only a few years ago and no "dedicated" nitrite oxidase was yet described to exist. Instead, eukaryotes seem to use several haemic proteins to oxidise nitrite to nitrate or to nitrogen dioxide (as described in sections 3.1.2. and 3.2.2.).

Presently it is not possible to discuss the molecular mechanism of nitrite oxidation, but only review the (still limited) knowledge on the bacterial MoNiOR enzymatic *machinery* and to speculate about its mechanism (section 4.3.1.).

4.3.1. Dissimilatory nitrite oxidation to nitrate

The dissimilatory nitrite oxidation to nitrate (eq. 65) is achieved with the molybdenum centre of the molybdenum-containing nitrite oxidoreductase (MoNiOR) enzymes (tentatively classified as nitrite:acceptor oxidoreductase EC 1.7.99.4).

$$NO_2^- + H_2O \xrightarrow{\text{pyranopterin Mo}} NO_3^- + 2e^- + 2H^+ \qquad (65)$$

$$E^{o'}(NO_3^-/NO_2^-) = +0.43V$$

4.3.1.1. Enzymatic *machinery*

The known MoNiOR (product of *nxr* genes) are membrane-bound proteins that can be divided into two groups depending on their subcellular localisation[1608]: (i) enzymes anchored on the periplasmatic side of the cytoplasmatic membrane (*Nitrospira*, *Nitrospina*, and "*Candidatus* Nitrotoga" MoNiOR)[1597,1601-1607] and (ii) enzymes anchored on the cytoplasmatic side of the intracytoplasmatic and cytoplasmatic membranes (*Nitrobacter* and *Nitrococcus* MoNiOR)[102,1598,1601,1609-1611]. Very recently, a new bacterium was added to the list of the "few" nitrifying organisms: *Nitrolancetus hollandicus* that belongs to the widespread phylum *Chloroflexi* and is believed to hold a cytoplasm-faced MoNiOR similar to the *Nitrobacter* and *Nitrococcus* ones.[1612]

Presently there is no structure of a MoNiOR, but there are evidences that MoNiOR must share several structural features with a NaR enzyme[1613], that catalyses the reverse reaction (nitrate reduction to nitrite(see[1613])), more precisely with the "respiratory"[1625] NaR.[1628] The *Nitrobacter hamburgensis* MoNiOR, a cytoplasm-faced enzyme, is an heterotrimer constituted by a catalytically active $\alpha\beta$-complex (≈115 and 65kDa) that interacts with a membranar *c* type haemic γ-subunit (≈32kDa).[1598,1601,1629,1630] The *Nitrospira moscoviensis* MoNiOR, a periplasm-faced enzyme, is also a catalytically active $\alpha\beta$-complex (≈130 and 46kDa)[1604], but the hypothetical transmembranar γ-subunit, responsible for the electron transfer between the β-subunit and the electron transport chain, was not yet identified and awaits experimental clarification[1604,1606]. However, for a periplasm-faced enzyme, the electrons from quinol oxidation do not need to pass back across the membrane and, perhaps, a membranar haemic subunit would not be needed.[1627]

The MoNiOR β-subunit (product of *nxrB* gene) of *Nitrobacter hamburgensis* displays a cysteines distribution identical to the one of the *E. coli* NaRH (with a total sequence identity of 45% [99]) and, accordingly, it probably holds three [4Fe-4S] and one [3Fe-4S] centres.[99] Four cysteine-rich binding motifs of Fe/S were also identified in *Nitrococcus* and *Nitrospira* β-subunits.[1606] Due to the similarities with NaRH, the MoNiOR β-subunit is believed to be responsible for the electron transfer from the α-subunit (where the nitrite oxidation should occur) to the γ-subunit or directly to the membrane electron transport chain. The α-subunit (product of *nxrA* gene) shows also a significant similarity to the C-terminal sequences of the *E. coli* NaRG and the biochemical characterisations

carried out point towards the presence of one Fe/S and one molybdenum centre in both MoNiOR groups.[99,1601,1604,1606,1630,1631] The molybdenum centre, where the nitrite oxidation should occur (the enzyme active site), is assumed to be coordinated by two pyranopterin cofactor molecules, as in NaRG (that belongs to the dimethylsulfoxide reductase family of the molybdoenzymes; Fig. 6-c; see section 4.). The molybdenum centre also should hold an oxo group (again as NaRG) that, in the course of the reaction, would be transferred to the nitrite molecule to yield nitrate.

4.3.1.2. Mechanism

Little is known about the MoNiOR-catalysed reaction. As in other molybdenum-containing enzymes, the "new" oxygen atom of nitrate is derived from water (eq. 65) and not from dioxygen[1632-1635], with the molybdenum centre probably intermediating the oxygen atom transfer (see the description of a molybdoenzyme catalytic mechanism in section 4.2.3). The elucidation of the structure and mechanistic strategies followed by cytoplasm- and periplasm-faced MoNiOR must wait for future experimental work.

space (=blank line)

It is interesting to note that nitrite-oxidising bacteria are versatile organisms that catalyse both nitrite oxidation and nitrate reduction, "switching" from aerobic nitrification to anaerobic grow by dissimilatory nitrate reduction (with pyruvate (*Nitrobacter* [1597,1598,1601,1636-1638]) or hydrogen (*Nitrospira*[1597]) as electron donors). If the bacteria employ the same enzyme or synthesise a different "version", is not known. However, the *Nitrobacter hamburgensis* (cytoplasm-faced) MoNiOR is able to catalyse also the nitrate reduction, at least *in vitro* [1601,1629,1630], while the *Nitrospira* (periplasm-faced) enzyme is not[1606].

Presently, the similarities between (i) "respiratory" cytoplasm-faced NaRGHI, (ii) periplasmatic NaR, (iii) cytoplasmatic anabolic NaR (nitrogen assimilation) and (iv) cytoplasm- and (v) periplasm-faced MoNiOR suggest that Biology had found one perfect "solution" to interconvert nitrate and nitrite: the pyranopterin-coordinated molybdenum centre.

5. OUTLOOK

Nitrite is long known as one of the players of the nitrogen biogeochemical cycle, participating in key pathways crucial to life on Earth and to the planetary "recycling" of nitrogen. More recently, nitrite is also being recognised as a molecule relevant to cell signalling and survival, virtually, in all forms of life. In spite of all those different biological functions, the nitrite handling seems remarkably similar.

Iron is probably the most used metal to handle nitrite. As discussed, prokaryotes reduce nitrite for assimilatory and dissimilatory purposes, using different haem types (*c*, sirohaem and d_1), with diverse axial coordinations (lysine, cysteine and histidine residues), in several protein arrangements (dimers/monomers, with several more haems, with only one additional haem or one Fe/S). Nevertheless, in spite of the differences, all the enzymes discussed (i) bind nitrite in the "nitro" mode to ferrous iron, (ii) activate the nitrite molecule, allegedly, through iron back-bonding and hydrogen bonding (to positively chargedresidues at the active site), (iii) promote the double protonation of one of the nitrite oxygen atoms (releasing a water molecule) and (iv) form an iron-NO complex. Subsequently, the iron-NO complex is cleaved -and the NO is released- or the complex is retained and further protonated and reduced -to yield ammonium.

The understanding of a biochemical pathway step requires the comprehension of the "chemical logic" behind the respective reaction and, to achieve that knowledge, a detailed comparison between the reaction mechanism of CcNiR, CSNiR and Cd_1NiR (and also CuNiR) is instructive: the wealth of information here discussed show, clearly, that still much remains to be understood, in particular about the unique characteristics of the d_1 haem and its ability to promptly release NO. Also the redox centres arrangements are still puzzling: the number of electrons needed to reduce nitrite (one or six) does not correlate at all with the number of intramolecular redox centres present in the enzymes (two or as many as eight). It is well accepted that it is kinetically more advantageous to have a protein with several redox centres than several associations of individual proteins (because in a protein with multi-centres there is no time delay due to the diffusion and collision of single proteins; thus, the electron transfer rate is effectively increased). A multi-centre protein also allows for a "fine-tuned" regulation mechanism, in particular through conformational regulation.

Are these the reasons for the five haems of CcNiR (electron "storage" to increase rate) and the two haems of Cd_1NiR (control the NO generation)? And what about the two redox centres of CSNiR and of CuNiR?

The prokaryotic haemic strategy to form NO was so successful that we can find several "vestiges" of the earlier, pre-aerobic, pathways on the nowadays mammalian and plant cells[742]: *e.g.*, under hypoxia/anoxia, the HG or Mb are taking advantage of the "old" haem redox chemistry, thoroughly explored on the prokaryotic haemic nitrite reductases, to form NO. The "lessons" from the ancestral prokaryotic world become more clear when it is realised that the chemistry behind an anaerobic activity is a perfect solution for the contemporary hypoxic/anoxic generation of NO. However, we can not fail to note that, if the aim was to generate NO, why do mammals lost the unique d_1 haem (or even the CuNiR enzyme) and developed only a specialised (complex) oxygen-dependent *b*-haem-containing NOS enzyme? As discussed, the strong binding of NO to ferrous haems imposes a severe mechanistic constraint to the proposed haem-containing nitrite reductases, as well as to NO receptors (*e.g.*, guanylate cyclase) and NO transporters, all of which have to avoid the *dogmatic* scavenging by haem iron; the d_1 haem ability to promptly release NO would have allowed the organism to overcome this "dilemma".

Remarkably, the mammalian cells are also using the haemic proteins to, under normoxic conditions, oxidise nitrite **-**in this case, it is the haem peroxidatic activity that is being "copied". Noteworthy, the usage of this chemistry in prokaryotes is not recognised and only a nitrite oxidoreductase enzyme is presently known to exist **-**the MoNiOR.

Besides haemic iron, also molybdenum can be widely used to handle nitrite. Its unique chemistry makes the molybdenum enzymatic centres excellent "oxygen atom exchangers", precisely what is needed to abstract/insert one oxygen atom from/into nitrite to generate NO/nitrate, or even to remove one oxygen from nitrate to yield nitrite. In fact, the molybdenum "solution" to interconvert nitrate and nitrite was so successful that three types of molybdenum-containing NaR enzymes and two types of MoNiOR enzymes were developed.

As was here discussed, several molybdoenzymes, with different molybdenum centre structures (Fig. 6-c(ii)) and active site pockets, are able to reduce nitrite to generate signalling NO. However, in spite of the differences, the mechanism "outline" of the molybdenum-mediated reactions

is not expected to be much different within the different molybdoenzymes, or even within different reaction types[1639]: the reduced Mo^{4+} cores are proposed to bind nitrite (or other oxo-molecule) through the oxygen atom, acting as a oxo group acceptors, whereas the Mo^{6+} cores are believed to act as oxo group donors (producing an "oxygenated" molecule, *e.g.*, water or urate); in a mechanism where the molybdenum atom intermediates the oxygen atom transfer from one substrate to the second substrate, as long as the thermodynamics of the reactions is favourable. Nevertheless, the structural differences among the several molybdoenzymes should impose different molecular mechanistic strategies (*e.g.*, a "sulfur shift"), most of which remain to be investigated **-**once again, it should be instructive to compare the reaction mechanisms of different molybdoenzymes in order to understand what controls the reactivity ("chemical logic") of the molybdenum centres.

In the context of this "double oxo transfer hypothesis", the nitrite reductase activity of the several molybdoenzymes here described (bacterial AOR and NaR, fungus NaR, plant C-NaR and mammalian XO/XD and AO) is not at all unexpected: these proteins generate NO using the molybdenum redox chemistry already "tested" in diverse oxo-transfer reactions of the carbon, sulfur and nitrogen metabolism. On the contrary, it is surprising that no "dedicated" molybdenum-containing nitrite reductase is known to exist. Are the molybdenum-containing nitrite reductases "disguised" under proteins that *we attribute* to other functions?

Biology also uses copper to reduce nitrite, although, comparatively, its utilisation is not so diversified (only one enzyme type is known, the CuNiR). Nonetheless, the copper-dependent nitrite reduction probably shares many features with the molybdenum handling: nitrite is bound in the "nitrito" mode to the reduced metal and the protonation of one of its oxygen atoms would yield a metal-hydroxo complex and release NO.

In summary, to handle nitrite, Biology developed several strategies, exploring different nitrite binding modes, metals and protein structures, but using a comparable "blueprint". Those strategies are the result of different evolutionary "assays". (i) CcNiR and CSNiR: two "solutions" based on the same basic *machinery* (an haem) to solve the same "problem" (form ammonium), but with distinct biological purposes, "respiration" and assimilation. (ii) Cd_1NiR and CuNiR: two *truly* different "solutions", developed in different geologic eras, to solve the same "problem", the "respiratory" NO formation,

in different organisms. (iii) CcNiR and Cd_1NiR: two "solutions" based on the same basic *machinery* (an haem) to solve two different "problems", "respiratory" formation of ammonium and NO. (iv) "Modular" evolution: penta- and octa-haemic CcNiR, CSNiR (ferredoxin-dependent) and FAD-containing CSNiR (NAD(P)-dependent), "classic" and extended CuNiR, and AOR and FAD-containing XO/XD/AO, all represent fascinating examples of how Biology, once had found a "solution", reuses it, introducing only minor adjustments to respond to specific cellular demands. (v) However, in the context of nitrite reduction, the most striking aspect is the evolutionary convergence, through which virtually all forms life are using a myriad of different metalloproteins to achieve the same objective: reduce nitrite to signalling NO.

To generate nitrite-dependent signalling NO, the organisms "reuse" different metalloproteins, present in the cells to accomplish other functions, and "switch" their activities to a nitrite reductase/NO synthase, when it is necessary. From a chemical point of view, the organisms are just using the redox chemistry of an available redox system and doing a "substrate adaptation" to generate NO. The biological use of a single protein to accomplish more than one function is not a new concept introduced with the nitrite/NO metabolism. This is a well recognised and common phenomenon **-**moonlighting**-** with important implications for systems biology and, in particular, for human physiology and pathology (see, *e.g.*, [1640]). In the nitrite reduction context, this phenomenon is carried out by several proteins and is triggered by the oxygen availability and/or cellular redox status, and, in some cases, by sophisticated (and very interesting) posttranslational modifications that regulate the *new* nitrite reductase activity of the protein. In accordance, it can be hypothesised that the "non-respiratory" nitrite reduction to NO is part of a conserved regulatory mechanism that "translates" the dioxygen availability/cellular redox imbalance into a differentiated flux of NO and, then, into a biological response that would overcome/repair the cellular changes. As F. Cutruzzolà *et al.* as pointed out, nitrite reduction to NO is a ubiquitous function, from a pre-aerobic past[742], that has been "reinvented" and employed ever since.

ABBREVIATIONS

AnAmmOx, anaerobic ammonium oxidation

AO, aldehyde oxidase

AOR, aldehyde oxidoreductase

Cb, cytoglobin

Cc, cytochrome *c*

CcNiR, cytochrome *c*-containing nitrite reductase (multi-*c*-haems-containing nitrite reductase)

CcO, cytochrome *c* oxidase (mitochondrial)

Cd_1NiR, cytochrome d_1-containing nitrite reductase (*c* and d_1 haems-containing nitrite reductase)

C-NaR, cytoplasmatic nitrate reductase of plants

CSNiR, cytochrome sirohaem-containing nitrite reductase (Fe/S and sirohaem-containing nitrite reductase)

CuNiR, copper-containing nitrite reductase (T1 and T2-containing nitrite reductase)

deoxy-Cb, deoxy-cytoglobin; $(Cb)Fe^{2+}$

deoxy-Hb, deoxy-haemoglobin; $(Hb)Fe^{2+}$

deoxy-Mb, deoxy-myoglobin; $(Mb)Fe^{2+}$

deoxy-Nb, deoxy-neuroglobin; $(Nb)Fe^{2+}$

deoxy-NS-HG, deoxygenated non-symbiotic haemic globin of plants; $(NS\text{-}HG)Fe^{2+}$

DNRA, dissimilatory nitrate reduction to ammonium

e-, electron(s)

EPR, electronic paramagnetic resonance spectroscopy

Fe/S, iron/sulfur centre

HAOR, hydroxylamine oxidoreductase

Hb, haemoglobin

HG, haemic globin

Mb, myoglobin

met-Cb, met-cytoglobin; $(Cb)Fe^{3+}$

met-Hb, met-haemoglobin; $(Hb)Fe^{3+}$

met-Mb, met-myoglobin; (Mb)Fe^{3+}

met-Nb, met-neuroglobin; (Nb)Fe^{3+}

MoNiOR, molybdenum-containing nitrite oxidoreductase

NaR, nitrate reductase (all types of enzymes that reduce nitrate to nitrite)

NaRGHI, "respiratory" nitrate reductase, after the name of the encoding genes, *narG*, *H* and *I*

NaRG, NaRH and NaRI, each of the three NaRGHI subunits

Nb, neuroglobin

NO, nitric oxide radical

NOS, NO synthase

NrfA, another designation for CcNiR, after the name of the encoding gene *nrf* (*nitrite reduction with formate*)

NrfB, NrfC, NrfD, NrfH, other proteins that interact with NrfA, belonging to the same encoding *nrf* gene

NS-HG, non-symbiotic haemic globin of plants

oxy-Cb, oxy-cytoglobin; (Cb)Fe^{2+}-O_2

oxy-Hb, oxy-haemoglobin; (Hb)Fe^{2+}-O_2

oxy-Mb, oxy-myoglobin; (Mb)Fe^{2+}-O_2

oxy-Nb, oxy-neuroglobin; (Nb)Fe^{2+}-O_2

oxy-NS-HG, oxygenated non-symbiotic haemic globin of plants; (NS-HG)Fe^{2+}-O_2

P_{50}, the oxygen concentration at which Hb or Mb are half-saturated

RPM-NaR, root specific plasma membrane-bound, succinate-dependent, nitrate reductase of plants

RPM-NiR, root specific plasma membrane-bound nitrite reductase of plants

RSH, thiol compound

RSNO, S-nitrosothiol

T1 and T2, type 1 and 2 copper centres of CuNiR, respectively

TTR, tetrathionate reductase

XD, xanthine dehydrogenase

XO, xanthine oxidase

AUTHOR INFORMATION

Corresponding Author

E-mails: jose.moura@fct.unl.pt

luisa.maia@fct.unl.pt

Biographies

Luisa Maia is a postdoctoral fellow in the group of José Moura, at the Universidade Nova de Lisboa (Portugal). She received her *Ph.D.* degree in Pharmaceutical and Clinical Biochemistry from the Universidade de Lisboa (Portugal) for work on the new catalytic activities of mammalian molybdoenzymes relevant to human patho/physiology. Her current research interests involve structure-activity relationships of metalloenzymes and reactive oxygen and nitrogen species biochemistry (formation, elimination and mediated mechanisms of diseases).

José J. G. Moura has a degree in Chemical Engineering and a Ph.D. in Chemistry and is Professor of Chemistry at Department of Chemistry, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa. Main field of research is Bioinorganic Chemistry and the role of Metals in Biology. 360 articles indexed in ISI Web of Knowledge and an h-index of 52. President of Chemistry Department and President of Scientific Council at FCT-UNL, Portuguese Delegate to COST and INTAS, a member of Scientific Panel in the Calouste Gulbenkian Foundation and FCT-MCTES, and of several scientific editorial boards. In 2006, he was elected Member of Academia das Ciências de Lisboa and in 2010, elected for 2 years as President of the Society of Biological Inorganic Chemistry. He is the Director of FCT-UNL Campus Library. Moura's current research interests include bioinorganic, biophysics, biocatalysis, energy bioconversion (hydrogen), role of metals in biology (haem and non-haem iron, molybdenum, tungsten, nickel, copper, vanadium and cobalt), inorganic systems as models for biocatalysis, spectroscopy (NMR, EPR and Mössbauer), (bio)electrochemistry and protein-protein interactions.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the funding from the projects PTDC/QUI-BIQ/100366/2008 and PEst-C/EQB/LA0006/2011 (FCT/MCTES).

REFERENCES AND NOTES

(1) Binkerd, E.F.; Kolari, O.E. *Food Cosmet. Toxicol.* **1975**, *13*, 661.

(2) Magee, P. H.; Barnes, J. M. *Br. J. Cancer* **1956**, *10*, 114.

(3) Tannenbaum, S. R.; Sisnkey, A. J.; Weisman, M.; Bishop, W. *J. Cancer Inst.* **1974**, *53*, 79.

(4) Craddock, V. M. *Nature* **1983**, *306*, 638.

(5) Bartsch, H.; Montesano, R. *Carcinogenesis* **1984**, *5*, 1381.

(6) Forman, D., Al-Dabbagh, S., and Doll, R. *Nature* **1985**, *313*, 620.

(7) Bartsch, H.; Ohshima, H.; Pignatelli, B. *Mutat. Res.* **1988**, *202*, 307.

(8) Ward, M. H., deKok, T.M. Levallois, P.; Brender, J.; Gulis, G.; Nolan, B.T.; VanDerslice, J. *Environ. Health Perspect.* **2005**, *113,* 1607.

(9) Fraústo da Silva, J.J.R.; Williams, R.J.P. *The Biological Chemistry of the Elements: The Inorganic Chemistry of Life*; Oxford University Press: Oxford, 2001.

(10) Richardson, D.J.; Watmough, N.J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 207.

(11) Galloway, J.N.; Dentener, F.J.; Capone, D.G.; Boyer, E.W.; Howarth, R.W.; Seitzinger, S.P.; Asner, G.P.; Cleveland, C.C.; Green, P.A.; Holland, E.A.; Karl, D.M.; Michaels, A.F.; Porter, J.H.; Townsend, A.R.; Vorosmarty, C.J. *Biogeochemistry* **2004**, *70*, 153.

(12) Bothe, H., Ferguson, S. J.; Newton, W. E. *Biology of the nitrogen cycle*; Elsevier: Amsterdam, 2007.

(13) Jetten, M.S.M. Environ. Microbiol. **2008**, *10*, 2903.

(14) Canfield, D.E.; Glazer, A.N.; Falkowski, P.G. *Science* **2010**, *330*, 192.

(15) Martínez-Espinosa, R.M.; Cole, J.A.; Richardson, D.J.; Watmough, N.J. *Biochem. Soc. Trans.* **2011**, *39*, 175.

(16) Fowler, D.; Coyle, M.; Skiba, U.; Sutton, M.A.; Cape, J.N.; Reis, S.; Sheppard, L.J.; Jenkins, A.; Grizzetti, B.; Galloway, J.N.; Vitousek, P.; Leach, A.; Bouwman, A.F.; Butterbach-Bahl, K.; Dentener, F.; Stevenson, D.; Amann, M.; Voss, M. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2013**,*368*, 20130164.

(17) Galloway, J.N.; Leach, A.M.; Bleeker, A.; Erisman, J.W. *Philos Trans. R. Soc. Lond. B Biol. Sci.* **2013**, *368*, 20130120.

(18) Besides the molybdenum/iron-dependent enzyme, there are also other nitrogenases, with different active site metal compositions.[19-21]

(19) Joerger, R.D.; Bishop, P.E. *Crit. Rev. Microbiol.* **1988**, *16*, 1.

(20) Kessler, P.S.; Mclarnan, J.; Leigh, J.A. *J. Bacteriol.* **1997**, *179*, 541.

(21) McGlynn, S.E.; Boyd, E.S.; Peters, J.W.; Orphan, V.J. *Front. Microbiol.* **2013**, *3*, 419.

(22) Boal, A.K.; Rosenzweig, A.C. *Science* **2012**, *337*, 1617.

(23) Lukoyanov, D.; Yang, Z.Y.; Barney, B.M.; Dean, D.R.; Seefeldt, L.C.; Hoffman, B.M. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 5583.

(24) Scheibel, M.G.; Schneider, S. *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 4529.

(25) Wiig, J.A.; Hu, Y.; Lee, C.C.; Ribbe, M.W. *Science* **2012**;*337*, 1672.

(26) Boyd, E.S.; Peters, W.J. *Front. Microbiol.* **2013**, *4*, 201.

(27) Hoffman, B.M.; Lukoyanov, D.; Dean, D.R.; Seefeldt, L.C. *Acc. Chem. Res.* **2013**, *46*, 587.

(28) Hu, Y.; Ribbe, M.W. *Biochim. Biophys. Acta.* **2013**, *1827*, 1112.

(29) Hu, Y.; Ribbe, M.W. *J. Biol. Chem.* **2013**, *288*, 13173.

(30) Lancaster, K.M.; Hu, Y.; Bergmann, U.; Ribbe, M.W.; DeBeer, S. *J. Am Chem Soc.* **2013**, *135*, 610.

(31) Spatzal, T.; Einsle, O.; Andrade, S.L. *Angew. Chem. Int. Ed. Engl.* **2013** (doi: 10.1002/anie.201303000).

(32) Wiig, J.A.; Lee, C.C.; Hu, Y.; Ribbe, M.W. *J. Am. Chem. Soc.* **2013**, *135*, 4982.

(33) Zhang, L.; Kaiser, J.T.; Meloni, G.; Yang, K.Y.; Spatzal, T.; Andrade, S.L.; Einsle, O.; Howard, J.B.; Rees, D.C. *Angew Chem. Int. Ed. Engl.* **2013** (doi: 10.1002/anie.201303877)

(34) Hewitt, E.J. *Annu. Rev. Plant Physiol.* **1975**, *26*, 73.

(35) Guerrero, M.G.; Vega, J.M.; Losada, M. *Ann. Rev. Plant Physiol.* **1981**, *32*, 169.

(36) Knaff, D.B.; Hirasawa, M. *Biochim. Biophys. Acta* **1991**, *1056*, 93.

(37) Lin, J.T.; Stewart, V. *Adv. Microb. Physiol.* **1997**, *39*, 1.

(38) Fischer, K.; Barbier, G.G.; Hecht, H.J.; Mendel, R.R.; Campbell, W.H.; Schwarz, G. *Plant Cell* **2005**, *17*, 1167.

(39) Flores, E.; Frias, J.E.; Rubio, L.M.; Herrero, A. *Photosynth. Res.* **2005**, *83*, 117.

(40) Moreno-Vivián, C.; Flores, E. *Biology of the nitrogen cycle*; Elsevier: Amsterdam, 2007; p263.

(41) Gates, A.J.; Luque-Almagro, V.M.; Goddard, A.D.; Ferguson, S.J.; Roldán, D.; Richardson, D.J. *Biochem. J.* **2011**, *435*, 743.

(42) Hirasawa, M.; Fukushima, K.; Tamura, G.; Knaff, D.B. *Biochim. Biophys. Acta* **1984**, *791*, 145.

(43) Olmo-Mira, M.F.; Cabello, P.; Pino, C.; Martínez-Luque, M.; Richardson, D.J.; Castillo, F.; Roldán, M.D.; Moreno-Vivián, C. *Arch. Microbiol.* **2006**, *186*, 339.

(44) Pino, C.; Olmo-Mira, F.; Cabello, P.; Martínez-Luque, M.; Castillo, F.; Roldán, M.D.; Moreno-Vivián, C. *Biochem. Soc. Trans.* **2006**, *34*, 127.

(45) Zumft, W.G. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 533.

(46) Moura, I.; Moura, J.J.G. *Curr. Opin. Chem. Biol.* **2001**, *5*, 168.

(47) Einsle, O.; Kroneck, P.M. *Biol. Chem.* **2004**, *385*, 875.

(48) The fungal (*Fusarium oxysporum*) denitrification system is found in the mitochondria, where the electron transport chain is coupled to the denitrification to produce ATP.[49-55] This fungal denitrification is carried out with a CuNiR enzyme (see text ahead). The existence of an anaerobic "respiration" pathway in an organelle devoted to aerobic "respiration" is very interesting for the origin and evolution theories of the mitochondrium (the protomitochondria[56]).

(49) Kobayashi, M.; Shoun, H. J, Biol. Chem. **1995**, *270*, 4146.

(50) Kobayashi, M.; Matsuo, Y.; Takimoto, A., Suzuki, S.; Maruo, F.; Shoun, H. *J. Biol. Chem.* **1996**, *271*, 16263.

(51) Takaya, N.; Kuwazaki, S.; Adachi, Y.; Suzuki, S.; Kikuchi, T.; Nakamura, H.; Shiro, Y.; Shoun, H. *J. Biochem.* **2003**, *133*, 461.

(52) Kim, S.-W.; Fushinobu, S., Zhou, S.; Wakagi, T.; Shoun, H. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 1403.

(53) Nakanishi, Y.;Zhou, S.; Kim, S.-W.; Fushinobu, S.; Maruyama, J.; Kitamoto, K.; Wakagi, T.; Shoun, H. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 984.

(54) Piña-Ochoa, E.; Høgslund, S.; Geslin, E.; Cedhagen, T.; Peter Revsbech, N.; Nielsen, L.P.; Schweizer, M.; Jorissen, F.; Rysgaard, S.; Risgaard-Petersen, N. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1148.

(55) Shoun, H.; Fushinobu, S.; Jiang, L.; Kim, S.-W.; Wakagi, T. Philos. Trans. R. Soc. Lond. B Biol. Sci. **2012**, *367*, 1186.

(56) Kim, S.-W.; Fushinobu, S.; Zhou, S.; Wakagi, T.; Shoun, H. *Appl. Environ. Microbiol*. **2009**, *75*, 2652.

(57) Takaya, N. *J. Biosci. Bioeng.* **2002**, *94*, 506.

(58) Risgaard-Petersen, N.; Langezaal, A.M.; Ingvardsen, S.; Schmid, M.C.; Jetten, M.S.M.; Op den Camp, H.J.M.; Derksen, J.W.M.; Piña-Ochoa, E.; Eriksson, S.P.; Nielsen, L.P.; Revsbech, N.P.; Cedhagen, T.; van der Zwaan, G.J. *Nature* **2006**, *443*, 93.

(59) Fujii, T.; Takaya, N. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 412.

(60) Demaneche, S.; Philippot, L.; David, M.M.; Navarro, E.; Vogel, T.M.; Simonet, P. *Appl. Environ. Microbiol.* **2009**, *75*, 534.

(61) IUPAC recommended nomenclature is nitrogen monoxide radical.

(62) Williams, P.A.; Fülöp, V.; Leung, Y.C.; Chan, C.; Moir, J.W.; Howlett, G.; Ferguson, S.J.; Radford, S.E.; Hajdu, J. *Nat. Struct. Biol.* **1995**, *2*, 975.

(63) The classification and nomenclature of this species has been frequently emended and presently is classified as *Achromobacter xylosoxidans* [64], although the old nomenclature will be here used.

(64) http://www.ncbi.nlm.nih.gov/mesh/68042441

(65) Dodd, F. E.; Hasnain, S.S.; Hunter, W.N.; Abraham, Z.H.L.; Debenham, M.; Kanzler, H.; Eldridge, M.; Eady, R.R.; Ambler, R.P.; Smith, B.E. *Biochemistry* **1995**, *34*, 10180.

(66) Deligeer; Kataoka, K.; Yamaguchi, K.; Suzuki, S. *Bull. Chem. Soc. Jpn*. **2000**, *73*, 1839.

(67) Nojiri, M.; Koteishi, H.; Nakagami, T.; Kobayashi, K.; Inoue, T.; Yamaguchi, K.; Suzuki, S. *Nature* **2009**, *462*, 117.

(68) Simon, J. *FEMS Microbiol. Rev.* **2002**, *26*, 285.

(69) Koops, H.P.; Pommerening-Roser, A. *FEMS Microbiol. Ecol.* **2001**, *37*, 1.

(70) Vlaeminck, S.E.; Hay, A.G.; Maignien, L.; Verstraete, W. *Environ. Microbiol.* **2011**, *13*, 283.

(71) Contrary to what might be expected at first glance, ammonium-oxidising bacteria (nitrifiers, *e.g.*, *N. europaea* and *Nitrosococcus oceani*) express also enzymes of the denitrification pathway: CuNiR and NO reductase (but not nitrate reductase, nor nitrous oxide reductase), forming a "nitrifier denitrification" pathway that prevents the accumulation of the highly toxic end product of ammonia oxidation **-**nitrite.[72-83] (Nitrite is toxic to nitrifiers (and other bacteria), inactivating, specifically and irreversibly, ammonium monooxygenase of *N. europaea*.[84]) Therefore, substantial quantities of the nitrous oxide are believed to be formed.[77,85-89] Nevertheless, the physiological function of CuNiR and

NO reductase in these bacteria is believed to be detoxification and not "respiratory".[76,78,84,90]

(72) Ritchie, G.A.; Nicholas, D.J. *Biochem. J.* **1974**, *138*, 471.

(73) DiSpirito, A.A.; Taaffe, L.R.; Lipscomb, J.D.; Hooper, A.B. *Biochim. Biophys. Acta* **1985**, *827*, 320.

(74) Beaumont, H.J.; Hommes, N.G.; Sayavedra-Soto, L.A.; Arp, D.J.; Arciero, D.M.; Hooper, A.B.; Westerhoff, H.V.; van Spanning, R.J.M. *J. Bacteriol.* **2002**, *184*, 2557.

(75) Chain, P.; Lamerdin, J.; Larimer, F.; Regala, W.; Lao, V.; Land, M.; Hauser, L.; Hooper, A.; Klotz, M.; Norton, J.; Sayavedra-Soto, L.; Arciero, D.; Hommes, N.; Whittaker, M.; Arp, D. *J. Bacteriol.* **2003**, *185*, 2759.

(76) Beaumont, H.J.E.; Lens, S.I.; Reijnders, W.N.M.; Westerhoff, H.V.; van Spanning, R.J.M. *Mol. Microbiol.* **2004**, *54*, 148.

(77) Schmidt, I.; van Spanning, R.J.; Jetten, M.S. Microbiology **2004**, *150*, 4107.

(78) Beaumont, H.J.E.; Lens, S.I.; Westerhoff, H.V.; van Spanning R.J.M. *J. Bacteriol.* **2005**, *187*, 6849.

(79) Cantera, J.J.; Stein, L.Y. *Arch. Microbiol.* **2007**, *188*, 349.

(80) Cantera, J.J.; Stein, L.Y. *Environ. Microbiol.* **2007**, *9*, 765.

(81) Schmidt, I. Curr. Microbiol. **2009**, *59*, 130.

(82) Yu, R.; Chandran, K. *BMC Microbiol.* **2010**, *10*, 70.

(83) Kondo, K.; Yoshimatsu, K.; Fujiwara, T. Microbes Environ. **2012**, *27*, 407.

(84) Stein, L.Y.;Arp, D.J. *Appl. Environ. Microb.* **1998**, *64*, 4098.

(85) Goreau, T.J.; Kaplan, W.A.; Wofsy, S.C.; McElroy, M.B.; Valois, F.W.; Watson, S.W. *Appl. Environ. Microbiol.* **1980**, *40*, 526.

(86) Lipschultz, F.; Zafiriou, O.C.; Wofsy, S.C. *Nature* **1981**, *294*, 641.

(87) Poth, M., Focht, D.D. *Appl. Environ. Microbiol.* **1985**, *49*, 1134.

(88) Yoshida, N.; Morimoto, H.; Hirano, M.; Koike, I.; Matsuo, S.; Wada, E.; Saino, T.; Hattori, A. *Nature* **1989**, *342*, 895.

(89) Shrestha, N.K.; Hadano, S.; Kamachi, T.; Okura, I. *Appl. Biochem. Biotechnol.* **2001**, *90*, 221.

(90) Tan, N.C.G.; Kampschreur, M.J.; Wanders, W.; van der Pol, W.L.; van de Vossenberg, J.; Kleerebezem, R.; van Loosdrecht, M.C.; Jetten, M.S. *Syst. Appl. Microbiol.* **2008**, *31*, 114.

(91) Könneke, M.; Bernhard, A.E.; de la Torre, J.R.; Walker, C.B.; Waterbury, J.B.; Stahl, D.A. *Nature* **2005**, *437*, 543.

(92) Schleper, C.; Jurgens, G.; Jonuscheit, M. *Nat. Rev. Microbiol.* **2005**, *3*, 479.

(93) Treusch, A.H.; Leininger, S.; Kletzin, A.; Schuster, S.C.; Klenk, H.P.; Schleper, C. *Environ. Microbiol.* **2005**, *7*, 1985.

(94) Hallam, S.J.; Konstantinidis, K.T.; Putnam, N.; Schleper, C.; Watanabe, Y.; Sugahara, J.;Preston, C.; de la Torre, J.; Richardson, P.M.; DeLong, E.F. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18296.

(95) Hatzenpichler, R.; Lebedeva, E.V.; Spieck, E.; Stoecker, K.; Richter, A.; Daims, H.; Wagner, M. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2134.

(96) Smith, A.J.; Hoare, D.S. *J. Bacteriol.* **1968**, *95*, 844.

(97) Bock, E. *Arch. Microbiol.* **1976**, *108*, 305.
(98) Bock, E.; Sundermeyer-Klinger, H.; Stackebrandt, E. *Arch. Microbiol.* **1983**, *136*, 281.
(99) Kirstein, K.; Bock, E. *Arch. Microbiol.* **1993**, *160*, 447.
(100) Teske, A.; Alm, E.; Regan, J.M.; Toze, S.; Rittmann, B.E.; Stahl, D.A. *J. Bacteriol.* **1994**, *176*, 6623.
(101) Dodsworth, J.A.; Hungate, B.; Torre, J.R.; Jiang, H.; Hedlund, B.P. *Methods Enzymol.* **2011**, *486*, 171.
(102) Spieck, E.; Lipski, A. *Methods Enzymol.* **2011**, *486*, 109.
(103) Jetten, M.S.M.; Strous, M.; van de Pas-Schoonen, K.T.; Schalk, J.; van Dongen, U.G.; van de Graaf, A.A.; Logemann, S.; Muyzer, G.; van Loosdrecht, M.C.; Kuenen, J.G. *FEMS Microbiol. Rev.* **1998**, *22*, 421.
(104) Strous, M.; Fuerst, J.A.; Kramer, E.H.M.; Logemann, S.; Muyzer, G.; van de Pas-Schoonen, K.T.; Webb, R.; Kuenen, J.G.; Jetten, M.S.M. *Nature* **1999**, *400*, 446.
(105) Strous, M.; Pelletier, E.; Mangenot, S.; Rattei, T.; Lehner, A.; Taylor, M.W.; Horn, M.; Daims, H.; Bartol-Mavel, D.; Wincker, P.; Barbe, V.; Fonknechten, N.; Vallenet, D.; Segurens, B.; Schenowitz-Truong, C.; Medigue, C.; Collingro, A.; Snel, B.; Dutilh, B.E.; Op den Camp, H.J.M.; van der Drift, C.; Cirpus, I.; van de Pas-Schoonen, K.T.; Harhangi, H.R.; van Niftrik, L.; Schmid, M.; Keltjens, J.; van de Vossenberg, J.; Kartal, B.; Meier, H.; Frishman, D.; Huynen, M.A.; Mewes, H.-W.; Weissenbach, J.; Jetten, M.S.M.; Wagner, M.; Le Paslier, D. *Nature* **2006**, *440*, 790.
(106) Klotz, M.G.; Stein, L.Y. *FEMS Microbiol. Lett.* **2008**, *278*, 146.
(107) Kuenen, J.G. *Nat. Rev. Microbiol.* **2008**, *6*, 320.
(108) Jetten, M.S.M.; van Niftrik, L.; Strous, M.; Kartal, B.; Keltjens, J.T.; Op den Camp, H.J.M. *Crit. Rev. Biochem. Mol. Biol.* **2009**, *44*, 65.
(109) de Almeida, N.M.; Maalcke, W.J.; Keltjens, J.T.; Jetten, M.S.; Kartal, B. *Biochem. Soc. Trans.* **2011**, *39*, 303.
(110) Kartal, B.; Maalcke, W.J.; de Almeida, N.M.; Cirpus, I.; Gloerich, J.; Geerts, W.; Op den Camp, H.J.M.; Harhangi, H.R.; Janssen-Megens, E.M.; Francoijs, K.-J.; Stunnenberg, H.G.; Keltjens, J.T.; Jetten, M.S.M; Strous, M. *Nature* **2011**, *479*, 127.
(111) Kartal, B.; van Niftrik, L.; Keltjens, J.T.; Op den Camp, H.J.; Jetten, M.S. *Adv. Microb. Physiol.* **2012**, *60*, 211.
(112) van de Vossenberg, J.; Woebken, D.; Maalcke, W.J.; Wessels, H.J.C.T.; Dutilh, B.E.; Kartal, B.; Janssen-Megens, E.M.; Roeselers, G.; Yan, J.; Speth, D.; Gloerich, J.; Geerts, W.; van der Biezen, E.; Pluk, W.; Francoijs, K.-J.; Russ, L.; Lam, P.; Malfatti, S.A.; Tringe, S.G.; Haaijer, S.C.M.; Op den Camp, H.J.M.; Stunnenberg, H.G.; Amann, R.; Kuypers, M.M.M.; Jetten, M.S.M. *Environ. Microbiol.* **2013**, *15*, 1275.
(113) Shimamura, M.; Nishiyama, T.; Shigetomo, H.; Toyomoto, T.; Kawahara, Y.; Furukawa, K.; Fujii, T.
Appl. Environ. Microbiol. **2007**, *73*, 1065.
(114) Li, M.; Ford, T.; Li, X.; Gu, J.-D. *Environ. Sci. Technol.* **2011**, *45*, 3547.

(115) Hira, D.; Toh, H.; Migita, C.T., Okubo, H.; Nishiyama, T.; Hattori, M.; Furukawa, K.; Fujii, T. *FEBS Lett.* **2012**, *586*, 1658.

(116) Kartal, B.; Kuypers, M.M.; Lavik, G.; Schalk, J.; Op den Camp, H.J.; Jetten, M.S.; Strous M. *Environ. Microbiol.* **2007**, *9*, 635.

(117) In addition to the aerobic nitrite oxidation (of nitrification), it was recently reported the occurrence of an anaerobic photosynthetically driven nitrite oxidation. In this not yet characterised process, anoxygenic phototrophic bacteria (closely related to *Thiocapsa* species) use nitrite as an electron donor for photosynthesis in the absence of oxygen.

(118) Griffin, B.M.; Schott, J.; Schink, B. *Science* **2007**, *316*, 1870.

(119) Besides aerobic ammonia-oxidizing bacteria and nitrite-oxidizing bacteria, there is recent evidence that nitrification can also be carried out in the suboxic zone by a number of crenarchaeota. These organisms could provide a direct local source of nitrite for AnAmmOx, in the suboxic zone, coupling nitrification and AnAmmOx.

(120) Francis, C.A.; Beman, J.M.; Kuypers, M.M. *ISME Journal* **2007**, *1*, 19.

(121) Lam, P.; Jensen, M.M.; Lavik, G.; McGinnis, D.F.; Muller, B.; Schubert, C.J.; Amann, R.; Thamdrup, B.; Kuypers, M.M.M. Proc. Natl. Acad. Sci. USA **2007**, *104*, 7104.

(122) van de Vossenberg, J.; Rattray, J.E.; Geerts, W.; Kartal, B.; van Niftrik, L.; van Donselaar, E.G.; Sinninghe Damsté, J.S.; Strous, M.; Jetten, M.S. *Environ. Microbiol.* **2008**, *10*, 3120.

(123) Yan, J.; Op den Camp, H.J.; Jetten, M.S.; Hu, Y.Y.; Haaijer, S.C. *Syst. Appl. Microbiol.* **2010**, *33*, 407.

(124) Mehta, M.P.; Baross, J.A. *Science* **2006**, *314*, 1783.

(125) Raghoebarsing, A.A.; Pol, A.; van de Pas-Schoonen, K.T.; Smolders, A.J.; Ettwig, K.F.; Rijpstra, W.I.; Schouten, S.; Damsté, J.S.; Op den Camp, H.J.; Jetten, M.S.; Strous, M. *Nature* **2006**, *440*, 918.

(126) Ettwig, K.F.; Shima, S.; Van De Pas-Schoonen, K.T.; Kahnt, J.; Medema, M.H.; Op Den Camp, H.J.M.; Jetten, M.S.M.; Strous, M. *Environ Microbiol* **2008**, *10*, 3164.

(127) Ettwig, K.F.; Butler, M.K.; Le Paslier, D.; Pelletier, E.; Mangenot, S.; Kuypers, M.M.; Schreiber, F.; Dutilh, B.E.; Zedelius, J.; de Beer, D.; Gloerich, J.; Wessels, H.J.; van Alen, T.; Luesken, F.; Wu, M.L.; van de Pas-Schoonen, K.T.; Op den Camp, H.J.; Janssen-Megens, E.M.; Francoijs, K.J.; Stunnenberg, H.; Weissenbach, J.; Jetten, M.S.; Strous, M. *Nature* **2010**, *464*, 543.

(128) Wu, M.L.; Ettwig, K.F.; Jetten, M.S.M.; Strous, M.; Keltjens, J.T.; van Niftrik, L. *Biochem. Soc. Trans.* **2011**, *39*, 243.

(129) Ettwig, K.F.; Speth, D.R.; Reimann, J.; Wu, M.L.; Jetten, M.S.M.; Keltjens, J.T. *Front. Microbiol.* **2012**, *3*, 273.

(130) Shen, L.-D.; He, Z.-F.; Zhu, Q.; Chen, D.-Q.; Lou, L.-P.; Xu, X.-Y.; Zheng, P.; Hu, B.-L. *Front. Microbiol.* **2012**, *3*, 269.

(131) Wu, M.L.; van Alen, T.A.; van Donselaar, E.G.; Strous, M.; Jetten, M.S.M.; van Niftrik, L. *FEMS Microbiol. Lett.* **2012**, *334*, 49.

(132) Wu, M.L.; van Teeseling, M.C.; Willems, M.J.; van Donselaar, E.G.; Klingl, A.; Rachel, R.;

Geerts, W.J.; Jetten, M.S.; Strous, M.; van Niftrik, L. *J. Bacteriol.* **2012**, *194*, 284.

(133) Wu, M.L.; de Vries, S.; van Alen, T.A.; Butler, M.K.; Op den Camp, H.J.; Keltjens, J.T.; Jetten, M.S.; Strous, M. *Microbiology* **2011**, *157*, 890.

(134) Luesken, F.A.; Wu, M.L.; Op den Camp, H.J.M.; Keltjens, J.T.; Stunnenberg, H.; Francoijs, K.-J.; Strous, M.; Jetten, M.S.M. *Environ. Microbiol.* **2012**, *14*, 1024.

(135) Ehrenreich, P.; Behrends, A.; Harder, J.; Widdel, F. *Arch. Microbiol.* **2000**, 173, 58.

(136) Zedelius, J.; Rabus, R.; Grundmann, O.; Werner, I.; Brodkorb, D.; Schreiber, F.; Ehrenreich, P.; Behrends, A. Wilkes, H.; Kube, M.; Reinhardt, R.; Widdel, F. *Environ. Microbiol. Rep.* **2010**, *3*, 125.

(137) In this context it should be noted that "intra-aerobic" pathways for hydrocarbon degradation are not a novelty: chlorite dismutases, that catalyse the conversion of the toxic chlorite into chloride plus dioxygen ($ClO_2^- \rightarrow Cl^- + O_2$)[138-144], have been suggested to provide dioxygen for chemo-organotrophic "respiration" and for monooxygenase-dependent biosynthesis, as well as to prevent cell damage (by chlorite), in several bacteria and archea (*e.g.*, *Pseudomonas chloritidismutans* [145], but also *Nitrospira* [146], *Nitrobacter* [129] or *Haloferax volcanii* [147]). Thus, presently it is clear that dioxygen formation can be driven, not only by photosynthesis, but also by chemical energy, using oxidants with reduction potentials more positive than the O_2/H_2O couple, as chloride or nitrite-derived NO.

(138) Rikken, G.B.; Kroon, A.G.M.; van Ginkel, C.G. *Appl. Microbiol. Biotechnol.* **1996**, *45*, 420.

(139) van Ginkel, C.G.; Rikken, G.B.; Kroon, A.G.M.; Kengen, S.W.M. *Arch. Microbiol.* **1996**, *166*, 321.

(140) de Geus, D.C.; Thomassen, E.A.J.; Hagedoorn, P.-L.; Pannu, N.S.; Van Duijn, E.; Abrahams, J.P. *J. Mol.Biol.* **2009**, *387*, 192.

(141) Mehboob, F.; Wolterink, A.F.M.; Vermeulen, A.J.; Jiang, B.; Hagedoorn, P.-L.; Stams, A.J.M.; Kengen, S. W.M. *FEMS Microbiol. Lett.* **2009**, *293*, 115.

(142) Goblirsch, B.R.; Streit, B.R.; DuBois, J.L.; Wilmot, C.M. *J. Biol. Inorg.Chem.* **2010**, *15*, 879.

(143) Kostan, J.; Sjöblom, B.; Maixner, F.; Mlynek, G.; Furtmüller, P.G.; Obinger, C.; Wagner, M.; Daims, H.; Djinovic-Carugo, K. *J. Struct.Biol.* **2010**, *172*, 331.

(144) Mlynek, G.; Sjöblom, B.; Kostan, J.; Füreder, S.; Maixner, F.; Gysel, K.; Furtmüller, P.G.; Obinger, C.; Wagner, M.; Daims, H.; Djinovic-Carugo, K. *J. Bacteriol.* **2011**, *193*, 2408.

(145) Mehboob, F.; Junca, H.; Schraa, G.; Stams, A.J.M. *Appl. Microbiol. Biotechnol.* **2009**, *83*, 739.

(146) Maixner, F.; Wagner, M.; Lücker, S.; Pelletier, E.; Schmitz-Esser, S.; Hace, K.; Spieck, E.; Konrat, R.; Le Paslier, D.; Daims, H. *Environ. Microbiol.* **2008**, *10*, 3043.

(147) Bab-Dinitz, E.; Shmuely,H.,Maupin-Furlow, J.; Eichler, J.; Shaanan,B. *Bioinformatics* **2006**, *22*, 671.

(148) Ducluzeau, A.L.; van Lis, R.; Duval, S.; Schoepp-Cothenet, B.; Russell, M.J.; Nitschke, W. *TIBS* **2009**, *34*, 9.

(149) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.

(150) Koppenol, W.H. *Free Radic. Biol. Med.* **1998**, *25*, 385.

(151) Eiserich, J.P.; Patel, R.P.; O'Donnell, V.B. *Mol. Aspects Med.* **1998**, *19*, 221.

(152) Koshland Jr., D. E. *Science* **1992**, *258*, 1861.
(153) Pfeiffer, S.; Mayer, B.; Hemmens, B. *Angew. Chem. Int. Edn. Engl.* **1999**, *38*, 1714.
(154) Stuehr, D.J. *Biochim. Biophys. Acta* **1999**, *1411*, 217.
(155) Alderton, W.K.; Cooper, C.E.; Knowles, R.G. *Biochem. J.* **2001**, *357*, 593.
(156) Carr, GJ; Ferguson, S.J. *Biochim. Biophys. Acta* **1990**, *1017*, 57.
(157) Jansen, A.; Drazen, J.; Osborne, J.A.; Brown, R.; Loscalzo, J.; Stamler, J.S. *J. Pharmacol. Exp. Ther.* **1992**, *261*, 154.
(158) Stamler, J.S.; Jaraki, O.; Osborne, J.; Simon, D.I.; Keaney, J.; Vita, J.; Singel, D.; Valeri, C.R.; Loscalzo. J. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 7674.
(159) Stamler, J.S.; Simon, D.I.; Osborne, J.A.; Mullins, M.E.; Jaraki, O.; Michel, T.; Singel, D.J.; Loscalzo, J. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 444.
(160) Brown, G.C., Cooper, C.E. **FEBS Lett. 1994**, *356*, 295.
(161) Cleeter, M.W.; Cooper, J.M.; Darley-Usmar, V.M.; Moncada, S.; Schapira, A.H. *FEBS Lett.* **1994**, *345*, 50.
(162) Kharitonov, V.G.; Sundquist, A.R.; Sharma, V.S. *J. Biol. Chem.* **1995**, *270*, 28158.
(163) Feelisch, M.; Stamler, J. S. *Methods in nitric oxide research*; John Wiley and sons Ltd.: Chichester, 1996; p3.
(164) Clementi, E.; Brown, G.C.; Feelisch, M.; Moncada, S. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7631.
(165) Feelisch, M.; Rassaf, T.; Mnaimneh, S.; Singh, N.; Bryan, N.S.; Jourd'Heuil, D.; Kelm, M. *FASEB J.* **2002**, *16*, 1775.
(166) Gaston, B.M.; Carver, J.; Doctor, A.; Palmer, L.A. *Mol. Interv.* **2003**, *3*, 253.
(167) Bryan, N.S.; Rassaf, T.; Maloney, R.E.; Rodriguez, C.M.; Saijo, F.; Rodriguez, J.R.; Feelisch, M. *Proc. Natl Acad. Sci. USA* **2004**, *101*, 4308.
(168) Liu, L.; Yan, Y.; Zeng, M.; Zhang, J.; Hanes, M. A.; Ahearn, G.; McMahon, T. J.; Dickfeld, T.; Marshall, H. E.; Que, L. G.; Stamler, J. S. *Cell* **2004**, *116*, 617.
(169) Lima, B.; Lam, G. K.; Xie, L.; Diesen, D. L.; Villamizar, N.; Nienaber, J.; Messina, E.; Bowles, D.; Kontos, C. D.; Hare, J. M.; Stamler, J. S.; Rockman, H. A. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 6297.
(170) Sanghani, P. C.; Davis, W. I.; Fears, S. L.; Green, S. L.; Zhai, L.; Tang, Y.; Martin, E.; Bryan, N. S.; Sanghani, S. P. *J. Biol. Chem.* **2009**, *284*, 24354.
(170) Toledo Jr., J.C.; Augusto, O. *Chem. Res. Toxicol.* **2012**, *25*, 975.
(171) Wink, D.A.; Darbyshire, J.F.; Nims, R.W.; Saavedra, J.E.; Ford, P.C. *Chem. Res. Toxicol.* **1993**, *6*, 23.
(172) Goldstein, S.; Czapski, G. *J. Am. Chem. Soc.* **1995**, *117*, 12078.
(173) Liu, X.; Liu, Q.; Gupta, E.; Zorko, N.; Brownlee, E.; Zweier, J. L. *Nitric Oxide* **2005**, *13*, 68.
(174) Shiva, S.; Wang, X.; Ringwood, L.A.; Xu, X.; Yuditskaya, S.; Annavajjhala, V.; Miyajima, H.; Hogg, N.; Harris, Z.L.; Gladwin, M.T. *Nat. Chem. Biol.* **2006**, *2*, 486.
(175) Reichert, E.T.; Mitchell, S. W. *Am. J. Med. Sci.* **1880**, *159*, 158.
(176) Ischaemia is a decrease/stop of the blood flow to a tissue (due to a blockage on the vessels leading to that tissue) that, consequently, leads to a decrease of the tissue dioxygen concentration

(hypoxia/anoxia) and pH (acidosis) -precisely the conditions that favour the nitrite reduction to NO, as will be described below.

(177) 3rd, G.; Tsao, P.S.; Mulloy, D.; Lefer A.M. J. Pharmacol. Exp. Ther. **1990**, *252*, 35.

(178) Demoncheaux, E.A.; Higenbottam, T.W.; Foster, P.J.; Borland, C.D.; Smith, A.P.; Marriott, H.M.; Bee, D.; Akamine, S.; Davies, M.B. *Clin. Sci.* (Lond) **2002**, *102*, 77.

(179) Hunter, C.J.; Dejam, A.; Blood, A.B.; Shields, H.; Kim-Shapiro, D.B.; Machado, R.F.; Tarekegn, S.; Mulla, N.; Hopper, A.O.; Schechter, A.N.; Power, G.G.; Gladwin, M.T. *Nat. Med.* **2004**, *10*, 1122.

(180) Webb, A.; Bond, R.; McLean, P.; Uppal, R.; Benjamin, N.; Ahluwalia, A. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13683.

(181) Duranski, M.R.; Greer, J.J.; Dejam, A.; Jaganmohan, S.; Hogg, N.; Langston, W.; Patel, R.P.; Yet, S.F.; Wang, X.; Kevil, C.G.; Gladwin, M.T.; Lefer, D. J. *J. Clin. Investig.* **2005**, *115*, 1232.

(182) Lu, P.; Liu, F.; Yao, Z.; Wang, C.Y.; Chen, D.D.; Tian, Y.; Zhang, J.H.; Wu, Y.H. *Hepatobiliary Pancreas. Dis. Int.* **2005**, *4*, 350.

(183) Lundberg, J.O.; Weitzberg, E.; *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 915.

(184) Pluta, R.M.; Dejam, A.; Grimes, G.; Gladwin, M.T.; Oldfield, E.H *JAMA* **2005**, *293*, 1477.

(185) Tsuchiya, K.; Kanematsu, Y.; Yoshizumi, M.; Ohnishi, H.; Kirima, K.; Izawa, Y.; Shikishima, M.; Ishida, T.; Kondo, S.; Kagami, S.; Takiguchi, Y.; Tamaki T. Am. J. Physiol. Heart. Circ. Physiol. **2005**, *288*, H2163.

(186) Baker, J.E.; Su, J.; Fu, X.; Hsu, A.; Gross, G.J. Tweddell, J.S.; Hogg, N. *J. Mol. Cell. Cardiol.* **2007**, *43*, 437.

(187) Bryan, N.S.; Calvert, J.W.; Elrod, J.W.; Gundewar, S.; Ji, S.Y.; Lefer, D.J. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 19144.

(188) Dezfulian, C.; Raat, N.; Shiva, S.; Gladwin, M.T. *Cardiovasc. Res.* **2007**, *75*, 327.

(189) Dejam, A.; Hunter, C.J.; Tremonti, C.; Pluta, R.M.; Hon, Y.Y.; Grimes, G.; Partovi, K.; Pelletier, M.M.; Oldfield, E.H.; Cannon 3rd., R.O.; Schechter, A.N.; Gladwin, M.T. *Circulation* **2007**, *116*, 1821.

(190) Shiva, S.; Sack, M.N.; Greer, J.J.; Duranski, M.; Ringwood, L.A.; Burwell, L.; Wang, X.; MacArthur, P.H.; Shoja, A.; Raghavachari, N.; Calvert, J.W.; Brookes, P.S.; Lefer, D.J.; Gladwin M.T. *J. Exp. Med.* **2007**, *204*, 2089.

(191) Bryan, N.S.; Calvert, J.W.; Gundewar, S.; Lefer, D.J. *Free Radic. Biol. Med.* **2008**, *45*, 468.

(192) Gonzalez, F.M.; Shiva, S.; Vincent, P.S.; Ringwood, L.A.; Hsu, L.Y.; Hon, Y.Y.; Aletras, A.H.; Cannon 3rd, R.O.; Gladwin, M.T.; Arai, A.E. *Circulation* **2008**, *117*, 2986.

(193) Maher, A.R.; Milsom, A.B.; Gunaruwan, P.; Abozguia, K.; Ahmed, I.; Weaver, R.A.; Thomas, P.; Ashrafian, H.; Born, G.V.; James, P.E.; Frenneaux, M.P. *Circulation* **2008**, *117*, 670.

(194) Sinha, S.S.; Shiva, S.; Gladwin, M.T. *Trends Cardiovasc. Med.* **2008**, *18*, 163.

(195) Raat, N.J.; Shiva, S.; Gladwin, M.T. *Adv. Drug. Deliv. Rev.* **2009**, *61*, 339.

(196) Zuckerbraun, B.S.; Shiva, S.; Ifedigbo, E.; Mathier, M.A.; Mollen, K.P.; Rao, J.; Bauer, P.M.; Choi, J.J.; Curtis, E.; Choi, A.M.; Gladwin, M. T. *Circulation* **2010**, *121*, 98.

(197) Blood, A.B.; Schroeder, H.J.; Terry, M.H.; Merrill-Henry, J.; Bragg, S.L.; Vrancken, K.; Liu, T.; Herring, J.L.; Sowers, L.C.; Wilson, S.M.; Power, G.G. *Circulation* **2011**, *123*, 605.

(198) It is generally accepted that hypoxia and anoxia corresponds to dioxygen concentrations lower than 20μM and 2μM, respectively.

(199) Kumar, D.; Branch, B.G.; Pattillo, C.B.; Hood, J.; Thoma, S.; Simpson, S.; Illum, S.; Arora, N.; Chidlow Jr., J.H.; Langston, W.; Teng, X.; Lefer, D.J.; Patel, R.P.; Kevil, C.G. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 7540.

(200) Bryan, N.S.; Fernandez, B.O.; Bauer, S.M.; Garcia-Saura, M.F.; Milsom, A.B.; Rassaf, T.; Maloney, R.E.; Bharti, A.; Rodriguez, J.; Feelisch, M. *Nat. Chem. Biol.* **2005**, *1*, 290.

(201) NO can (i) inhibit the mitochondrial "respiration", thus limiting the dioxygen consumption, reactive oxygen species formation and cytochrome *c* release, and, consequently, preventing further oxidative stress damage; (ii) at the same time, NO can increase the blood flow, modulating the dioxygen diffusion, and maintain an antiapoptotic and anti-inflammatory environment.[161,188,190,202-207] Therefore, it seems to be crucial to link the limited dioxygen supply to higher levels of NO formation, what can be difficult to achieve with the oxygen-dependent NOS.

(202) Torres, J.; Darley Usmar, V.; Wilson, M. T. *Biochem. J.* **1995**, *312*, 169.

(203) Shiva, S.; Brookes, P.S.; Patel, R. P.; Anderson, P.G.; Darley-Usmar, V.M. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 7212.

(204) Thomas, D.D.; Liu, Z.P.; Kantrow, S.P.; Lancaster, J.R. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 355.

(205) Frost, M.T.; Wang, Q.; Moncada, S.; Singer, M. *Am. J. Physiol. Reg. I.* **2005**, *288*, R394.

(206) Burwell, L.S.; Nadtochiy, S.M.; Tompkins, A.J.; Young, S.; Brookes, P. S. *Biochem. J.* **2006**, *394*, 627.

(206) Mason, M.G.; Nicholls, P.; Wilson, M.T.; Cooper,C.E. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 708.

(207) Nadtochiy, S.M.; Burwell, L.S.; Brookes, P.S. *J. Mol. Cell. Cardiol.* **2007**, *42*, 812.

(208) Larsen, F.J.; Weitzberg, E.; Lundberg, J.O.; Ekblom, B. *Acta Physiol. (Oxf)* **2007**, *191*, 59.

(209) Shiva, S.; Huang, Z.; Grubina, R.; Sun, J.; Ringwood, L.A.; MacArthur, P.H.; Xu, X.; Murphy, E.; Darley-Usmar, V.M.; Gladwin, M.T. *Circ. Res.* **2007**, *100*, 654.

(210) Larsen, F.J.; Weitzberg, E.; Lundberg, J.O.; Ekblom, B. *Free Radic. Biol. Med.* **2010**, *48*, 342.

(211) Li, H.; Liu, X.; Cui, H.; Chen, Y.R.; Cardounel, A.J.; Zweier J.L. J. Biol. Chem. **2006**, *281*, 12546.

(212) Castello, P.R.; David, P.S.; McClure, T.; Crook, Z.; Poyton R.O. *Cell Metab.* **2006**, *3*, 277.

(213) Benamar, A.; Rolletschek, H.; Borisjuk, L.; Avelange-Macherel, M.-H.; Curien, G.; Mostefai, H.A.; Andriantsitohaina, R.; Macherel, D. *Biochim. Biophys. Acta* **2008**, *1777*, 1268.

(214) Castello, P.R.; Woo, D.K.; Ball, K.; Wojcik, J.; Liu, L.; Poyton, R.O. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 8203.

(215) Gautier, C., van Faassen, E., Mikula, I., Martasek, P., Slama-Schwok, A. *Biochem. Biophys. Res. Commun.* **2006**, *341*, 816.

(216) Vanin, A.F.; Bevers, L.M.; Slama-Schwok, A.; van Faassen, E.E. *Cell Mol. Life Sci.* **2006**, *64*, 96.

(217) Kozlov, A.V.; Staniek, K.; Nohl, H. *FEBS Lett.* **1999**, *454*, 127.

(218) Badejo Jr., A.M.; Hodnette, C.; Dhaliwal, J.S.; Casey, D.B.; Pankey, E.; Murthy, S.N.; Nossaman, B.D.; Hyman, A.L.; Kadowitz, P. J. *Am. J. Physiol. Heart Circ. Physiol.* **2010**, *299*, H819.

(219) He, C.M.; Knipp, M. *J. Am. Chem. Soc.* **2009**, *131*, 12042.

(220) Knipp, M.; He, C. *IUBMB Life* **2011**, *63*, 304.

(221) Wittenberg, B.A.; Wittenberg, J.B. *Annu. Rev. Physiol.* **1989**, *51*, 857.

(222) Qiu, Y.; Sutton, L.; Riggs, A.F. *J. Biol. Chem.* **1998**, *273*, 23426.

(223) Salhany, J.M. *Biochemistry* **2008**, *47*, 6059.

(224) Doyle, M.P.; Pickering, R.A.; DeWeert, T.M.; Hoekstra, J.W.; Pater, D. *J. Biol. Chem.* **1981**, *256*, 12393.

(225) Cosby, K.; Partovi, K.S.; Crawford, J.H., Patel, R.P.; Reiter, C.D.; Martyr, S.; Yang, B.K.; Waclawiw, M.A.; Zalos. G.; Xu, X.; Huang, K.T.; Shields, H.; Kim-Shapiro, D.B.; Schechter, A.N.; Cannon 3rd, R.O.; Gladwin, M.T. *Nat. Med.* **2003**, *9*, 1498.

(226) Huang, Z.; Shiva, S.; Kim-Shapiro, D.B.; Patel, R.P.; Ringwood, L.A.; Irby, C.E.; Huang, K.T.; Ho, C.; Hogg, N.; Schechter, A.N.; Gladwin, M.T. *J. Clin. Invest.* **2005**, *115*, 2099.

(227) Haldane, J. *J. Hyg.* **1901**, *1*, 115.

(228) Brooks, J. *Proc. R. Soc. Lond. B Biol. Sci.* **1937**, *123*, 368.

(229) Sulc, F.; Immoos, C.E.; Pervitsky, D.; Farmer, P.J. *J. Am. Chem. Soc.* **2004**, *126*, 1096.

(230) Gladwin, M.T.; Kim-Shapiro, D.B. *Blood* **2008**, *112*, 2636.

(231) Ward, J.P.T. *Biochim. Biophys. Acta* **2008,** *1777*, 1.

(232) Tsai, A.G.; Cabrales, P.; Hangai-Hoger, N.; Intaglietta, M. *Antioxid. Redox Signal.* **2004**, *6*, 1011.

(233) Bertuglia, S.; Giusti, A. *Am. J. Physiol. Heart Circ. Physiol.* **2005**, *288*, H525.

(234) Crawford, J.H.; Isbell, T.S.; Huang, Z.; Shiva, S.; Chacko, B.K.; Schechter, A.N.; Darley-Usmar, V.M.; Kerby, J.D.; Lang Jr., J.D.; Kraus, D.; Ho, C.; Gladwin, M.T.; Patel, R.P. *Blood* **2006**, *107*, 566.

(235) Gladwin, M.T.; Raat, N.J.; Shiva, S.; Dezfulian, C.; Hogg, N.; Kim-Shapiro, D.B.; Patel, R.P. *Am. J. Physiol. Heart Circ. Physiol.* **2006**, *291*, H2026.

(236) Larsen, F.J.; Ekblom, B.; Sahlin, K.; Lundberg, J.O.; Weitzberg, E. *N. Engl. J.Med.* **2006**, *355*, 2792.

(237) Webb, A.J.; Patel, N.; Loukogeorgakis, S.; Okorie, M.; Aboud, Z.; Misra, S.; Rashid, R.; Miall, P.; Deanfield, J.; Benjamin, N.; MacAllister, R.; Hobbs, A.J.; Ahluwalia, A. *Hypertension* **2008**, *51*, 784.

(238) Raat, N.J.; Noguchi, A.C.; Liu, V.B.; Raghavachari, N.; Liu, D.; Xu, X.; Shiva, S.; Munson, P.J.; Gladwin, M.T. *Free Radic. Biol. Med.* **2009**, *47*, 510.

(239) Rifkind, J.M.; Nagababu, E.; Cao, Z.; Barbiro-Michaely, E.; Mayevsky, A. *Adv. Exp. Med. Biol.* **2009**, *645*, 27.

(240) Kapil, V.; Milsom, A.B.; Okorie, M.; Maleki-Toyserkani, S.; Akram, F., Rehman, F.; Arghandawi. S.; Pearl, V.; Benjamin, N.; Loukogeorgakis, S.; Macallister, R.; Hobbs, A.J.; Webb, A.J.; Ahluwalia, A. *Hypertension* **2010**, *56*, 274.

(241) Doyle, M.P. Hoekstra, J.W. *J. Inorg. Biochem.* **1981**, *14*, 351.

(242) Parks, N.J.; Krohn, K.J.; Mathis, C.A.; Chasko, J.H.; Geiger, K.R.; Gregor, M.E.; Peek, N.F.

Science **1981**, *212*, 58.
(243) Cassoly, R.; Gibson, Q.H. *J. Mol. Biol.* **1975**, *91*, 301.
(244) Moore, E.G.; Gibson, Q.H. *J. Biol. Chem.* **1976**, *251*, 2788.
(245) Rose, E. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1983**, *105*, 2866.
(246) Sharma, V. S.; Taylor, T. G.; Gardiner, R. *Biochemistry* **1987**, *26*, 3837.
(247) Traylor, T. G.; Sharma, V. S. *Biochemistry* **1992**, *31*, 2847.
(248) Hoshino, M.; Ozawa, K.; Seki, H.; Ford, P. C. *J. Am. Chem. Soc.* **1993**, *115*, 9568.
(249) Radi, R. *Chem. Res. Toxicol.* **1996**, *9*, 828.
(250) Kharitonov, V.G.; Sharma, V.S.; Magde, D.; Koesling, D. *Biochemistry* **1997**, *36*, 6814.
(251) Cooper, C. E. *Biochim. Biophys. Acta* **1999**, *1411*, 290.
(252) Azizi, F.; Kielbasa, J. E.; Adeyiga, A. M.; Maree, R. D.; Frazier, M.; Yakubu, M.; Shields, H.; King, S. B.; Kim-Shapiro, D. B. *Free Radical Biol. Med.* **2005**, *39*, 145.
(253) Sharma, V.S.; Ranney, H.M. *J. Biol. Chem.* **1978**, *253*, 6467.
(254) Luchsinger, B.P.; Rich, E.N.; Yan, Y.; Williams, E.M.; Stamler, J.S.; Singel, D.J. *J. Inorg. Biochem.* **2005**, *99*, 912.
(255) Olson, J.S.; Phillips Jr., G.N. *J. Biol. Chem.* **1996**, *271*, 17593.
(256) Ionascu, D.; Gruia, F.; Ye, X.; Yu, A.; Rosca, F.; Beck, C.; Demidov, A.; Olson, J.S.; Champion, P.M. *J. Am. Chem. Soc.* **2005**, *127*, 16921.
(257) Nienhaus, K.; Palladino, P.; Nienhaus, G.U. *Biochemistry* **2008**, *47*, 935.
(258) Morris, R.J.; Gibson, Q.H. *J. Biol. Chem.* **1980**, *255*, 8050.
(259) Doyle, M. P.; Pickering, R. A.; Cook, B. R. *J. Inorg. Biochem.* **1983**, *19*, 329.
(260) Eich, R.F.; Li, T.; Lemon, D.D.; Doherty, D.H.; Curry, S.R.; Aitken, J.F.; Mathews, A.J.; Johnson, K.A.; Smith, R.D.; Phillips Jr., G.N.; Olson, J.S. *Biochemistry* **1996**, *35*, 6976.
(261) Gow, A.J.; Luchsinger, B.P.; Pawloski, J.R.; Singel, D.J.; Stamler, J.S. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9027.
(262) Herold, S.; Exner, M.; Nauser, T. *Biochemistry* **2001**, *40*, 3385.
(263) Joshi, M.S.; Ferguson Jr., T.B.; Han, T.H.; Hyduke, D.R.; Liao, J.C.; Rassaf, T.; Bryan, N.; Feelisch, M.; Lancaster Jr., J.R. *Proc. Natl. Acad. Sc. USA* **2002**, *99*, 10341.
(264) Gardner, P.R.; Gardner, A.M.; Brashear, W.T.; Suzuki, T.; Hvitved, A.N.; Setchell, K.D.R.; Olson, J.S. *J. Inorg. Biochem.* **2006**, *100*, 542.
(265) Under normoxia, these oxidation reactions are thought to be crucial for the NO homeostasis[266-273]: (i) the endothelial NOS-dependent NO, that diffuses towards the blood stream, should be rapidly scavenged and oxidised by the oxy-Hb in the intravascular space; (ii) n the cardiac muscle, is the oxidation by oxy-Mb that constitutes the major mechanism to scavenge NO and, thus, to avoid the unwanted NO effects (*e.g.*, inhibition of CcO, to protect the mitochondrial "respiration"). The met-Hb/Mb formed (eq. 16) are, then, regenerated (reduced) by specific met-Hb/Mb reductases and oxygenated to yield oxy-Hb/Mb; therefore, resulting in a "catalytic" NO scavenging under normoxia. In this way, the NO life time is controlled and its concentration is kept with the characteristics of a local signalling molecule.

(266) Kelm, M.; Schrader, J. *Circ. Res.* **1990**, *66*, 1561.

(267) Lancaster Jr., J.R. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 8137.

(268) Liu, X.; Miller, M.J.S.; Joshi, M.S.; Sadowska-Krowicka, H.; Clark, D.A.; Lancaster, J.R. *J. Biol. Chem.* **1998**, *273*, 18709.

(269) Brunori, M. *Trends Biochem. Sci.* **2001**, *26*, 21.

(270) Flögel, U.; Merx, M.W.; Gödecke, A.; Decking, U.K.M.; Schrader, J. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 735.

(271) Huang, K.-T.; Han, T.H.; Hyduke, D.R.; Vaughn, M.W.; Herle, H.V.; Hein, T.W.; Zhang, C.; Kuo, L.; Liao, J.C. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 11771.

(272) Thomas, D.D.; Liu, X.; Kantrow, S.P.; Lancaster Jr., J.R. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 355.

(273) Witting, P.K.; Douglas, D.J.; Mauk, A.G. *J. Biol. Chem.* **2001**, *276*, 3991.

(274) Gladwin, M.T.; Shelhamer, J.H.; Schechter, A.N.; Pease-Fye, M.E.; Waclawiw, M.A.; Panza, J.A.; Ognibene, F.P.; Cannon III, R. O. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 11482.

(275) Nagababu, E.; Ramasamy, S.; Abernethy, D.R.; Rifkind, J.M. *J. Biol. Chem.* **2003**, *278*, 46349.

(276) Jeffers, A.; Xu, X.; Huang, K.T.; Cho, M.; Hogg, N.; Patel, R.P.; Kim-Shapiro, D.B. *Comp. Biochem. Physiol. A* **2005**, *142*, 130.

(277) Kozlov, A.V.; Costantino, G.; Sobhian, B.; Szalay, L.; Umar, F.; Nohl, H.; Bahrami, S.; Redl, H. *Antioxid. Redox Signal.* **2005**, *7*, 515.

(278) Tsuchiya, K.; Kanematsu, Y.; Yoshizumi, M.; Ohnishi, H.; Kirima, K.; Izawa, Y.; Shikishima, M.; Ishida, T.; Kondo, S.; Kagami, S.; Takiguchi, Y.; Tamaki, T. *Am. J. Physiol. Heart Circ. Physiol.* **2005**, *288*, H2163.

(279) Hataishi, R.: Rodrigues, A.C.; Neilan, T.G.; Morgan, J.G.; Buys, E.; Shiva, S.; Tambouret, R.; Jassal, D.S.; Raher, M.J.; Furutani, E.; Ichinose, F.; Gladwin, M.T.; Rosenzweig, A.; Zapol, W.M.; Picard, M.H.; Bloch, K. D.; Scherrer-Crosbie, M. *Am. J. Physiol. Heart Circ. Physiol.* **2006**, *291*, H379.

(280) Dalsgaard, T.; Simonsen, U.; Fago, A. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *292*, H3072.

(281) Isbell, T.S.; Gladwin, M.T.; Patel, R.P. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *293*, H2565.

(282) Rassaf, T.; Flogel, U.; Drexhage, C.; Hendgen-Cotta, U.; Kelm, M.; Schrader, J. *Circ. Res.* **2007**, *100*, 1749.

(283) Alzawahra, W.F.; Talukder, M.A.H.; Liu, X.; Samouilov, A.; Zweier, J.L. *Am. J. Physiol. Heart Circ. Physiol.* **2008**, *295*, H499.

(284) Cossins, A.; Berenbrink, M. *Nature* **2008**, *454*, 416.

(285) Hendgen-Cotta, U.B.; Merx, M.W.; Shiva, S.; Schmitz, J.; Becher, S.; Klare, J.P.; Steinhoff, H.J.; Goedecke, A.; Schrader, J.; Gladwin, M.T.; Kelm, M.; Rassaf, T. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 10256.

(286) Minneci, P.C.; Deans, K.J.; Shiva, S.; Zhi, H., Banks, S.M.; Kern, S.; Natanson, C.; Solomon, S.B.; Gladwin, M.T. *Am. J. Physiol. Heart Circ. Physiol.* **2008**, *295*, H743.

(287) Yu, B.; Raher, M.J.; Volpato, G.P.; Bloch, K.D.; Ichinose, F.; Zapol, W.M. *Circulation* **2008**, *117*, 1982.

(288) Dufour, S.P.; Patel, R.P.; Brandon, A.; Teng, X.; Pearson, J.; Barker, H.; Ali, L.; Yuen, A.H.; Smolenski, R.T.; Gonzalez-Alonso, J. *Am. J. Physiol. Heart. Circ. Physiol.* **2010**, *299*, H1936.

(289) Hendgen-Cotta, U. B.; Kelm, M.; Rassaf, T. *Nitric Oxide* **2010**, *22*, 75.

(290) Cantu-Medellin, N.; Vitturi, D.A.; Rodriguez, C.; Murphy, S.; Dorman, S.; Shiva, S.; Zhou, Y.; Jia, Y.; Palmer, A.F.; Patel, R.P. *Nitric Oxide* **2011**, *25*, 59.

(291) Ormerod, J.O.M.; Ashrafian, H.; Maher, A.R.; Arif, S.; Steeples, V.; Born, G.V.R.; Egginton, S.; Feelisch, M.; Watkins, H.; Frenneaux, M.P. *Cardiovasc. Res.* **2011**, *89*, 560.

(292) Patel, R.P.; Hogg, N.; Kim-Shapiro, D.B. *Cardiovasc. Res.* **2011**, *89*, 507.

(293) Shiva, S.; Rassaf, T.; Patel, R.P.; Gladwin, M.T. Cardiovasc. Res. **2011**, *89*, 566.

(294) Presently, there are several evidences that Mb exerts multifaceted functions. Mb has a clearly defined oxygen transporter function (facilitate diffusion) in smooth, skeletal and cardiac muscles.[221,222,295-298] However, Mb knockout mouse models do not have the respiration or dioxygen consumption compromised[299,300]; in addition, under steady state normoxic condition, the slow Mb diffusion seems to oppose a predominant role in facilitating oxygen transport in myocardium[301,302]. These (and other) studies suggest that the Mb function **-**thought to be fully understood half a century ago**-** has to be reassessed. One important "novel" function of Mb is certainly the control of the cardiac NO homeostasis, not only via its NO scavenging activity (as mentioned in[265]), but also as a nitrite reductase/NO synthase. Other "unconventional" functions of Mb include oxygen sensing (as will be discussed below), reactive oxygen species scavenging[303] and intracellular fatty acid transporter[304].

(295) Millikan, G.A. *Proc. R. Soc. Lond. B* **1937**, *123*, 218.

(296) Wittenberg, J.B. *Physiol. Rev.* **1970**, *50*, 559.

(297) Wittenberg, J.B., Wittenberg, *J. Exp. Biol.* **2003**, *206*, 2011.

(298) Garry, D.J.; Mammen, P.P. *Adv. Exp. Med. Biol.* **2007**, *618*, 181.

(299) Garry, D.J.; Ordway, G.A.; Lorenz, J.N.; Radford, N.B.; Chin, E.R.; Grange, R.W.; Bassel-Duby, R.; Williams, R.S. *Nature* **1998**, *395*, 905.

(300) Godecke, A.; Flogel, U.; Zanger, K.; Ding, Z.; Hirchenhain, J.; Decking, U.K.; Schrader, J. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10495.

(301) Lin, P.C.; Kreutzer, U.; Jue, T. *Biophys. J.* **2007**, *92*, 2608.

(302) Lin, P.C.; Kreutzer, U.; Jue, T. *J. Physiol.* **2007**, *578*, 595.

(303) Flogel, U.; Godecke, A.; Klotz, L.O.; Schrader, J. *FASEB J.* **2004**, *18*, 1156.

(304) Sriram, R.; Kreutzer, U.; Shih, L.; Jue, T. *FEBS Lett.* **2008**, *582*, 3643.

(305) Ignarro, L.J. *Biosci. Rep.* **1999**, *19*, 51.

(306) Gladwin, M.T.; Ognibene, F.P.; Pannell, L.K.; Nichols, J.S.; Pease-Fye, M.E.; Shelhamer, J.H.; Schechter, A.N. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 9943.

(307) Cannon III, R.O.; Schechter, A.N.; Panza, J.A.; Ognibene, F.P.; Pease-Fye, M.E.; Waclawiw, M.A.; Shelhamer, J.H.; Gladwin, M.T. *J. Clin. Investig.* **2001**, *108*, 279.

(308) Piknova, B.; Gladwin, M.T.; Schechter, A N.; Hogg, N. *J. Biol. Chem.* **2005**, *280*, 40583.

(309) McMahon, T.J.; Stone, A.E.; Bonaventura, J.; Singel, D.J.; Stamler, J.S. *J. Biol. Chem.* **2000**, *275*, 16738.

(310) McMahon, T.J.; Moon, R.E.; Luschinger, B.P.; Carraway, M.S;, Stone, A.E.; Stolp, B.W.; Gow, A.J.; Pawloski, J.R.; Watke, P.; Singel, D.J.; Piantadosi, C.A.; Stamler, J. S. *Nat. Med.* **2002**, *8*, 711.

(311) Luchsinger, B.P.; Rich, E. N., Gow, A.J.; Williams, E.M.; Stamler, J.S.; Singel, D.J. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 461.

(312) Angelo, M.; Singel, D.J.; Stamler, J.S. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 8366.

(313) Grubina, R.; Huang, Z.; Shiva, S.; Joshi, M.S.; Azarov, I.; Basu, S.; Ringwood, L.A.; Jiang, A.; Hogg, N.; Kim-Shapiro, D.B.; Gladwin, M.T. *J. Biol. Chem.* **2007**, *282*, 12916.

(314) Moshage, H.; Kok, B.; Huizenga, J.R.; Jansen, P.L. *Clin. Chem.* **1995**, *41*, 892.

(315) Kleinbongard, P; Dejam, A.; Lauer, T.; Rassaf, T.; Schindler, A.; Picker, O.; Scheeren, T.; Godecke, A.; Schrader, J.; Schulz, R.; Heusch, G.; Schaub, G.A.; Bryan, N.S.; Feelisch, M.; Kelm, M. *Free Radic. Biol. Med.* **2003**, *35*, 790.

(316) Dejam, A.; Hunter, C.J.; Pelletier, M.M.; Hsu, L.L.; Machado, R.F.; Shiva, S.; Power, G.G.; Kelm, M.; Gladwin, M.T.; Schechter, A.N. *Blood* **2005**, *106*, 734.

(317) Grau, M.; Hendgen-Cotta, U.B.; Brouzos, P.; Drexhage, C.; Rassaf, T.; Lauer, T.; Dejam, A.; Kelm, M.; Kleinbongard, P. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2007**, *851*, 106.

(318) Hopmann, K.H.; Cardey, B.; Gladwin, M-T.; Kim-Shapiro, D.B.; Ghosh, A. *Chem. Eur. J.* **2011**, *17*, 6348.

(319) Fernandez, B.O.; Ford, P. C. *J. Am. Chem. Soc.* **2003**, *125*, 10510.

(320) Fernandez, B.O.; Lorkovic, I.M.; Ford, P.C. *Inorg. Chem.* **2003**, *42*, 2.

(321) Jee, J.-E.; van Eldik, R. *Inorg. Chem.* **2006**, *45*, 6523.

(322) Nagababu, E.; Ramasamy, S.; Rifkind, J.M. *Nitric Oxide* **2006**, *15*, 20.

(323) Basu, S.; Grubina, R.; Huang, J.; Conradie, J.; Huang, Z.; Jeffers, A.; Jiang, A.; He, X.; Azarov, I.; Seibert, R.; Mehta, A.; Patel, R.; King, S.B.; Hogg, N.; Ghosh, A.; Gladwin, M.T.; Kim-Shapiro, D.B. *Nat. Chem. Biol.* **2007**, *3*, 785.

(324) Navati, M.S.; Friedman, J.M. *J. Am. Chem. Soc.* **2009**, *131*, 12273.

(325) Ford, P.C. *Inorg. Chem.* **2010**, *49*, 6226.

(326) Roche, C.J.; Friedman, J.M. *Nitric Oxide* **2010**, *22*, 180.

(327) Tejero, J.; Basu, S.; Helms, C., Hogg, N.; King, B.; Kim-Shapiro, D.B.; Gladwin, M.T. . *J. Biol. Chem.* **2012**, *287*, 18262.

(328) Rodkey, F.L. *Clin. Chem.* **1976**, *22*, 1986.

(329) Wanat, A.; Gdula-Argasinka, J.; Rutkowsha-Zbik, D.; Witko, M.; Stochel, G.;van Eldik, R. *J. Biol. Inorg. Chem.* **2002**, *7*, 165.

(330) Schwab, D.E.; Stamler, J.S.; Singel, D.J. *Nat. Chem. Biol.* **2009**, *5*, 367.

(331) The nitrite binding to met-Hb was found to be complex (not involving a single association and dissociation rate), being affected by allosteric effectors, relative concentrations of reactants and pH. The K_d value of the (Hb)Fe^{3+}-NO_2^- complex was recently shown to decrease as nitrite/met-Hb ratio, temperature and pH decrease, reaching a minimum of ≈5x10^{-5}M at pH 6.5 and nitrite/Met-Hb of 1/1.[323,330,332] This pH dependence suggests that nitrite has a higher affinity for T-state met-Hb than for R-state met-Hb.[332] Nevertheless, those results anticipate that also the (Hb)Fe^{2+}-NO_2^- complex

would depend on the same factors.

(332) Goetz, B.I.; Shields, H.W.; Basu, S.; Wang, P.; King, S.B.; Hogg, N.; Gladwin; M.T.; Kim-Shapiro, D.B. *Nitric Oxide* **2010**, *22*, 149.

(333) Grätzel, M; Taniguchi, S.; Henglein, A. *Ber. Bunsenges. Phys. Chem.* **1970**, *74*, 488.

(334) Treinin, A.; Hayon, E. *J. Am. Chem. Soc.* **1970**, *92*, 5821.

(335) Kojima, H.; Nakatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. *Anal. Chem.* **1998**, *70*, 2446.

(336) Nagano, T.; Yoshimura, T *Chem. Rev.* **2002**, *102*, 1235.

(337) Wink, D.A.; Darbyshire, J.F.; Nims, R.W.; Saavedra, J.E.; Ford, P.C. *Chem. Res. Toxicol.* **1993**, *6*, 23.

(338) Kharitonov, V.G.; Sundquist, A.R.; Sharma, V.S. J. Biol. Chem. **1995**, *270*, 28158.

(339) Beckman, J.S.; Koppenol, W.H. *Am. J. Physiol.* **1996**, *271*, C1424.

(340) Williams, D.L.H *Nitrosation reactions and the chemistry of nitric oxide*; Elsevier: Amsterdam, 2004; p1.

(341) Huang, K.T.; Keszler, A.; Patel, N.; Patel, R.P.; Gladwin, M.T.; Kim-Shapiro, D.B.; Hogg, N. *J. Biol. Chem.* **2005**, *280*, 31126.

(342) Roche, C.J.; Dantsker, D.; Samuni, U.; Friedman, J.M. *J. Biol. Chem.* **2006**, *281*, 36874.

(343) Jensen, F.B. *FEBS J.* **2008**, *275*, 3375.

(344) Blood, A.B.; Tiso, M.; Verma, S.T.; Lo, J.; Joshi, M.S.; Azarov, I.; Longo, L.D.; Gladwin, M.T.; Kim-Shapiro, D.B.; Power, G.G. *Am. J. Physiol. Heart Circ. Physiol.* **2009**, *296*, H237.

(345) Rifkind, J.M.; Nagababu, E.; Ramasamy, S. *Nitric Oxide* **2011**, *24*, 102.

(346) Nagababu, E.; Ramasamy, S.; Rifkind, J.M. *Biochemistry* **2007**, *46*, 11650.

(347) Salgado, M.T.; Nagababu, E.; Rifkind, J.M. *J. Biol. Chem.* **2009**, *284*, 12710.

(348) Bonaventura, C.; Henkens, R.; Alayash, A.I.; Crumbliss, A.L. *IUBMB Life* **2007**, *59*, 498.

(349) Grubina, R.; Basu, S.; Tiso, M.; Kim-Shapiro, D.B.; Gladwin, M.T. *J. Biol. Chem.* **2008**, *283*, 3628.

(350) Salhany, J.M. *Blood Cells Mol. Dis.* **2010**, *44*, 111.

(351) Sharma, V.S.; Isaacson, R.A.; John, M.E.; Waterman, M.R.; Chevion, M. *Biochemistry* **1983**, *22*, 3897.

(352) The non-nucleated red blood cell is unique among human cells in that the plasma membrane is its only structural component. Presently, the red blood cell membrane is thought as a composite structure in which a membrane envelope is secured to an elastic network of skeletal proteins via transmembrane proteins.[353] The membrane proteins comprise transporters, signalling receptors, adhesion proteins (involved in the interaction with endothelial and other blood cells) and other proteins of still undefined function. Some of the membrane proteins are assembled into super-macromolecular complexes termed "metabolons", like the band 3. The band 3 comprises an anion transporter, various glycolytic enzymes, the presumptive carbon dioxide transporter and carbonic anhydrase, that, together, seem to play key role in regulating the cell metabolism and ion and gas transport function. In addition, deoxygenated Hb binds to the band 3 cytoplasmatic face, in

the same region as the glycolytic enzymes.
(353) Mohandas, N.; Gallagher, P.G. *Blood* **2008**, *112*, 3939.
(354) Lancaster Jr.; J.R., *Methods Enzymol.* **1996**, *268*, 31.
(355) Lancaster Jr., J.R. *Nitric Oxide* **1997**, *1*, 18.
(356) The percentage of met-Hb in the blood is normally very low (<1% of total Hb), however its local relative concentration in the cell membrane could be much higher (within a metabolon that favours the binding of deoxygenated Hb). Furthermore, the reaction with nitrite should increase it even more (eq. 13).
(357) Vaughn, M.W.; Huang, K.T.; Kuo, L.; Liao, J.C. *J. Biol. Chem.* **2000**, *275*, 2342.
(358) Herold, S.; Rock, G. *J. Biol. Chem.* **2003**, *278*, 6623.
(359) Chien, J.C. *J. Am. Chem. Soc.* **1969**, *91*, 2166.
(360) Hoshino, M.; Maeda, M.; Konishi, R.; Seki, H.; Ford, P.C. *J. Am. Chem. Soc.* **1996**, *118*, 5702.
(361) Reductive nitrosylation, known for almost a century[362], is the process by which met-Hb is reduced and nitrosylated in the presence of excess of NO, and it involves (i) binding of NO (eq. 20), (ii) intramolecular haem reduction (eq. 25b), (iii) water nucleophilic attack to yield deoxy-Hb (eq. 26) and (iv) the subsequent nitrosylation of the ferrous haem by NO (eq. 15).
(362) Keilin, D.; Hartree, E.F. *Nature* **1937**, *139*, 548.
(363) It should be noted that the reductive nitrosylation is formally the reverse of the nitrite reduction, as represented in eq. 13c+13b+13a; accordingly, also the reductive nitrosylation is expected to be controlled by the Hb conformation.
(364) Ascenzi, P.; Bocedi, A.; Antonini, G.; Bolognesi, M.; Fasano, M. *FEBS J.* **2007**, *274*, 551.
(365) Ascenzi, P.; Cao, Y.; di Masi, A.; Gullotta, F.; De Sanctis, G.; Fanali, G.; Fasano, M.; Coletta, M. *FEBS J.* **2010**, *277*, 2474.
(366) Ascenzi, P.; di Masi, A.; Gullotta, F.; Mattu, M.; Ciaccio, C., Coletta, M. *Biochem. Biophys. Res. Commun.* **2010**, *393*, 196.
(367) Jia, L.; Bonaventura, C.; Bonaventura, J.; Stamler, J.S. *Nature* **1996**, *380*, 221.
(368) Stamler, J.S.; Jia, L.; Eu, J.P.; McMahon, T.J.; Demchenko, I.T.;, Bonaventura, J.; Gernert, K.; Piantadosi, C.A. *Science* **1997**, *276*, 2034.
(369) Pawloski, J.R.; Hess, D.T.; Stamler, J.S. *Nature* **2001**, *409*, 622.
(370) Xu, X.; Cho, M.; Spencer, N.Y.; Patel, N.; Huang, Z.; Shields, H.; King, S.B.; Gladwin, M.T.; Hogg, N.; Kim-Shapiro, D.B. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 11303.
(371) Huang, K.T.; Azarov, I.; Basu, S.; Huang, J.; Kim-Shapiro, D. B. *Blood* **2006**, *107*, 2602.
(372) Salgado, M.T.; Ramasamy, S.; Tsuneshige, A.; Manoharan, P.T.; Rifkind, J.M. *. Am. Chem. Soc.* **2011**, *133*, 13010.
(373) Feelisch, M.; Rassaf, T.; Mnaimneh, S.; Singh, N.; Bryan, N.S.; Jourd'Heuil, D.; Kelm, M. *FASEB J.* **2002**, *16*, 1775.
(374) Crawford, J.H.; Chacko, B.K.; Pruitt, H.M.; Piknova, B.; Hogg, N.; Patel, R.P. *Blood* **2004**, *104*, 1375.
(375) Shiva, S.; Frizzell, S.; Gladwin M.T. *Nitric Oxide: Biology and Pathobiology*; Elsevier: San Diego,

2010; p605.
(376) Feelisch, M.; Fernandez, B.O.; Bryan, N.S.; Garcia-Saura, M.F.; Bauer, S.; Whitlock, D.R.; Ford, P.C.; Janero, D.R.; Rodriguez, J.; Ashrafian, H. *J. Biol. Chem.* **2008**, *283*, 33927.
(377) Li, H.; Cui, H.; Kundu, T.K.; Alzawahra, W.; Zweier, J.L. J. Biol. Chem. **2008**, *283*, 17855.
(378) Mikulski, R; Tu, C; Swenson, E.R.; Silverman, D.N. *Free Radic. Biol. Med.* **2010**, *48*, 325.
(379) Buerk, D.G-; Barbee, K.A.; Jaron, D. *Adv. Exp. Med. Biol.* **2011**, *701*, 271.
(380) Rodriguez, J.; Maloney, R.E.; Rassaf, T.; Bryan, N.S.; Feelisch, M. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 336.
(381) Shiva, S.; Gladwin, M.T. J. Mol. Cell Cardiol. **2009**, *46*, 1.
(382) Brunori, M. Trends Biochem. Sci. **2001**, *26*, 209.
(383) Wegener, J.W.; Godecke, A.; Schrader, J.; Nawrath, H. *Br. J. Pharmacol.* **2002**, *136*, 415.
(384) Godecke, A.; Molojavyi, A.; Heger, J.; Flogel, U.; Ding, Z.; Jacoby, C.; Schrader, J. *J. Biol. Chem.* **2003**, *278*, 21761.
(385) Wunderlich, C.; Flogel, U.; Godecke, A.; Heger, J.; Schrader, J. *Circ. Res.* **2003**, *92*, 1352.
(386) Merx, M.W.; Gödecke, A.; Flögel, U.; Schrader, J. *FASEB J.* **2005**, *19*, 1015.
(387) Burmester, T.; Weich, B.; Reinhardt, S.; Hankeln, T. *Nature* **2000**, *407*, 520.
(388) Reuss, S.; Saaler-Reinhardt, S.; Weich, B.; Wystub, S.; Reuss, M.H.; Burmester, T.; Hankeln, T. *Neuroscience* **2002**, *115*, 645.
(389) Geuens, E.; Brouns, I.; Flamez, D.; Dewilde, S.; Timmermans, J.-P.; Moens, L. *J. Biol. Chem.* **2003**, *278*, 30417.
(390) Schmidt, M.; Giessl, A.; Laufs, T.; Hankeln, T.; Wolfrum, U.; Burmester, T. *J. Biol. Chem.* **2003**, *278*, 1932.
(391) Schmidt, M.; Laufs, T.; Reuss, S.; Hankeln, T.; Burmester, T. *Neurosci. Lett.* **2005**, *374*, 207.
(392) Ostojić, J.; Sakaguchi, D.S.; de Lathouder, Y.; Hargrove, M.S.; Trent 3rd, J.T.; Kwon, Y.H.; Kardon, R.H.; Kuehn, M.H.; Betts, D.M.; Grozdanić , S. *Invest. Ophthalmol. Vis. Sci.* **2006**, *47*, 1016.
(393) Awenius, C.; Hankeln, T.; Burmester, T. *Biochem. Biophys. Res. Commun.* **2001**, *287*, 418.
(394) Trent III, J.T.; Watts, R.A.; Hargrove, M.S. *J. Biol. Chem.* **2001**, *276*, 30106.
(395) Zhang, C.; Wang, C.; Deng, M.; Li, L.; Wang, H.; Fan, M.; Xu, W.; Meng, F.; Qian, L.; He, F. *Biochem. Biophys. Res. Commun.* **2002**, *290*, 1411.
(396) Burmester, T.; Haberkamp, M.; Mitz, S.; Roesner, A.; Schmidt, M.; Ebner, B.; Gerlach, F.; Fuchs, C.; Hankeln, T. *IUBMB Life* **2004**, *56*, 703.
(397) Hankeln, T.; Ebner, B.; Fuchs, C.; Gerlach, F.; Haberkamp, M.; Laufs, T.L.; Roesner, A.; Schmidt, M.; Weich, B.; Wystub, S.; Saaler-Reinhardt, S.; Reuss, S.; Bolognesi, M.; De Sanctis, D.; Marden, M.C.; Kiger, L.; Moens, L.; Dewilde, S.; Nevo, E.; Avivi, A.; Weber, R.E.; Fago, A.; Burmester, T. *J. Inorg. Biochem.* **2005**, *99*, 110.
(398) Brunori, M.; Vallone, B. Cell. Mol. Life Sci. **2007**, *64*, 1259.
(399) Nb displays all determinants of the globin fold, typically eight α-helices, named "A" to "H", and three key amino acid residues, namely the proximal and distal histidine residues that coordinate the

haem, at the helical positions F8 and E7, respectively (*i.e.*, the eighth and seventh residues on the F and E helices).[400,401] For human Nb, these topological positions correspond to His_{96} and His_{64}, respectively.

(400) Bashford, D.; Chothia, C.; Lesk, A.M. *J. Mol. Biol.* **1987**, *196*, 199.

(401) Moens, L.; Vanfleteren, J.; Van de Peer, Y.; Peeters, K.; Kapp, O.; Czeluzniak, J.; Goodman, M.; Blaxter, M.; Vinogradov, S. *Mol. Biol. Evol.* **1996**, *13*, 324.

(402) Couture, M.; Burmester, T.; Hankeln, T.; Rousseau, D.L. *J. Biol. Chem.* **2001**, *276*, 36377.

(403) Dewilde, S.; Kiger, L.; Burmester, T.; Hankeln, T.; Baudin-Creuza, V.; Aerts, T.; Marden, M.C.; Caubergs, R.; Moens, L. *J. Biol. Chem.* **2001**, *276*, 38949.

(404) Kriegl, J.M.; Bhattacharyya, A.J.; Nienhaus, K.; Deng, P.; Minkow, O.; Nienhaus, G.U. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 7992.

(405) Pesce, A.; Dewilde, S.; Nardini, M.; Moens, L.; Ascenzi, P.; Hankeln, T.; Burmester, T.; Bolognesi, M. *Structure* **2003**, *11*, 1087.

(406) Nienhaus, K.; Kriegl, J.M.; Nienhaus, G.U. *J. Biol. Chem.* **2004**, *279*, 22944.

(407) Pesce, A.; Dewilde, S.; Nardini, M.; Moens, L.; Ascenzi, P.; Hankeln, T.; Burmester, T.; Bolognesi, M. *Micron* **2004**, *35*, 63.

(408) Uno, T.; Ryu, D.; Tsutsumi, H.; Tomisugi, Y., Ishikawa, Y.; Wilkinson, A. J.; Sato, H.; Hayashi, T. *J. Biol. Chem.* **2004**, *279*, 5886.

(409) Uzan, J.; Dewilde, S.; Burmester, T.; Hankeln, T.; Moens, L.; Hamdane, D.; Marden, M.C.; Kiger, L. *Biophys. J.* **2004**, *87*, 1196.

(410) Vallone, B.; Nienhaus, K.; Brunori, M.; Nienhaus, G.U. *Proteins* **2004**, *56*, 85.

(411) The human Nb coordination state (hexa- *versus* penta-coordination) is controlled through a redox mechanism that involves the formation, or cleavage, of an intramolecular disulfide bond between the surface Cys_{CD7} and Cys_{D5} (respectively, the seventh residue on the inter-helix region between helices C and D (Cys_{46}) and the fifth residue on helix D (Cys_{55})).[405,410,412-416] The formation of the CD7-D5 disulfide bond should perturb the positioning of the distal histidine E7 on the neighbouring E helix and, thus, modulate the haem "internal" hexa-coordination, controlling in this way the populations of penta- and hexa-coordinated Nb, as was confirmed by theoretical studies.[417,418] Actually, Nb holds (besides a large channel connecting the haem with the bulk) two small internal cavities on the haem distal side that enable the distal histidine side chain to move either towards the interior or the exterior of the distal pocket[405,410]; moreover, "external" ligand (carbon monoxide) biding was shown to be accompanied by conformational changes involving distal histidine reposition and haem "sliding" towards a preformed crevice, while the overall protein structure is not significantly altered[405,410,414,416]. In this conversion mechanism, the Phe_{B10} residue was shown to be essential to "translate" the disulfide bond formation into the conformational change of the haem pocket.[419-422] Supporting this mechanism of hexa- to penta-coordination conversion is the observation that the disulfide-containing Nb displays an increased histidine-haem dissociation rate (k_{off}≈0.6 *versus* $7 s^{-1}$ [412,413,423,424]) and a concomitantly decreased histidine affinity (K≈3300 *versus* 280 [412,413,423]), relatively to the reduced or mutated ($Cys_{46}Gly$, $Cys_{55}Ser$, $Cys_{120}Ser$) Nb.

The consequent increase of the population of penta-coordinated molecules in disulfide-containing Nb leads to an increase in the global affinity of "external" ligands.[412,413,425,426] That is, for dioxygen, *e.g.*, its global affinity increases by the same factor as the histidine affinity decreases (≈10), even though the dioxygen-penta-coordinated Nb k_{on} and k_{off} do not change significantly with the disulfide bond formation.[412,425]

(412) Hamdane, D.; Kiger, L.; Dewilde, S.; Green, B.N.; Pesce, A.; Uzan, J.; Burmester, T.; Hankeln, T.; Bolognesi, M.; Moens, L.; Marden, M.C. *J. Biol. Chem.* **2003**, *278*, 51713.

(413) Hamdane, D.; Kiger, L.; Dewilde, S.; Green, B.N.; Pesce, A.; Uzan, J.; Burmester, T.; Hankeln, T.; Bolognesi, M.; Moens, L.; Marden, MC. *Micron.* **2004**, *35*, 59.

(414) Vallone, B.; Nienhaus, K.; Matthes, A.; Brunori, M.; Nienhaus, G.U. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17351.

(415) Nadra, A.D.; Marti, M.A.; Pesce, A.; Bolognesi, M.; Estrin, D.A. *Proteins* **2008**, *71*, 695.

(416) Bocahut, A.; Bernad, S.; Sebban, P.; Sacquin-Mora, S. *J. Phys. Chem. B* **2009**, *113*, 16257.

(417) Nadra, A.D.; Martí, M.A.; Pesce, A.; Bolognesi, M.; Estrin, D.A. *Proteins Struct. Funct. Bioinf.* **2007**, *71*, 695.

(418) Bocahut, A.; Derrien, V.; Bernad, S.; Sebban, P.; Sacquin-Mora, S.; Guittet, E.; Lescop, E. *J. Biol. Inorg. Chem.* **2013**, *18*, 111.

(419) Vinck, E.; Van Doorslaer, S.; Dewilde, S.; Moens, L. *J. Am. Chem. Soc.* **2004**, *126*, 4516.

(420) Nienhaus, K.; Nienhaus, G.U. *J. Biol. Phys.* **2007**, *33*, 357.

(421) Lutz, S.; Nienhaus, K.; Nienhaus, G.U.; Meuwly, M. *J. Phys. Chem. B* **2009**, *113*, 15334.

(422) Ezhevskaya, M.; Trandafir, F.; Moens, L.; Dewilde, S.; Van Doorslaer, S. *J. Inorg. Biochem.* **2011**, *105*, 1131.

(423) Kiger, L.; Uzan, J.; Dewilde, S.; Burmester, T.; Hankeln, T.; Moens, L.; Hamdane, D.; Baudin-Creuza, V.; Marden, M.C. *IUBMBLife* **2004**, *56*, 709.

(424) Smagghe, B.J.; Sarath, G.; Ross, E.; Hilbert, J.L.; Hargrove, M.S. *Biochemistry* **2006**, *45*, 561.

(425) Fago, A.; Hundahl, C.; Dewilde, S.; Gilany, K.; Moens, L.; Weber, R.E. *J. Biol. Chem.* **2004**, *279*, 44417.

(426) Astudillo, L.; Bernad, S.; Derrien, V.; Sebban, P.; Miksovska. J. *Biophys. J.* **2010**, *99*, L16.

(427) Moens, L.; Dewilde, S. *Nature* **2000**, *407*, 461.

(428) Hargrove, M.; Brucker, E.; Stec, B.; Sarath, G.; Arredondo-Peter, R.; Klucas, R.; Olson, J.; Phillips, G. *Struct. Fold. Des.* **2000**, *8*, 1005.

(429) Trent III, J.T.; . Hargrove, M.S. *J. Biol. Chem.* **2002**, *277*, 19538.

(430) Dioxygen transporters with very high affinities would facilitate dioxygen diffusion only under very low dioxygen concentration. *E.g.*, plant leghaemoglobins (see section 3.2.1.3. for details about these proteins), with dioxygen affinities ≈10 times higher than Mb, are believed to facilitate dioxygen transport in hypoxic root nodules. The Nb, with an affinity two orders of magnitude higher[394,403], would serve this role only under an oxygen gradient in the nanomolar range.

(431) Fago, A.; Hundahl, C.; Malte, H.; Weber, R.E. *IUBMB Life* **2004**, *56*, 689.

(432) Weiland, T.R.; Kundu, S.; Trent III, J.T.; Hoy, J.A.; Hargrove, M.S. *J. Am. Chem. Soc.* **2004**,

126, 11930.

(433) Sun, Y.; Jin, K.; Mao, X.O.; Zhu, Y.; Greenberg, D.A. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 15306.

(434) Sun, Y.; Jin, K.; Peel, A.; Mao, X.O.; Xie, L.; Greenberg, D.A *Proc. Natl. Acad. Sci. USA* **2003** *100*, 3497.

(435) Herold, S.; Fago, A.; Weber, R.E.; Dewilde, S.; Moens, L. *J. Biol. Chem.* **2004**, *279*, 22841.

(436) Fordel, E.; Thijs, L.; Martinet, W.; Schrijvers, D.; Moens, L.; Dewilde, S. *Gene* **2007**, *398*, 114.

(437) Fordel, E.; Thijs, L.; Moens, L.; Dewilde, S. *FEBS J.* **2007**, *274*, 1312.

(438) Nicolis, S.; Monzani, E.; Ciaccio, C.; Ascenzi, P.; Moens, L.; Casella, L. *Biochem. J.* **2007**, *407*, 89.

(439) Li, R.C.; Morris, M.W.; Lee, S.K.; Pouranfar, F.; Wang, Y.; Gozal, D. *Brain Res.* **2008**, *1190*, 159.

(440) Duong, T.T.; Witting, P.K.; Antao, S.T.; Parry, S.N.; Kennerson, M.; Lai, B.; Vogt, S.; Lay, P.A.; Harris, H.H. *J. Neurochem.* **2009**, *108*, 1143.

(441) Liu, J.; Yu, Z.; Guo, S.; Lee, S.R.; Xing, C.; Zhang, C.; Gao, Y.; Nicholls, D.G.; Lo, E. H.; Wang, X. *J. Neurosci. Res.* **2009**, *87*, 164.

(442) Nayak, G.; Prentice, H.M.; Milton, S.L. *J. Neurochem.* **2009**, *110*, 603.

(443) Li, L.; Moore, P.K. *Biochem. Soc. Trans.* **2007**, *35*, 1138.

(444) Li, X.; Bazer, F.W.; Gao, H.; Jobgen, W.; Johnson, G.A.; Li, P.; McKnight, J.R.; Satterfield, C.; Spencer, T.E.; Wu, G. *Amino Acids* **2009**, *37*, 65.

(445) Kajimura, M.; Fukuda, R.; Bateman, R.M.; Yamamoto,T.; Suematsu, M. *Antioxidants Redox Sign.* **2010**, *13*, 157.

(446) Motterlini, R.; Otterbein, L. E. *Nat. Rev. Drug Discov.* **2010**, *9*, 728.

(447) Burmester, T.; Ebner, B.; Weich, B.;Hankeln, T. *Mol. Biol. Evol.* **2002**, *19*, 416.

(448) Garry, D.J.; Mammen, P.P.A. *Lancet* **2003**, *362*, 342.

(449) Van Doorslaer, S.; Dewilde, S.; Kiger, L.; Nistor, S.V.; Goovaerts, E.; Marden, M.C.; Moens, L. *J. Biol. Chem.* **2003**, *278*, 4919.

(450) Wakasugi, K.; Nakano, T.; Morishima, I. *J. Biol. Chem.* **2003**, *278*, 36505.

(451) Brunori, M.; Giuffrè, A.; Nienhaus, K.; Nienhaus, G.U.; Scandurra, F.M.; Vallone, B. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 8483.

(452) Herold, S.; Fago, A. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2005**, *142*, 124.

(453) Hundahl, C.; Stoltenberg, M.; Fago, A.; Weber, R.; Dewilde, S.; Fordel, E.; Danscher, G. *Neuropathol. Appl. Neurobiol.* **2005**, *31*, 610.

(454) Schmidt-Kastner, R.; Haberkamp, M.; Schmitz, C.; Hankeln, T.; Burmester, T. *Brain Res.* **2006**, *1103*, 173.

(455) Burmester, T.; Hankeln, T. *J. Exp. Biol.* **2009**, *212*, 1423.

(456) Jin, K.; Mao, Y.; Mao, X.; Xie, L.; Greenberg, D. *Stroke* **2010**, *41*, 557.

(457) Fago, A.; Mathews, A.; Moens, L.; Dewilde, S.; Brittain, T. *FEBS Lett.* **2006**, *580*, 4884.

(458) Fago, A.; Mathews, A.; Brittain, T. *IUBMB Life* **2008**, *60*, 398.

(459) Raychaudhuri, S.; Skommer, J.; Henty, K.; Birch, N.; Brittain, T. *Apoptosis* **2010**, *15*, 401.

(460) Sowa, A.W.; Guy, P.A.; Sowa, S.; Hill, R.D. *Acta Biochim. Pol.* **1999**, *46*, 431.

(461) Pesce, A.; Bolognesi, M.; Bocedi, A.; Ascenzi, P.; Dewilde, S.; Moens, L.; Hankeln, T.; Burmester, T. *EMBO Rep.* **2002**, *3*, 1146.

(462) Wakasugi, K.; Kitatsuji, C.; Morishima, I. *Ann. N.Y. Acad. Sci.* **2005**, *1053*, 220.

(463) Wakasugi, K.; Morishima, I. *Biochemistry* **2005**, *44*, 2943.

(464) Kitatsuji, C.; Kurogochi, M.; Nishimura, S.; Ishimori, K.; Wakasugi, K. *J. Mol. Biol.* **2007**, *368*, 150.

(465) Burmester, T.; Hankeln, T. *News Physiol. Sci.* **2004**, *19*, 110.

(466) Brunori, M.; Vallone, B. *FASEB J.* **2006**, *20*, 2192.

(467) Jung, K.H.; Chu, K.; Ko, S.Y.; Lee, S.T.; Sinn, D.I.; Park, D.K.; Kim, J.M.; Song, E.C.; Kim, M.; Roh, J.K. *Stroke* **2006**, *37*, 2744.

(468) Khan, A.A.; Wang, Y.; Sun, Y.; Mao, X.O.; Xie, L.; Miles, E.; Graboski, J.; Chen, S.; Ellerby, L.M.; Jin, K.; Greenberg, D.A. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17944.

(469) Greenberg, D.A.; Jin, K.; Khan, A.A. *Curr. Opin. Pharmacol.* **2008**, *8*, 20.

(470) Wang, X.; Liu, J.; Zhu, H.; Tejima, E.; Tsuji, K.;Murata, Y.; Atochin, D.N.; Huang, P.L.; Zhang, C.; Lo, E.H. *Stroke* **2008**, *39*, 1869.

(470) Avivi, A.; Gerlach, F.; Joel, A.; Reuss, S.; Burmester, T.; Nevo, E.; Hankeln, T. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 21570.

(471) Lechauve, C.; Chauvierre, C.; Dewilde, S.; Moens, L.; Green, B.N.; Marden, M.C.; Célier, C.; Kiger, L. *FEBS J.* **2010**, *277*, 2696.

(472) Sanctis, D.; Dewilde, S.; Pesce, A.; Moens, L.; Ascenzi, P.; Hankeln, T.; Burmester, T.; Bolognesi, M. *Biochem. Biophys. Res. Commun.* **2004**, *316*, 1217.

(473) Sanctis, D.; Dewilde, S.; Pesce, A.; Moens, L.; Ascenzi, P.; Hankeln, T.; Burmester, T.; Bolognesi, M. *J. Mol. Biol.* **2004**, *336*, 917.

(474) Schmidt, M.; Gerlach, F.; Avivi, A.; Laufs, T.; Wystub, S.; Simpson, J.C.; Nevo, E.; Saaler-Reinhardt, S.; Reuss, S.; Hankeln, T.; Burmester, T. *J. Biol. Chem.* **2004**, *279*, 8063.

(475) Burmester, T.; Gerlach, F.; Hankeln, T. *Adv. Exp. Med. Biol.* **2007**, *618*, 169.

(476) Gardner, A.M.; Cook, M.R.; Gardner, P.R. *J. Biol. Chem.* **2010**, *285*, 23850.

(477) Liu, X.; Follmer, D.; Zweier, J.R.; Huang, X.; Hemann, C.; Liu, K.; Druhan, L.J.; Zweier, J.L. *Biochemistry* **2012**, *51*, 5072.

(478) Kawada, N.; Kristensen, D.B.; Asahina, K.; Nakatani, K.; Minamiyama, Y.; Seki, S.; Yoshizato, K. *J. Biol. Chem.* **2001**, *276*, 25318.

(479) Fordel, E.; Geuens, E.; Dewilde, S.; Rottiers, P.; Carmeliet, P.; Grooten, J.; Moens, L. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 342.

(480) Halligan, K.E.; Jourd'heuil, F.L.; Jourd'heuil, D. *J. Biol. Chem.* **2009**, *284*, 8539.

(481) Singh, S.; Manda, S.M.; Sikder, D.; Birrer, M.J.; Rothermel, B.A.; Garry, D.J.; Mammen, P.P. *J. Biol. Chem.* **2009**, *284*, 10409.

(482) Stagner, J.I.; Parthasarathy, S.N.; Wyler, K.; Parthasarathy, R.N. *Transplant. Proc.* **2005**, *37*,

3452.
(483) Fordel, E.; Thijs, L.; Martinet, W.; Lenjou, M.; Laufs, T.; Van Bockstaele, D.; Moens, L.; Dewilde, S. *Neurosci. Lett.* **2006**, *410*, 146.
(484) Xu, R.; Harrison, P.M.; Chen, M.; Li, L.; Tsui, T.Y.; Fung, P.C.; Cheung, P.T.; Wang, G.; Li, H.; Diao, Y.; Krissansen, G.W.; Xu, S.; Farzaneh, F. *Mol. Ther.* **2006**, *13*, 1093.
(485) Guo, X.; Philipsen, S.; Tan-Un, K.C. Biochem. Biophys. Res. Commun. **2007**, *364*, 145.
(486) Li, D.; Chen, X.Q.; Li, W.J.; Yang, Y.H.; Wang, J.Z.; Yu, A.C. *Neurochem. Res.* **2007**, *32*, 1375.
(487) Hodges, N.J.; Innocent, N.; Dhanda, S.; Graham, M. *Mutagenesis* **2008**, *23*, 293.
(488) Emara, M.; Turner, A.R.; Allalunis-Turner, J. *Cancer Cell Int.* **2010**, *10*, 33.
(489) Nakatani, K.; Okuyama, H.; Shimahara, Y.; Saeki, S.; Kim, D.H.; Nakajima, Y.; Seki, S.; Kawada, N.; Yoshizato, K. *Lab. Invest.* **2004**, *84*, 91.
(490) Shivapurkar, N.; Stastny, V.; Okumura, N.; Girard, L.; Xie, Y.; Prinsen, C.; Thunnissen, F.; Wistuba, I.; Czerniak, B.; Frenkel, E.; Roth, J.; Liloglou, T.; Xinarianos, G.; Field, J.; Minna, J.; Gazdar, A. *Cancer Res.* **2008**, *68*, 7448.
(491) Tiso, M.; Tejero, J.; Basu, S.; Azarov, I.; Wang, X.; Simplaceanu, V.; Frizzell, S.; Jayaraman, T.; Geary, L.; Shapiro, C.; Ho, C.; Shiva, S.; Kim-Shapiro, D.B.; Gladwin, M.T. *J. Biol. Chem.* **2011**, *286*, 18277.
(492) Li, H.; Hemann, C.; Abdelghany, T.M.; El-Mahdy, M.A.; Zweier, J.L. *J. Biol. Chem.* **2012**, *287*, 36623.
(493) Ikeda-Saito, M.; Hori, H.; Andersson, L.A.; Prince, R.C.; Pickering, I.J.; George, G.N.; Sanders 2nd, C.R.; Lutz, R.S.; McKelvey, E.J.; Mattera, R. *J. Biol. Chem.* **1992**, *267*, 22843.
(494) Brancaccio, A.; Cutruzzolá, F.; Allocatelli, C. T.; Brunori, M.; Smerdon, S.J.; Wilkinson, A.J.; Dou, Y.; Keenan, D.; Ikeda-Saito, M.; and Brantley Jr.; R.E. *J. Biol. Chem.* **1994**, *269*, 13843.
(495) Petersen, M.G.; Dewilde, S.; Fago, A. *J. Inorg. Biochem.* **2008**, *102*, 1777.
(496) Gardner, P.R. *J. Inorg. Biochem.* **2005**, *99*, 247.
(497) Oxidative stress (term introduced by H. Sies, in 1985) is "a disturbance in the pro-oxidant/antioxidant balance in favour of the former, leading to potential damage"[498]; it refers to a serious imbalance between the antioxidant defences and the formation of reactive oxygen and nitrogen species.
(498) Sies, H. *Oxidative stress II: Oxidants and Antioxidants*; Academic Press: London, 1991.
(499) MacMunn. C. Philos. Trans. R. Soc. London **1886**, *177*, 267.
(500) Liu, X.; Kim, C.N.; Yang ,J.; Jemmerson, R.; Wang, X. *Cell* **1996**, *86*, 147.
(501) Kluck, R.M.; Bossy-Wetzel, E.; Green, D.R.; Newmeyer, D.D. *Science* **1997**, *275*, 1132.
(502) Yang, J.; Liu, X. S.; Bhalla, K.; Kim, C.N.; Ibrado, A.M.; Cai, J.Y.; Peng, T.I.; Jones, D.P.; Wang, X.D. *Science* **1997**, *275*, 1129.
(503) Cai, J.; Yang, J.; Jones, D.P. *Biochim. Biophys. Acta* **1998**, *1366*, 139.
(504) Bossy-Wetzel, E.; Green, D.R. *Mutat. Res.* **1999**, *434*, 243.
(505) McMillin, J.B.; Dowhan, W. Biochem. Biophys. Acta **2002**, *1585*, 97.
(506) Ott, M.; Robertson, J.D.; Zhivotovsky, B.; Orrenius, S. *Proc. Natl. Acad. Sci. USA* **2002**, *99*,

1259.

(507) Fadeel, B.; Orrenius, S. *J. Intern. Med.* **2005**, *258*, 479.

(508) Yu, X.; Acehan, D.; Ménétret, J.-F.; Booth, C.R.; Ludtke, S.J.; Riedl, S.J.; Shi, Y.; Wang, X.; Akey, C.W. *Structure* **2005**, *13*, 1725.

(509) Garrido, C.; Galluzzi, L.; Brunet, M.; Puig, P.E.; Didelot, C.; Kroemer, G. *Cell Death Differ.* **2006**, *13*, 1423.

(510) Gogvadze, V.; Orrenius, S.; Zhivotovsky, B. *Biochim. Biophys. Acta* **2006**, *1757*, 639.

(511) Ow, Y.-L.P.; Green, D.R.; Hao, Z.; Mak, T.W. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 532.

(512) Bertini, I.; Chevance, S.; Del Conte, R.; Lalli, D.; Turano, P *PLoS ONE* **2011**, *6*, e18329.

(513) The reduction of Cc is being used for almost 50 years as a laboratory assay to quantify the formation of superoxide radical.[514] In addition, the reactivity of Cc with superoxide has been explored for the construction of biosensors (*e.g.* [515]).

(514) Fridovich, I. *CRC Handbook of methods for oxygen radical research*; CRC Press: Boca Raton, 1986; p213.

(515) Wegerich, F.; Turano, P.; Allegrozzi, M.; Mohwald, H.; Lisdat, F. *Anal. Chem.* **2009**, *81*, 2976.

(516) Pereverzev, M.O.; Vygodina, T.V.; Konstantinov, A.A.; Skulachev, V.P. *Biochem. Soc. Trans.* **2003**, *31*, 1312.

(517) Kagan, V.E.; Tyurin, V.A.; Jiang, J.; Tyurina, Y.Y.; Ritov, V.B.; Amoscato, A.A.; Osipov, A.N.; Belikova, N.A.; Kapralov, A.A.; Kini, V.; Vlasova, I.I.; Zhao, Q.; Zou, M.; Di, P.; Svistunenko, D.A.; Kurnikov, I. V.; Borisenko, G.G. *Nat. Chem. Biol.* **2005**, *1*, 223.

(518) Belikova, N.A.; Vladimirov, Y.A.; Osipov, A.N.; Kapralov, A.A.; Tyurin, V.A.; Potapovich, M.V.; Basova, L.V.; Peterson, J.; Kurnikov, I.V.; Kagan, V.E. *Biochemistry* **2006**, *45*, 4998.

(519) Kapralov, A.A.; Kurnikov, I.V.; Vlasova, I.I.; Belikova, N.A.; Tyurin, V.A.; Basova, L.V.; Zhao, Q.; Tyurina, Y.Y.; Jiang, J.F.; Bayir, H.; Vladimirov, Y.A.; Kagan, V.E. *Biochemistry* **2007**, *46*, 14232.

(520) Chen, Y.R.; Deterding, L.J.; Sturgeon, B.E.; Tomer, K.B.; Mason, R.P. *J. Biol. Chem.* **2002**, *277*, 29781.

(521) Estevam, M.L.; Nascimento, O.R.; Baptista, M.S.; Di Mascio, P.; Prado, F.M.; Faljoni-Alario, A.; Zucchi, M.D.; Nantes, I.L. *J. Biol. Chem.* **2004**, *279*, 39214.

(522) Thomson, L.; Trujillo, M.; Telleri, R.; Radi, R. *Arch. Biochem. Biophys.* **1995**, *319*, 491.

(523) Cassina, A.M.; Hodara, R.; Souza, J.M.; Thomson, L.; Castro, L.; Ischiropoulos, H.; Freeman, B.A.; Radi, R. *J. Biol. Chem.* **2000**, *275*, 21409.

(524) Batthyany, C.; Souza, J.M.; Duran, R.; Cassina, A.; Cervenansky, C.; Radi, R. *Biochemistry* **2005**, *44*, 8038.

(525) Rodríguez-Roldán, V.; García-Heredia, J.M.; Navarro, J.A.; De la Rosa, M.A.; Hervás, M. *Biochemistry* **2008**, *47*, 12371.

(526) García-Heredia, J.M.; Díaz-Moreno, I.; Nieto, P.M.; Orzáez, M.; Kocanis, S.; Teixeira, M.; Pérez-Payá, E.; Díaz-Quintana, A.; De la Rosa, M.A. *Biochim. Biophys. Acta* **2010**, *1797*, 981.

(527) Spooner, P.J.R.; Watts, A. *Biochemistry* **1992**, *31*, 10129.

(528) Tuominen, E.K.J.; Wallace, C.J.A.; Kinnunen, P.K.J. *J. Biol. Chem.* **2002**, *277*, 8822.

(529) Basova, L.V.; Kurnikov, I.V.; Wang, L.; Ritov, V.B.; Belikova, N.A.; Vlasova, I.I.; Pacheco, A.A.; Winnica, D.E.; Peterson, J.; Bayir, H.; Waldeck, D.H.; Kagan, V.E. *Biochemistry* **2007**, *46*, 3423.
(530) Jori, G.; Tamburro, A.M.; Azzi, A. *Photochem. Photobiol.* **1974**, *19*, 337.
(531) Brown, L.R.; Wuthrich, K. *Biochim. Biophys. Acta* **1977**, *468*, 389.
(532) Soussi, B.; Bylund-Fellenius, A.C.; Schersten, T.; Angstrom, J. *Biochem. J.* **1990**, *265*, 227.
(533) Pinheiro, T.J. *Biochimie* **1994**, *76*, 489.
(534) Pinheiro, T.J.; Cheng, H.; Seeholzer, S.H.; Roder, H. *J. Mol. Biol.* **2000**, *303*, 617.
(535) Jing, W.G.; Liu, C.W.; Tang, J.L.; Wu, Z.Y.; Dong, S.J.; Wang, E.K. *Chin. J. Chem.* **2003**, *21*, 544.
(536) Wackerbarth, H.; Hildebrandt, P. *Chem. Phys. Chem.* **2003**, *4*, 714.
(537) Oellerich, S.; Lecomte, S.; Paternostre, M.; Heimburg, T.; Hildebrandt, P. *J. Phys. Chem. B* **2004**, *108*, 3871.
(538) Domanov, Y.A.; Molotkovsky, J.G.; Gorbenko, G.P. *Biochim. Biophys. Acta* **2005**, *1716*, 49.
(539) Petrovic, J.; Clark, R. A.; Yue, H.; Waldeck, D. H.; and Bowden, E. F. *Langmuir* **2005**, *21*, 6308.
(540) Paes de Sousa, P.M.; Pauleta, S.R.; Gonçalves, M.L.S.; Pettigrew, G.W.; Moura, I.; Moura, J.J.G.; Santos, M.M.C. *J. Biol. Inorg. Chem.* **2011**, *16*, 209.
(541) Letellier, L.; Shechter, E. *Eur. J. Biochem.* **1973**, *40*, 507.
(542) Nantes, I.L.; Zucchi, M.R.; Nascimento, O.R.; Faljoni-Alario, A. *J. Biol. Chem.* **2001**, *276*, 153.
(543) Rytomaa, M.; Kinnunen, P.K.J. *J. Biol. Chem.* **1995**, *270*, 3197.
(544) Gonzalvez, F.; Gottlieb, E. *Apoptosis* **2007**, *12*, 877.
(545) Ott, M.; Gogvadze, V.; Orrenius, S.; Zhivotovsky, B. *Apoptosis* **2007**, *12*, 913.
(546) Basu, S.; Azarova, N.A.; Font, M.D.; King, S.B.; Hogg, N.; Gladwin, M.T.; Shiva, S.; Kim-Shapiro, D.B. *J. Biol. Chem.* **2008**, *283*, 32590.
(547) Forman, H.J.; Azzi, A. *FASEB J.* **1997**, *11*, 374.
(548) van Beek-Harmsen, B.J.; van der Laarse, W.J. *J. Histochem. Cytochem.* **2005**, *53*, 803.
(549) Casalini, S.; Battistuzzi, G.; Borsari, M.; Ranieri, A.; Sola, M. *J. Am. Chem. Soc.* **2008**, *130*, 15099.
(550) Rose, E.J.; Hoffman, B.M. *J. Am. Chem. Soc.* **1983**, *105*, 2866.
(551) Hoshino, M.; Ozawa, K.; Seki, H.; Ford, P.C. *J. Am. Chem. Soc.* **1993**, *115*, 9568.
(552) Ascenzi, P.; Coletta, M.; Santucci, R.; Polizio, F.; Desideri, A. *J. Inorg. Biochem.* **1994**, *53*, 273.
(553) Bønding, S.H.; Henty, K.; Dingley, A.J.; Brittain, T *Int. J. Biol. Macromol.* **2008**, *43*, 295.
(554) In accordance with this efficient Cc binding and reduction, the Nb neuronal protective role has been suggested be due to (i) its ability to rapidly bind the Cc that is released from the mitochondria during apoptosis (thus blocking the Cc binding to the apoptotic protease activating factor-1), and (ii) to its ability to reduce the Cc with an high intermolecular electron transfer rate ($\approx 2000 s^{-1}$), which is in the range of the one of Cc reduction of CcO[555]. In this way, Nb would "reset the Cc level" that triggers apoptosis, *i.e.*, Nb would "reprogram" the onset of Cc-induced apoptosis.[440,441,457,458,459,553,555]
(555) Brittain, T.; Skommer, J.; Raychaudhuri, S.; Birch, N. *Int. J. Mol. Sci.* **2010**, *11*, 2306.

(556) Shiva, S.; Sack, M.N.; Greer, J.J.; Duranski, M.; Ringwood, L.A.; Burwell, L.; Wang, X.D.; MacArthur, P.H.; Shoja, A.; Raghavachari, N.; Calvert, J.W.; Brookes, P.S.; Lefer, D.J.; Gladwin, M.T. *J. Exp. Med.* **2007**, *204*, 2089.

(557) Shen, Y.H., Wang, X.L.; Wilcken, D.E.L. *FEBS Lett.* **1998**, *433*, 125.

(558) Boyd, C.S.; Cadenas, E. *Biol. Chem.* **2002**, *383*, 411.

(559) Lee, V.Y.; McClintock, D.S.; Santore, M.T.; Budinger, G.R.S.; Chandel, N.S. *J. Biol. Chem.* **2002**, *277*, 16067.

(560) Moncada, S.; Erusalimsky, J.D. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 214.

(561) Schonhoff, C.M.; Gaston, B.; Mannick, J.B. *J. Biol. Chem.* **2003**, *278*, 18265.

(562) Li, C.Q.; Wogan, G.N. *Cancer Lett.* **2005**, *226*, 1.

(563) Castro, L.; Eiserich, J.P.; Sweeney, S.; Radi, R.; Freeman, B.A. *Arch. Biochem. Biophys.* **2004**, *421*, 99.

(564) Pecina, P.; Borisenko, G.G.; Belikova, N.A.; Tyurina, Y.; Pecinova, A.; Lee, I.; Samhan-Arias, A.K.; Przyklenk, K.; Kagan, V.E.; Huttemann, M. *Biochemistry* **2010**, *49*, 6705.

(565) Garcia-Heredia, J.M.; Díaz-Quintana, A.; Salzano, M.; Orzáez, M.; Párez-Payá, E.; Teixeira, M.; De la Rosa, M.A.; Dáaz-Moreno, I. *J. Biol. Inorg. Chem.* **2011**, *16*, 1155.

(566) The enzymes localisation has been subjected to a great controversy, due, at least in part, to the different methodologies employed: while enzymatic assays evaluate the xanthine and dimethylaminocinnamaldehyde oxidation activities in tissue homogenates (for XO/XD and AO, respectively), immunohistochemical methods do not discriminate between both enzymes and identify the proteins, in intact tissues, in any of its forms (sulfo-, desulfo- and demolybdo-forms; see section 4.2.3. for details). The differences between the two types of methodologies are mostly pertinent in tissues where XO can be found in desulfo- or demolybdo-forms (that do not hydroxylate xanthine, but are able to oxidise other substrates, such as NADH[567-573]), like human milk[574,575] and heart[576]. In general, it is accepted that human XO is mostly present in liver, intestine, mammal gland, small vessels endothelial cells and in plasma, while AO can be found mostly in the liver, but also in heart, lung, kidney, brain and eye.[577-584]

(567) Murray, K.N.; Chaykin, S. *J. Biol. Chem.* **1966**, *241*, 3468.

(568) Landon, E.J.; Myles, M. *Biochem. Biophys. Acta* **1967**, *143*, 429.

(569) Massey, V.; Brumby, P.E.; Komai, H.; Palmer, G. *J. Biol. Chem.* **1969**, *244*, 1682.

(570) Mira, L.; Maia, L.; Barreira, L.; Manso, C.F. *Arch. Biochem. Biophys.* **1995**, *318*, 53.

(571) Maia, L.; Vala, A.; Mira, L. *Free Rad. Res.* **2005**, *39*, 979.

(572) Maia, L.; Duarte, R.O.; Ponces-Freire, A.; Moura, J.J.G.; Mira, L. *J. Biol. Inorg. Chem.* **2007**, *12*, 777.

(573) Kundu, T.K.; Velayutham, M.; Zweier, J.L. *Biochemistry* **2012**, *51*, 2930.

(574) Abadeh, S.; Killacky, J.; Benboubetra, M.; Harrison, R. *Biochim. Biophys. Acta* **1992**, *1117*, 25.

(575) Godber, B.L.; Sanders, S.A.; Harrison, R.; Eisenthal, R.; Braughler, J.M. *Biochem. Soc. Trans.* **1997**, *25*, 519s.

(576) Abadeh, S.; Case, P.C.; Harrison, R. *Biochem. Soc. Trans.* **1993**, *21*, 99S.

(577) Della Corte E.; Gozzetti, G.; Novello, F.; Stirpe, F. *Biochim. Biophys. Acta* **1969**, *191*, 164.
(578) Parks, D.A.; Granger, D.N. *Acta Physiol. Scand. Suppl.* **1986**, *548*, 87.
(579) Beedham C. *Prog. Med. Chem.* **1987**, *24*, 85.
(580) Eddy, L.J.; Stewart, J.R.; Jones, H.P.; Engerson, T.D.; McCord, J.M.; Downey, J.M. *Am. J. Physiol.* **1987**, *253*, H709.
(581) Moriwaki, Y.; Yamamoto, T.; Yamaguchi, K.; Takahashi, S.; Higashino, K. *Histochem. Cell Biol.* **1996**, *105*, 71.
(582) Sarnesto, A.; Linder, N.; Raivio, K. O. *Lab. Invest.* **1996**, *74*, 48.
(583) Linder, N.; Rapola, J.; Raivio, K.O. *Lab. Invest.* **1999**, *79* , 967.
(584) Marti, R.; Varela, E.; Pascual, C.; Segura, R.M. *Clin. Chim. Acta* **2001**, *303*, 117.
(585) Hille, R. e Nishino, T. *FASEB J.* **1995**, *9*, 995.
(586) Hille, R. *Chem. Rev.* **1996**, *96*, 2757.
(587) Hille, R. *Arch. Biochem. Biophys.* **2005**, *433*, 107.
(588) Hille, R. *Eur. J. Inorg. Chem.* **2006**, 1913.
(589) Nishino, T.; Okamoto, K.; Eger, B.T.; Pai, E.F.; Nishino, T. *FEBS J.* **2008**, *275*, 3278.
(590) Hille, R.; Nishino, T.; Bittner, F. *Coord. Chem. Rev.* **2011**, *255*, 1179.
(591) Okamoto, K.; Kusano, T.; Nishino, T. *Curr. Pharm. Des.* **2013**, *19*, 2606.
(592) Jarasch, E.D.; Grund, C.; Bruder, G.; Heid, H.W.; Keenan, T.W.; Franke, W.W. *Cell* **1981**, *25*, 67.
(593) Ichikawa, M.; Nishino, T.; Ichikawa, A. *J. Histochem. Cytochem.* **1992**, *40*, 1097.
(594) Adachi, T.; Fukushima, T.; Usami, Y.; Hirano, K. *Biochem. J.* **1993**, *289*, 523.
(595) Fukushima, T.; Adachi, T.; Hirano, K. *Biol. Pharm. Bull.* **1995**, *18*, 156.
(596) White, C.R.; Darley-Usmartt, V.; Berrington, W.R.; Mcadams, M.; Gore, J.Z.; Thompsont, J.A.; Parkst, D.A.; Tarpeyt, M.M.; Freemant, B.A. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8745.
(597) Radi, R.; Rubbo, H.; Bush, K.; Freeman, B.A. *Arch. Biochem. Biophys.* **1997**, *339*, 125.
(598) Rouquette, M.; Page, S.; Bryant, R.; Benboubetra, M.; Stevens, C.R.; Blake, D.R.; Whish, W.D.; Harrison, R.; Tosh, D. *FEBS Lett.* **1998**, *426*, 397.
(599) Vickers, S.; Schiller, H.J.; Hildreth, J.E.; Bulkley, G.B. *Surgery* **1998**, *124*, 551.
(600) Houston, M.; Estevez, A.; Chumley, P.; Aslan, M.; Marklundi, S.; Parks, D.A.; Freeman, B.A. *J. Biol. Chem.* **1999**, *274*, 4985.
(601) Kelley, E.E.; Hock, T.; Khoo, N.K.; Richardson, G.R.; Johnson, K.K.; Powell, P.C.; Giles, G.I.; Agarwal, A.; Lancaster Jr., J.R.; Tarpey, M.M. *Free Radic. Biol. Med.* **2006**, *40*, 952.
(602) Angermuller, S.; Bruder, G.; Volkl, A.; Wesch, H.; Fahimi, H.D. *Eur. J. Cell Biol.* **1987**, *45*, 137.
(603) Dikov, A.; Alexandrov, I.; Russinova, A.; Boyadjieva-Michailova, A. *Acta Histochem.* **1988**, *83*, 107.
(604) Turner, N.A.; Doyle, W.A.; Ventom, A.M.; Bray, R.C. *Eur. J. Biochem.* **1995**, *232*, 646.
(605) Garattini, E.; Terao, M. *Drug Metab. Rev.* **2011**, *43*, 374.
(606) Garattini, E.; Fratelli, M.; Terao, M. *Cell. Mol. Life Sci.* **2008**, *65*, 1019.
(607) Pryde, D.C.; Dalvie, D.; Hu, Q.; Jones, P.; Obach, R.S.; Tran, T.D. *J. Med. Chem.* **2010**, *53*,

8441.
(608) Swenson, T.L.; Casida, J.E. *Toxicol. Sci.* **2013**, *133*, 22.
(609) Krenitsky, T.A.; Neil, S.M.; Elion, G.B.; Hitchings, G.H. *Arch. Biochem. Biophys.* **1972**, *150*, 585.
(610) Hall, W.W.; Krenitsky, T.A. *Arch. Biochem. Biophys.* **1986**, *251*, 36.
(611) Dixon, M.; Thurlow, S. *Biochem. J.* **1924**, *18*, 989.
(612) Fridovich, I.; Handler, P. *J. Biol. Chem.* **1957**, *228*, 67.
(613) Fridovich, I.; Handler, P. *J. Biol. Chem.* **1962**, *237*, 916.
(614) Sergeev, N.S.; Ananiadi, L.I., L'Vov N,P.; Kretovich, W.L. *J. Appl. Biochem.* **1985**, 86.
(615) Millar, T.M.; Stevens, C.R.; Benjamin, N.; Eisenthal, R.; Harrison, R.; Blake, D.R. *FEBS Lett.* **1998**, *427*, 225.
(616) Zhang, Z.; Naughton, D.; Winyard, P.G.; Benjamin, N.; Blake, D.R.; Symons, M.C. *Biochem. Biophys. Res. Commun.* **1998**, *249*, 767.
(617) Godber, H.L.J.; Doel, J.J.; Sapkota, G.P.; Blake, D.R.; Stevens, C.R.; Eisenthal, R.; Harrison, R. *J. Biol. Chem.* **2000**, *275*, 7757.
(618) Li, H.; Samouilov, A.; Liu, X.; Zweier, J.L. *J. Biol. Chem.* **2001**, *276*, 24482.
(619) Li, H.; Samouilov, A.; Liu, X.; Zweier, J.L. *Biochemistry* **2003**, *42*, 1150.
(620) Li, H.; Samouilov, A.; Liu, X.; Zweier, J.L. J. Biol. Chem. **2004**, *279*, 16939.
(621) Li, H.; Cui, H.; Kundu, T.K.; Alzawahra, W.; Zweier, J.L. J. Biol. Chem. **2008**, *283*, 17855.
(622) Li, H.; Kundu, T.K.; Zweier, J.L. J. Biol. Chem. **2009**, *284*, 33850.
(623) Maia, L.; Moura, J.J.G. *J. Biol. Inorg. Chem.* **2011**, *16*, 443.
(624) Yee, S.B.; Pritsos, C.A. *Arch. Biochem. Biophys.* **1997**, *347*, 235.
(625) Dambrova, M.; Uhlén, S.; Welch, C.J.; Wikberg, J.E.S. *Eur. J. Biochem.* **1998**, *257*, 178.
(626) Stoddart, A.M.; Levine, W.G. *Biochem. Pharmacol.* **1992**, *43*, 2227.
(627) Harrison R. *Biochem. Soc. Trans.* **1997**, *25*, 786.
(628) Wright, R.M.; Repine, J.E. *Biochem. Soc. Trans.* **1997**, *25*, 799.
(629) Beckman, K.B.; Ames, B.N. *Physiol. Rev.* **1998**, *78*, 547.
(630) Suzuki, H.; Delano, F.A.; Parks, D.A., Jamshidi, N.; Granger, D.N.; Ishii, H.; Suematsu, M.; Zweifach, B.W.; Schmid-Schonbein, G.W. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 4754.
(631) Harrison R. *Free Radic. Biol. Med.* **2002**, *33*, 774.
(632) Stirpe, F.; Ravaioli, M.; Battelli, MG.; Musiani, S.; Grazi, G.L. *Am. J. Gastroenterol.* **2002**, *97*, 2079.
(633) Small, D.M.; Coombes, J.S.; Bennett, N.; Johnson, D.W.; Gobe, G.C. *Nephrology* **2012**, *17*, 311.
(634) Bachschmid, M.M.; Schildknecht, S.; Matsui, R.; Zee, R.; Haeussler, D.; Cohen, RA.; Pimental, D.; Loo, B. *Ann Med.* **2013**, *45*, 17.
(635) Madamanchi, N.R.; Runge, M.S. *Free Radic. Biol. Med.* **2013**, *61*, 473.
(636) Granger, D.N.; Rutili, G.; McCord, J.M. *Gastroenterology* **1981**, *81*, 22.
(637) McCord, J.M., *N Engl J Med*. **1985**, *312*, 159.
(638) Zweier, J.L.; Kuppusamy, P.; Lutty, G.A. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 4046.

(639) Zweier, J.L.; Broderick, R.; Kuppusamy, P.; Thompson-Gorman, S.; Lutty, G.A. *J. Biol. Chem.* **1994**, *269*, 24156.

(640) Nishino, T.; Nakanishi, S.; Okamoto, K.; Mizushima, J.; Hori, H.; Iwasaki, T.; Nishino, T.; Ichimori, K.; Nakazawa. H. *Biochem. Soc. Trans.* **1997**, *25*, 783.

(641) Berry, C.E.; Hare, J.M. *J. Physiol.* **2004**, *555*, 589.

(642) Lieber, C.S. *N. Engl. J. Med.* **1988**, *319*, 1639.

(643) Cederbaum, A.I. *Free Radic. Biol. Med.* **1989**, *7*, 537.

(644) Kato, S.; Kawase, T.; Alderman, J.; Inatomi, N.; Lieber, C., *Gastroenterology* **1990**, *98*, 203.

(645) Nordmann, R.; Ribière, C.; Rouach, H. *Free Radic. Biol. Med.* **1992**, *12*, 219.

(646) Wright, R.M.; McManaman, J.L.; Repine, J.E. Free Radic. Biol. Med. **1999**, *26*, 348.

(647) Wu, D.; Cederbaum, A.I. *Alcohol. Res. Health* **2003**, *27*, 277.

(648) Alikulov, Z.A.; L'vov, N.P.; Kretovich, V.L. *Biokhimiia* **1980**, *45*, 1714.

(649) Millar, T.M.; *FEBS Lett.* **2004**, *562*, 129.

(650) Himmel, H.M.; Sadony, V.; Ravens, U. *J. Chromatogr.* **1991**, *568*, 105.

(651) Pesonen, E.J.; Linder, N.; Raivio, K.O.; Sarnesto, A.; Lapatto, R.; Hockerstedt, K.; Makisalo, H.; Andersson, S. *Gastroenterology* **1998**, *114*, 1009.

(652) Williamson, J.R. *J. Biol. Chem.* **1966**, *241*, 5026.

(653) Williamson, J.R.; Steenbergen, C.; Rich, T.; Deleeuw, G.; Barlow, C.; Chance, B. *Pathophysiology and Therapeutics of Myocardial Ischemia*; Spectrum Publications, Inc.: New York, 1977; p193.

(654) Kobayashi, K.; Neely, J.R. *J. Mol. Cell. Cardiol.* **1983**, *15*, 359.

(655) Varadarajan, S.G.; Novalija, E.; Smart, S.C.; Stowe, D.F. *Am. J. Physiol.* **2001**, *280*, H280.

(656) Zhou, L.; Stanley, W.C.; Saidel, G.M.; Yu, X.; Cabrera, M.E. *J. Physiol.* **2005**, *569*, 925.

(657) Stirpe, F.; Della Corte E. *J. Biol. Chem.* **1969**, *244*, 3855.

(658) Waud, W. R.; Rajagopalan, K. V. *Arch. Biochem. Biophys.* **1976**, *172*, 354.

(659) Waud, W. R.; Rajagopalan, K. V. *Arch. Biochem. Biophys.* **1976**, *172*, 365.

(660) Roy, R.S.; McCord, J.M. *Fed. Proc.* **1982**, *41*, 767.

(661) Engerson, T.D.; McKelvey, T.G.; Rhyne, D.B.; Boggio, E.B.; Snyder, S.J.; Jones, H.P. *J. Clin. Invest.* **1987**, *79*, 1564.

(662) Parks, D.A.; Williams, T.K.; Beckman, J.S. *Am. J. Physiol.* **1988**, *254*, G768.

(663) Frederiks, W.M.; Bosch, K. S. *Hepatology* **1996**, *24*, 1179.

(664) Webb, A.; Bond, R.; McLean, P.; Uppal, R.; Benjamin, N.; Ahluwalia, A. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 13683.

(665) Lundberg, J.O.; Weitzberg, E. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 915.

(666) Tripatara, P.; Patel, N.S.; Webb, A.; Rathod, K.; Lecomte, F.M.; Mazzon, E.; Cuzzocrea, S.; Yaqoob, M.M.; Ahluwalia, A.; Thiemermann, C. *J. Am. Soc. Nephrol.* **2007**, *18*, 570.

(667) Webb, A.J., Milsom, A.B., Rathod, K.S., Chu, W. ., Qureshi, S., Lovell, M.J.; Lecomte, F.M.J.; Perrett, D.; Raimondo, C.; Khoshbin, E.; Ahmed, Z.; Uppal, R.; Benjamin, N.; Hobbs, A.J.; Ahluwalia, A.; *Circ. Res.* **2008**, *103*, 957.

(668) Alef, M.J.; Vallabhaneni, R.; Carchman, E.; Morris Jr., S.M.; Shiva, S.; Wang, Y.; Kelley, E.E.; Tarpey, M.M.; Gladwin, M.T.; Tzeng, E.; Zuckerbraun, B.S. *J. Clin. Invest.* **2011**, *121*, 1646.

(669) Pickerodt, P.A.; Emery, M.J.; Zarndt, R.; Martin, W.; Francis, R.C., Boemke, W.; Swenson, E.R. *Anesthesiology* **2012**, *117*, 592.

(670) Samal, A.A.; Honavar, J.; Brandon, A.; Bradley, K.M.; Doran, S.; Liu, Y.; Dunaway, C.; Steele, C.; Postlethwait, E.M.; Squadrito, G.L.; Fanucchi, M.V.; Matalon, S.; Patel, R.P. *Free Radic. Biol. Med.* **2012**, *53*, 1431.

(671) Sugimoto, R.; Okamoto, T.; Nakao, A.; Zhan, J.; Wang, Y.; Kohmoto, J.; Tokita, D.; Farver, C.F.; Tarpey, M.M.; Billiar, T.R.; Gladwin, M.T.; McCurry, K.R. *Am. J. Transplant.* **2012**, *12*, 2938.

(672) Olson, J.S.; Ballow, D.P.; Palmer, G.; Massey, V. *J. Biol. Chem.* **1974**, *249*, 4350.

(673) Cighetti, G.; Del Puppo, M.; Paroni, R.; Galli, Kienle M. *FEBS Lett.* **1990**, *274*, 82.

(674) Xia, Y.; Zweier, J.L. *J. Biol. Chem.* **1995**, *270*, 18797.

(675) Jacobson, K.B.; Kaplan, N.O. *J. Biol. Chem.* **1957**, *226*, 603.

(676) Lowry, O.H.; Passonneau, J.V.; Schulz, D.W.; Rock, M.K. *J. Biol. Chem.* **1961**, *236*, 2746.

(677) Stubbs, M.; Veech, R.L.; Krebs, H.A. *Biochem. J.* **1972**, *126*, 59.

(678) Crow, K. *Trends Biochem. Sci.* **1983**, *8*, 310.

(679) Harris, C. M.; Massey, V. *J. Biol. Chem.* **1997**, *272*, 28335.

(680) Harris, C. M.; Massey, V. *J. Biol. Chem.* **1997**, *272*, 8370.

(681) Terada, L.S.; Dormish, J.J.; Shanley, P.F.; Leff, J.A.; Anderson, B.O.; Repine, J.E. *Am. J. Physiol.* **1992**, *263*, L394.

(682) Cantu-Medellin, N.; Kelley, E.E. Redox Biology **2013**, *1*, 353.

(683) Saito, T.; Nishino, T. *J. Biol. Chem.* **1989**, *264*, 10015.

(684) Sanders, S.A.; Eisenthal, R.; Harrison, R. *Eur. J. Biochem.* **1997**, *245*, 541.

(685) Okamoto, K.; Eger, B.T.; Nishino, T.; Kondo, S.; Pai, E.F.; Nishino, T. *J. Biol. Chem.* **2003**, *278*, 1848.

(686) Becker, M.A.; Schumacher Jr., H.R.; Wortmann, R.L.; Eustace, D.; Eustace, D.; Palo, W.A.; Streit, J.; Joseph-Ridge, N. *N. Engl. J. Med.* **2005**, *353*, 2450.

(687) Takano, Y.; Hase-Aoki, K.; Horiuchi, H.; Zhao, L.; Kasahara, Y.; Kondo, S.; Becker, M.A. *Life Sci.* **2005**, *76*, 1835.

(688) Okamoto, K.; Nishino, T. *J. Nippon. Med. Sch.* **2008**, *75*, 2.

(689) Obach, R.S. *Drug Metab. Dispos.* **2004**, *32*, 89.

(690) Friedl, H.P.; Smith, D.J.; Till, G.O.; Thomson, P.D.; Louis, D.S.; Ward, P.A. *Am. J. Pathol.* **1990**, *136*, 491.

(691) Yokoyama, Y.; Beckman, J.S.; Beckman, T.K.; Wheat, J.K.; Cash, T.G.; Freeman, B.A.; Parks, D.A. *Am. J. Physiol.* **1990**, *258*, G564.

(692) Tan, S.; Yokoyama, Y.; Dickens, E.; Cash, T.G.; Freeman, B.A.; Parks, D.A. *Free Radic. Biol. Med.* **1993**, *15*, 407.

(693) Weinbroum, A.; Nielsen, V.G.; Tan, S.; Gelman, S.; Matalon, S.; Skinner, K.A.; Bradley Jr., E.; Parks, D.A. *Am. J. Physiol.* **1995**, *268*, G988.

(694) Landmesser, U.; Spiekermann, S.; Dikalov, S.; Tatge, H.; Wilke, R.; Kohler, C.; Harrison, D.G.; Hornig, B.; Drexler, H. *Circulation* **2002**, *106*, 3073.

(695) Kelley, E.E.; Trostchansky, A.; **Rubbo**, H.; **Freeman**, B.A.; **Radi**, R.; Tarpey, M.M. *J. Biol. Chem.* **2004**, *279*, 37231.

(696) Knowles, M.E.; McWeeny, D.J.; Couchman, L. Thorogood, M. *Nature* **1973**, *247*, 288.

(697) Green, L.C.; Wagner, D.A.; Glogowski, J.; Skipper, P.L.; Wishnok, J.S.; Tannenbaum, S.R. *Anal. Biochem.* **1982**, *126*, 131.

(698) Benjamin, N.; O'Driscoll, F.; Dougall, H.; Duncan, C.; Smith, L.;Golden, M. *Nature* **1994,** *368*, 502.

(699) Lundberg, J.O.N.; Weitzberg, E.; Lundberg, J. M.; Alving, K. *Gut* **1994**, *35*, 1543.

(700) McKnight, G.M.; Smith, L.M.; Drummond, R.S.; Duncan, C.W.; Benjamin, N. *Gut* **1997**, *40*, 211.

(701) Bjorne, H.; Govoni, M.; Tornberg, DC.; Lundberg, JO.; Weitzberg, E. *Crit. Care Med.* **2005**, *33*, 1722.

(702) Gago, B.; Lundberg, J.O.; Barbosa, R.M.; Laranjinha, J. *Free Radic. Biol. Med.* **2007**, *43*, 1233.

(703) Weitzberg, E.; Hezel, M.; Lundberg, J.O. *Anesthesiology* **2010**, *113*, 1460.

(704) Weller, R.; Pattullo, S.; Smith, L.; Golden, M.; Ormerod, A.; Benjamin, N. *J. Invest. Dermatol.* **1996**, *107*, 327.

(705) Benjamin, N.; Pattullo, S.; Weller, R.; Smith, L.; Ormerod, A. *Lancet* **1997**, *349*, 1776.

(706) Lundberg, J.O.N.; Carlsson, S.; Engstrand, L.; Morcos, E.; Wiklund, N.P.; Weitzberg, E. *Urology* **1997**, *50*, 189.

(707) Tannenbaum, S.R., Weisman, M.; Fett, D. *Food Cosmet. Toxicol.* **1976**, *14*, 549.

(708) Duncan, C.; Douglas, H.; Johnston, P.; Green, S.; Brogan, R.; Leifert, C.; Smith, L.; Golden, M.; Benjamin, N. *Nature Med.* **1995**, *1*, 546.

(709) Kitagawa, H.; Takeda, F.; Kohei, H. *J. Pharmacol. Exp. Ther.* **1990**, *253*, 1133.

(710) Whittle, B.J.R. *Br. J. Pharmacol.* **1993**, *110*, 3.

(711) Mohammad, A.; Ali, N.; Reza, B.; Ali, K. *Indian J. Pharmacol.* **2010**, *42*, 78.

(712) Brown, J.F.; Hanson, P.J.; Whittle, B.J.R. *Eur. J. Pharmacol.* **1992**, *223*, 103.

(713) Kono, Y.; Shibata, H.; Adachi, K.; Tanaka, K. *Arch. Biochem. Biophys.* **1994**, *311*, 153.

(714) Dykhuizen, R.; Frazer, R.; Duncan, C.; Smith, C.; Golden, M.; Benjamin, N.; Leifert, C. *Antimicrob. Agents Chemother.* **1996**, *40*, 1422.

(715) Kaplan, S.; Lancaster, J.; Basford, R.; Simmons, R. *Infect. Immun.* **1996**, *64*, 69.

(716) Lundberg, J.O.; Govoni, M. *Free Radic. Biol. Med.* **2004**, *37*, 395.

(717) Govoni, M.; Jansson, E.A.; Weitzberg, E.; Lundberg J. O. *Nitric Oxide* **2008**, *19*, 333.

(718) Webb, A.J.; Patel, N.; Loukogeorgakis, S.; Okorie, M.; Aboud, Z.; Misra, S.; Rashid, R.; Miall, P.; Deanfield, J.; Benjamin, N.; MacAllister, R.; Hobbs, AJ.; Ahluwalia, A. *Hypertension* **2008**, *51*, 784.

(719) Petersson, J.; Carlstrom, M.; Schreiber, O.; Phillipson, M.; Christoffersson, G.; Jagare, A.; Roos, S.; Jansson, E.A.; Persson, A.E.; Lundberg, J.O.; Holm, L. *Free Radic. Biol. Med.* **2009**, *46*, 1068.

(720) Human saliva contains both nitrate and nitrite[707,708]: nitrate is originated from diet sources and circulation (up to 25% of circulating nitrate is actively taken up by the salivary glands and secreted

into saliva[708,718,721]); nitrite, on the other hand, is present in diet only in small amounts.

(721) Spiegelhalder, B.; Eisenbrand, G.; Preussman, R. *Food Cosmet. Toxicol.* **1976**, *14*, 545.

(722) Sasaki, T.; Matano, K. J. *Food Hygiene Soc. Jap.* **1979**, *20*, 363.

(723) Lundberg, J.O.; Weitzberg, E.; Cole, J.A.; Benjamin, N. *Nat. Rev. Microbiol.* **2004**, *2*, 593.

(724) Kapil, V.; Haydar, S.M.; Pearl, V.; Lundberg, J.O.; Weitzberg, E.; Ahluwalia, A. *Free Radic. Biol. Med.* **2012**, *55*, 93.

(725) Schreiber, F.; Stief, P.; Gieseke, A.; Heisterkamp, I.N.; Verstraete, W.; de Beer, D.; Stoodley, P. *BMC Biology* **2010**, *8*, 24.

(726) Lee, K.Y.; Kuchynka, D.J., Kochi, J.K. *Inorg. Chem.* **1990**, *29*, 4196.

(727) Ignarro, L.J.; Fukuto, J.M.; Griscavage, J.M.; Rogers, N.E.; Byrns, R.E. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 8103.

(728) Ford, P.C.; Lorkovic, I.M. *Chem. Rev.* **2002**, *102*, 993.

(729) Peri, L.; Pietraforte, D.; Scorza, G.; Napolitano, A.; Fogliano, V.; Minetti, M. *Free Radic. Biol. Med.* **2005**, *39*, 668.

(730) Rocha, B.S.; Gago, B.; Barbosa, R.M.; Laranjinha, J. *Toxicology* **2009**, *265*, 41.

(731) Samouilov, A.; Kuppusamy, P.; Zweier, J.L. *Arch. Biochem. Biophys.* **1998**, *357*, 1.

(732) Modin, A.; Björne, H.; Herulf, M.; Alving, K.; Weitzberg, E.; Lundberg, J.O.N. *Acta Physiol. Scand.* **2001**, *171*, 9.

(733) Opie, L.H. *Circ. Res.* **1976**, *38*, 152.

(734) Cobbe, S.M.; Poole-Wilson, P.A. *J. Mol. Cell Cardiol.* **1980**, *12*, 761.

(735) Momomura, S.; Ingwall, J.S.; Parker, J.A.; Sahagian, P.; Ferguson, J.J.; Grossman, W. *Circ. Res.* **1985**, *57*, 822.

(736) Zweier, J.L.; Wang, P.; Samouilov, A.; Kuppusamy, P. *Nat. Med.* **1995**, *1*, 804.

(737) Zweier, J.L.; Samouilov, A.; Kuppusamy, P. *Biochim. Biophys. Acta* **1999**, *1411*, 250.

(738) Aamand, R.; Dalsgaard, T.; Jensen, F.B.; Simonsen, U.; Roepstorff, A.; Fago, A. *Am. J. Physiol. Heart Circ. Physiol.* **2009**, *297*, H2068.

(739) Jensen, F.B. *Acta Physiol. Scand.* **2004**, *182*, 215.

(740) Khersonsky, O.; Tawfik, D.S. *Annu. Rev. Biochem.* **2010**, *79*, 471.

(741) Feelisch, M.; Martin, J.F. Trends Ecol. Evol. **1995**, *10*, 496.

(742) Cutruzzolà, F.; Rinaldo, S.; Castiglione, N.; Giardina, G., Pecht, I.; Brunori, M. *Bioessays* **2009**, *31*, 885.

(743) van Faassen, E.E.; Bahrami, S.; Feelisch, M.; Hogg, N.; Kelm, M.; Kim-Shapiro, D.B.; Kozlov, A.V.; Li, H.; Lundberg, J.O.; Mason, R.; Nohl, H.; Rassaf, T.; Samouilov, A.; Slama-Schwok, A.; Shiva, S.; Vanin, A.F.; Weitzberg, E.; Zweier, J.; Gladwin, M.T. *Med. Res. Rev.* **2009**, *29*, 683.

(744) Vitturi, D.A.; Patel, R.P. *Free Radic. Biol. Med.* **2011**, *51*, 805.

(745) Huie, R.E. *Toxicology* **1994**, *89*, 193.

(746) Pryor, W.; Lightsey, J.W. *Science* **1981**, *214*, 435.

(746) O'Donnell, V.B.; Eiserich, J.P.; Bloodsworth, A.; Chumley, P.H.; Kirk, M.; Barnes, S.; Darley-Usmar, V.M.; Freeman, B.A. *Methods Enzymol.* **1999**, *301*, 454.

(747) Grzelak, A.; Balcerczyk, A.; Mateja, A.; Bartosz, G. *Biochim. Biophys. Acta* **2001**, *1528*, 97.
(748) Alvarez, B.; Radi, R. *Amino Acids* **2003**, *25*, 295.
(749) Nicolis, S.; Monzani, E.; Roncone, R.; Gianelli, L.; Casella, L. *Chem. Eur. J.* **2004**, *10*, 2281.
(750) Nakai, K.; Mason, R. P. *Free Radic. Biol. Med.* **2005**, *39*, 1050.
(751) Keszler, A.; Mason, R.P.; Hogg, N. *Free Radic. Biol. Med.* **2006**, *40*, 507.
(752) Akaike, T.; Fujii, S.; Sawa, T.; Ihara, H. *Nitric Oxide* **2010**, *23*, 166.
(753) Khoo, N.K.; Freeman, B.A. *Curr. Opin. Pharmacol.* **2010**, *10*, 179.
(754) Gamgee, A. *Philos. Trans. R. Soc. London* **1868**, *158*, 589.
(755) Martin, H.; Huisman, T.H.J. *Nature* **1963**, *200*, 898.
(756) Cohen, G.; Martinez, M.; Hochstein, P. *Biochemistry* **1964**, *3*, 901.
(757) Wallace, W.J.; Caughey, W.S. *Biochem. Biophys. Res. Commun.* **1975**, *62*, 561.
(758) Rodkey, F.L. *Clin. Chem.* **1976**, *22*, 1986.
(759) Kosaka, H.; Imaizumi, K.; Imai, K.; Tyuma, I. *Biochim. Biophys. Acta* **1979**, *581*, 184.
(760) Doyle, M.P.; Pickering, R.A.; Dykstra, R.L.; Nelson, C.L.; Boyer, R.F. *Biochem. Biophys. Res. Commun.* **1982**, *105*, 127.
(761) Kosaka, H.; Imaizumi, K.; Tyuma, I. *Biochim. Biophys. Acta* **1982**, *702*, 237.
(762) Doyle, M.P.; Herman, J.G.; Dykstra, R.L. *J. Free Radic. Biol. Med.* **1985**, *1*, 145.
(763) Lissi, E. *Free Radic. Biol. Med.* **1998**, *24*, 1535.
(764) Herold, S.; Exner, M.; Nauser, T. *Biochemistry* **2001**, *40*, 3385.
(765) Titov, V.Y.; Petrenko, Y. M. *Biochemistry (Mosc.)* **2005**, *70*, 473.
(766) Keszler, A.; Piknova, B.; Schechter, A.N.; Hogg, N. *J. Biol. Chem.* **2008**, *283*, 9615.
(767) As a consequence of poisoning, the nitrite concentration in plasma may increase to values of 100-400μM; such high concentrations of nitrite certainly overwhelm the cellular antioxidant defences and trigger the autocatalytic oxidation of Hb, leading to severe methaemoglobinemia[765,768-770]. Besides the fast decrease in the vital ferrous Hb, nitrite poisoning leads to protein and lipid damage (tyrosine and lipid nitration, protein radical formation and lipid peroxidation[745-751]) through the nitrogen dioxide radical formed.
(768) Huang, A.; Terry, W.; Guido, F.; Torres, J.C.; Lipsman, J.; DeRobertis, N.; Cola, C.; DiDio, V.; Vincent, C.; Long, H.; Nelson, L.S.; Hoffman, R.S.; Leib, H.; Devine, B.; Woron, R.; Smith, P.; Wekell, M.; Noviello, S. *Morb. Mortal. Wkly. Rep.* **2002**, *51*, 639.
(769) Kohn, M.C.; Melnick, R.L.; Ye, F.; Portier, C.J. *Drug Metab. Dispos.* **2002**, *30*, 676.
(770) Reeder, B.J.; Wilson, M. T. *Curr. Med. Chem.* **2005**, *12*, 2741.
(771) George, P.; Irvine, D.H. *Nature* **1951**, *168*, 164.
(772) Harel, S.; Kanner, J. *Free Rad. Res. Commun.* **1988**, *5*, 21.
(773) Galaris, D.; Eddy, L.; Arduini, A.; Cadenas, E.; Hochstein, P. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 1162.
(774) Alayash, A.I.; Ryan, B.A.; Eich, R.F.; Olson, J.S.; Cashon, R.E. *J. Biol. Chem.* **1999**, *274*, 2029.
(775) Lardinois, O.M.; Ortiz de Montellano, P.R. *Biochemistry (Mosc.)* **2004**, *43*, 4601.

(776) Hewitt, J.; Reardon, D.N. *Blood* **1991**, *78*, 1891.

(777) Khoo, U.Y.; Mewman, D.J.; Miller, W.K.; Price, C.P. *Eur. J. Clin. Chem. Clin. Biochem.* **1994**, *32*, 435440.

(778) Giardina, B.; Messana, I.; Scatena, R.; Castagnola, M. *Crit. Rev. Biochem. Mol. Biol.* **1995**, *30*, 165.

(779) Kilinc, K.; Kilinc, A.; Wolf, R.E.; Grisham, M.B. *Biochem. Biophys. Res. Commun.* **2001**, *285*, 273.

(780) Herold, S. *Free Radic. Biol. Med.* **2004**, *36*, 565.

(781) Wang, P.; Zweier, J.L. *J. Biol. Chem.* **1996**, *271*, 29223.

(782) Kooy, N.W.; Lewis, S.J.; Royall, J.A.; Ye, Y.Z.; Kelly, D.R.; Beckman, J.S. *Crit. Care Med.* **1997**, *25*, 812.

(783) Liu, P.; Hock, C.E.; Nagele, R.; Wong, P.Y. *Am. J. Physiol.* **1997**, *272*, H2327.

(784) Ferdinandy, P.; Danial, H.; Ambrus, I.; Rothery, R.A.; Schulz, R. *Circ. Res.* **2000**, *87*, 241.

(785) Vinten-Johansen, J. *Circ. Res.* **2000**, *87*, 170.

(786) Kikugawa, K.; Nakauchi, K.; Beppu, M.; Hiramoto, K.; Ando, K.; Hayakawa, M. *Biol. Pharm. Bull.* **2000**, *23*, 379.

(787) Casella, L. *Chem. Eur. J.* **2006**, *12*, 749.

(788) Herold, S.; Rehmann, F.J.; *J. Biol. Inorg. Chem.* **2001**, *6*, 543.

(789) Herold, S.; Rehmann, F.J. *Free Radic. Biol. Med.* **2003**, *34*, 531.

(790) Grisham, M.B. *Free Radic. Biol. Med.* **1985**, *1*, 227.

(791) Galaris, D.; Sevanian, A.; Cadenas, E.; Hochstein, P. *Arch. Biochem. Biophys.* **1990**, *281*, 163.

(792) Newman, E.S.; Rice-Evans, C.A.; Davies, M.J. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 1414.

(793) Osawa, Y. Williams, M.S. *Free Radic. Biol. Med.* **1996**, *21*, 35.

(794) Gunther, M.R.; Sampath, V.; Caughey, W.S. *Free Radic. Biol. Med.* **1999**, *26*, 1388.

(795) Vuletich, J.L.; Osawa, Y.; Aviram, M. *Biochem. Biophys. Res. Commun.* **2000**, *269*, 647.

(796) Reeder, B.J.; Svistunenko, D.A.; Sharpe, M.A.; Wilson, M.T. *Biochemistry (Mosc.)* **2002**, *41*, 367.

(797) Although it should be noted that also NO may reduce the ferryl-haem through a reaction similar to the reductive nitrosylation described in 3.1.1.1.; $k \approx 2x10^7$ $M^{-1}s^{-1}$.

(798) Anni, H.; Yonetani, T. Met. Ions Biol. Syst. **1992**, *28*, 219.

(799) Dunford, H.B. *Heme Peroxidases*; Wiley- VCH: NewYork, 1999.

(800) Alfonso-Prieto, M.; Biarnés, X.; Vidossich, P.; Rovira, C. *J. Am. Chem. Soc.* **2009**, *131*, 11751.

(801) Poulos, T.L. *Arch. Biochem. Biophys.* **2010**, *500*, 3.

(802) Díaz, A.; Loewen, P.C.; Fita, I.; Carpena, X. *Arch. Biochem. Biophys.* **2012**, *525*, 102.

(803) Klebanoff, S.J. *Free Radical Biol. Med.* **1993**, *14*, 351.

(804) van der Vliet, A.; Eiserich, J.P.; Halliwell, B.; Cross, C.E. *J. Biol. Chem.* **1997**, *272*, 7617.

(805) Eiserich, J.P.; Hristova, M.; Cross, C.E.; Jones, A.D.; Freeman, B.A.; Halliwell, B.; van der Vliet, A. *Nature* **1998**, *391*, 393.

(806) Sampson, J.B.; Ye, Y.Z.; Rosen, H.; Beckman, J.S. *Arch. Biochem. Biophys.* **1998**, *356*, 207.

(807) Burner, U.; Furtmuller, P.G.; Kettle, A.J.; Koppenol, W.H.; Obinger, C. *J. Biol. Chem.* **2000**, *275*, 20597.

(808) Van Dalen, C.J..; Winterbourn, C.C.; Senthilmohan, R.; Kettle, A.J. *J. Biol. Chem.* **2000**, *275*, 11638.

(809) Brennan, M.-L.; Wu, W.; Fu, X.; Shen, Z.; Song, W.; Frost, H.; Vadseth, C.; Narine, L.; Lenkiewicz, E.; Borchers, M.T.; Lusis, A.J. *J. Biol. Chem.* **2002**, *277*, 17415.

(810) In addition to the chloride or thiocyanate oxidation, the nitrite oxidation catalysed by a peroxidase could constitute a defensive mechanism against pathological microorganisms. *E.g.*, nitration of virulence factors of bacterial pathogens such as *P. aeruginosa* decreases the ability of the factors to induce airway inflammation and cytotoxicity *in vivo* in mice.[811] This natural cytoprotective role is being taken further, as acidified nitrite is being examined as a therapy for highly refractory infections by mucoid *P. aeruginosa* in patients with cystic fibrosis.[812,813]

(811) Reszka, K.J.; Xiong, Y.; Sallans, L.; Pasula, R.; Olakanmi, O.; Hassett, D.J.; Britigan, B.E. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2012**, *302*, L1044.

(812) Yoon, S.S.; Coakley, R.; Lau, G.W.; Lymar, S.V.; Gaston, B.; Karabulut, A.C.; Hennigan, R.F.; Hwang, S.H.; Buettner, G.; Schurr, M.J.; Mortensen, J.E.; Burns, J.L.; Speert, D.; Boucher, R.C.; Hassett, D.J. *J. Clin. Invest.* **2006**, *116*, 436.

(813) Major, T.A.; Panmanee, W.; Mortensen, J.E.; Gray, L.D.; Hoglen, N.; Hassett, D.J. *Antimicrob. Agents Chemother.* **2010**, *54*, 4671.

(814) Farrel, A.; Blake, D.; Palmer, R.; Moncada, S. *Ann. Rheum. Dis.* **1992**, *51*, 1219.

(815) Kaur, H.; Halliwell, B. *FEBS Lett.* **1994**, *350*, 9.

(816) Torre, D.; Ferrario, G.; Sperenza, F.; Orani, A.; Fiori, G.; Zeroli, C. *J. Clin. Pathol.* **1996**, *49*, 574.

(817) Wu, W.; Chen, Y.; Hazen, S.L. *J. Biol. Chem.* **1999**, *274*, 25933.

(818) Duguet, A.; Iijima, H.; Eum, S.Y.; Hamid, Q.; Eidelman, D.H. *Am. J. Respir. Crit. Care Med.* **2001**, *164*, 1119.

(819) Iijima, H.; Duguet, A.; Eum, S.Y.; Hamid, Q.; Eidelman, D.H. *Am. J. Respir. Crit. Care Med.* **2001**, *163*, 1233.

(820) MacPherson, J.C.; Comhair, S.A.; Erzurum, S.C.; Klein, D.F.; Lipscomb, M.F.; Kavuru, M.S.; Samoszuk, M. K.; and Hazen, S.L. *J. Immunol.* **2001**, *166*, 5763.

(821) Chance,B. *Arch. Biochem. Biophys.* **1952**, *41*, 416

(822) Shibata, H.; Kono, Y.; Yamashita, S.; Sawa, Y.; Ochiai, H.; and Tanaka, K. *Biochim. Biophys. Acta* **1995**, *1230*, 45.

(823) van Dalen, C.J.; Winterbourn, C.C.; Kettle, A.J. *Biochem. J.* **2006**, *394*, 707.

(824) Bruck, T.B.; Fielding, R.J.; Symons, M.C.R.; Harvey, P.J. *Eur. J. Biochem.* **2001**, *268*, 3214.

(825) Palmerini, C.A.; Marmottini, F.; Arienti, G. *J. Biochem. Mol. Toxicol.* **2012**, *26*, 87.

(826) Heppel, L.A.; Porterfield, V.T. *J. Biol. Chem.* **1949**, *178*, 549.

(827) Chance, B. *J. Biol. Chem.* **1950**, *182*, 649.

(828) Monzani, E.; Roncone, R.; Galliano, M.; Koppenol, W.H.; Casella, L. *Eur. J. Biochem.* **2004**, *271*, 895.

(829) It is noteworthy that several penta-coordinated haemic proteins (and also free haems) display a considerable ($\approx 10^3 M^{-1}s^{-1}$) peroxidatic activity, even though *circa* four orders of magnitude lower than the true peroxidases[518,830]; as expected, the activity of "closed" hexa-coordinated haemic proteins is comparatively negligible. This anticipates that other haemic proteins could contribute to oxidise nitrite and nitrate proteins.

(830) Diederix, R.E.M.; Ubbink, M.; Canters, G.W. *Biochemistry* **2002**, *41*, 13067.

(831) Radi, R.; Thomson, L.; Rubbo, H.; Prodanov, E. *Arch. Biochem. Biophys.* **1991**, *288*, 112.

(832) Radi, R.; Turrens, J.F.; Freeman, B.A. *Arch. Biochem. Biophys.* **1991**, *288*, 118.

(833) Radi, R.; Bush, K.M.; Freeman, B.A. *Arch. Biochem. Biophys.* **1993**, *300*, 409.

(834) Barr, D.P.; Gunther, M.R.; Deterding, L.J.; Tomer, K.B.; Mason, R.P. *J. Biol. Chem.* **1996**, *271*, 15498.

(835) Deterding, L.J.; Barr, D.P.; Mason, R.P.; Tomer, K.B. *J. Biol. Chem.* **1998**, *273*, 12863.

(836) Nantes, I.L.; Faljoni-Alario, A.; Vercesi, A.E.; Santos, K.E.; Bechara, E.J. *Free Radic. Biol. Med.* **1998**, *25*, 546.

(837) Spector, A.; Zhou, W.; Ma, W.; Chignell, C.F.; Reszka, K.J. *Exp. Eye Res.* **2000**, *71*, 183.

(838) Lawrence, A.; Jones, C.M.; Wardman, P.; Burkitt, M.J. *J. Biol. Chem.* **2003**, *278*, 29410.

(839) Velayutham, M.; Hemann, C.; Zweier, J.L. *Free Radic. Biol. Med.* **2011**, *51*, 160.

(840) Castro, L.; Eiserich, J.P.; Sweeney, S.; Radi, R.; Freeman, B.A. *Arch. Biochem. Biophys.* **2004**, *421*, 99.

(841) Kagan, V.E.; Borisenko, G.G., Tyurina, Y.Y.; Tyurin, V.A.; Jiang, J.; Potapovich, A.I.; Kini, V.; Amoscato, A.A.; Fujii, Y. *Free Radical Biol. Med.* **2004**, *37*, 1963.

(842) Roberg, K.; Ollinger, K. *Am. J. Pathol.* **1998**, *152*, 1151.

(843) Bobba, A.; Atlante, A.; Giannattasio, S.; Sgaramella, G.; Calissano, P.; Marra, E. *FEBS Lett.* **1999**, *457*, 126.

(844) Roberg, K.; Johansson, U.; Ollinger, K. *Free Radic. Biol. Med.* **1999**, *27*, 1228.

(845) Roberg, K. *Lab. Invest.* **2001**, *81*, 149.

(846) Sedlák, E.; Fabian, M.; Robinson, N.C.; Musatov, A. *Free Radic. Biol. Med.* **2010**, *49*, 1574.

(847) O'Rourke, B.; Cortassa, S.; Aon, M.A. *Physiology* **2005**, *20*, 303.

(848) Chance, B.; Sies, H.; Boveris, A. *Physiol. Rev.* **1979**, *59*, 527.

(849) Freeman, B.A.; Crapo, J.D. *J. Biol. Chem.* **1981**, *256*, 10986.

(850) Darley-Usmar, V.M.; Stone, D.; Smith, D.; Martin, J.F. *Ann. Med.* **1991**, *23*, 583.

(851) Turrens, J.F. Biosci. Rep. **1997**, *17*, 3.

(852) Eiserich, J.P.; Cross, C.E.; Jones, A.D.; Halliwell, B.; Van der Vliet, A. *J. Biol. Chem.* **1996**, *271*, 19199.

(853) S-nitrosation and nitrosylation in the presence of nitrite that is not changed by NO scavengers or dioxygen does not proof the direct involvement of nitrite, because the NO formed would likely be inaccessible for scavenging or detection. The same reasoning holds for nitration reactions.

(854) Cassens, R.G.; Ito, I.; Lee, M.; Buege, D. *Bioscience* **1978**, *28*, 633.
(855) Bartholomew, B.; Hill, M.J. *Food Chem. Toxicol.* **1984**, *22*, 789.
(856) Pennington, J.A,T. *Food Control* **1998**, *9*, 385.
(857) Bryan, N.S. *Free Radic. Biol. Med.* **2006**, *41*, 691.
(858) Green, L.C., Luzuriaga, K.R., Wagner, D.A., Rand, W.; Istfan, N.; Young, V.R.; Tannenbaum, S.R. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 7764.
(859) Rhodes, P.M.; Leone, A.M.; Francis, P.L.; Struthers, A.D.; Moncada, S. *Biochem. Biophys. Res. Commun.* **1995**, *209*, 590.
(860) Lauer, T.; Preik, M.; Rassaf, T.; Strauer, B.E.; Deussen, A.; Feelisch, M.; Kelm, M. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 12814.
(861) Shingles, R.; Roh, M.H.; McCarty, R.E. *J. Bioenerg. Biomembr.* ***1997****, 29*, 611.
(862) Jensen, F.B. *Acta Physiol. Scand.* **2005**, *184*, 243.
(863) Samouilov, A.; Woldman, Y.Y.; Zweier, J.L.; Khramtsov, V.V. *Nitric Oxide* **2007**, *16*, 362.
(864) Vitturi, D.A.; Teng, X.; Toledo, J.C.; Matalon, S.; Lancaster Jr., J.R.; Patel, R. P. *Am. J. Physiol. Heart Circ. Physiol.* **2009**, *296*, H1398.
(865) Jensen, F.B.; Rohde, S. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2010**, *298*, R972.
(866) Butler, A.R.; Feelisch, M. *Circulation* **2008**, *117*, 2151.
(867) Lundberg, J.O.; Weitzberg, E.; Gladwin, M.T. *Nat. Rev. Drug Discovery* **2008**, *7*, 156.
(868) Kevil, C.G.; Kolluru, G.K.; Pattillo, C.B.; Giordano, T. *Free Radic. Biol. Med.* **2011**, *51*, 576.
(869) Larsen, F.J.; Schiffer, T.A.; Borniquel, S.; Sahlin, K.; Ekblom, B.; Lundberg, J.O.; Weitzberg E. *Cell Metab.* **2011**, *13*, 149.
(870) Lundberg, J.O.; Carlstrom, M.; Larsen, F.J.; Weitzberg, E. *Cardiovasc. Res.* **2011**, *89*, 525.
(871) Pluta, R.M.; Oldfield, E.H.; Bakhtian, K.D.; Fathi, A.R.; Smith, R.K.; Devroom, H.L.; Nahavandi, M.; Woo, S.; Figg, W.D.; Lonser, R.R. *PLoS One* **2011**, *6*, e14504.
(872) Lefer, D.J. *Am. J. Physiol. Renal Physiol.* **2006**, *290*, F777.
(873) Casey, D.B.; Badejo, A,M.; Dhaliwal, J.S.; Murthy, S.N.; Hyman, A.L.; Nossaman, B.D.; Kadowitz, P.J. *Am. J. Physiol. Heart Circ. Physiol.* **2009**, *296*, H524.
(874) Calvert, J.W.; Lefer, D.J. *Cardiovasc. Res.* **2009**, *83*, 195.
(875) Lefer, D.J. *Arch. Pharm. Res.* **2009**, *32*, 1127.
(876) Calvert, J.W.; Lefer, D.J. Nitric Oxide **2010**, *22*, 91.
(877) Ingram, T.E.; Pinder, A.G.; Bailey, D.M.; Fraser, A.G.; James, P.E. *Am. J. Physiol. Heart Circ. Physiol.* **2010**, *298*, H331.
(878) Jung, K.H.; Chu, K.; Lee, S.T.; Sunwoo, J.S.; Park, D.K.; Kim, J.H.; Kim, S.; Lee, S.K.; Kim, M.; Roh, J.K. *Biochem. Biophys. Res. Commun.* **2010**, *403*, 66.
(879) Nossaman, B.D.; Akuly, H.A.; Lasker, G.F.; Nossaman, V.E.; Rothberg, P.A.; Kadowitz, P.J. *Asian J. Exp. Biol. Sci.* **2010**, *1*, 451.
(880) Gilchrist, M.; Shore, A.C.; Benjamin, N. *Cardiovasc. Res.* **2011**, *89*, 492.
(881) Lundberg, J.O.; Carlstrom, M.; Larsen, F.J.; Weitzberg, E. *Cardiovasc. Res.* **2011**, *89*, 525.
(882) Machha, A.; Schechter, A.N. *Eur. J. Nutr.* **2011**, *50*, 293.

(883) Murillo, D.; Kamga, C.; Mo, L.; Shiva, S. *Nitric Oxide* **2011**, *25*, 70.
(884) Pattillo, C.B.; Bir, S.; Rajaram, V.; Kevil, C.G. *Cardiovasc. Res.* **2011**, *89*, 533.
(885) Sindler, A.L.; Fleenor, B.S.; Calvert, J.W.; Marshall, K.D.; Zigler, M.L.; Lefer, D.J.; Seals, D.R. *Aging Cell* **2011**, *10*, 429.
(886) Dykhuizen, R.S.; Fraser, A.; McKenzie, H.; Golden, M.; Leifert, C.; Benjamin, N. *Gut* **1998**, *42*, 334.
(887) Phillips, R.; Kuijper, S.; Benjamin, N.; Wansbrough-Jones, M.; Wilks, M.; Kolk, A.H. *Antimicrob. Agents Chemother.* **2004**, *48*, 3130.
(888) Anyim, M., Benjamin, N.; Wilks, M. *Int. J. Antimicrob. Agents* **2005**, *26*, 85.
(889) Rao, A.; Jump, R.L.; Pultz, N.J.; Pultz, M.J.; Donskey, C.J. *Antimicrob. Agents Chemother.* **2006**, *50*, 3901.
(890) Cueto, M.; Herandez-Perers, O.; Martin, R.; Bentura, M.L.; Rodrigo, J.; Lamas, S.; Golvano, M.P. *FEBS Lett.* **1996**, *398*, 159.
(891) Leshem, T.Y.; Haramaty, E. *J. Plant Physiol.* **1996**, *148*, 258.
(892) Leshem, Y.Y.; Kuiper, P.J.C. *Biol. Plant* **1996**, *38*, 1.
(893) Noritake, T.; Kawakita, K.; Doke, N. *Plant Cell Physiol.* **1996**, *37*, 113.
(894) Ninnemann, H.; Maier, J. *Photochem. Photobiol.* **1996**, *64*, 393.
(895) Delledonne, M.; Xia, Y.; Dixon, R.A.; Lamb, C. Nature **1998**, *394*, 585.
(896) Durner, J.; Wendehenne, D.; Klessig, D.F. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10328.
(897) Mathieu, C.; Moreau, S.; Frendo, P.; Puppo, A.; Davies, M.J. *Free Radic. Biol. Med.* **1998**, *24*, 1242.
(898) Beligni, M.V.; Lamattina, L. *Planta.* **2000**, *210*, 215.
(899) Clarke, A.; Desikan, R.; Hurst, R.D.; Hancock, J.T.; Neill, S.J. *Plant J.* **2000**, *24*, 667.
(900) Foissner, I.; Wendehenne, D.; Langebartels, C.; Durner, J. *Plant J.* **2000**, *23*, 817.
(901) Klessig, D.F.; Durner, J.; Noad, R. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8849.
(902) Beligni, M.V.; Lamattina, L. *Plant Cell Environ.* **2001**, *24*, 267.
(903) Delledonne M.; Zeier J.; Marocco A.; Lamb C. *Proc. Natl. Acad. Sci. USA* **2001** ,*98*, 13454.
(904) Garcia-Mata, C.; Lamattina, L. *Plant Physiol.* **2001**, *126*, 1196.
(905) Tun, N.N.; Holk, A.; Scherer, G.F. *FEBS Lett.* **2001**, *509*, 174.
(906) Wendehenne, D.; Pugin, A.; Klessig, D.F.; Durner, J. *Trends Plant Sci.* **2001**, *6*, 177.
(907) Yamasaki, H.; Shimoji, H.; Ohshiro, Y.; Sakihama, Y. *Nitric Oxide* **2001**, *5*, 261.
(908) Beligni, M.V.; Fath, A.; Bethke, P.C.; Lamattina, L.; Jones, R.L. *Plant Physiol.* **2002**, *129*, 1642.
(909) Desikan, R.; Griffiths, R.; Hancock, J.; Neill, S. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16314.
(910) Garcia-Mata, C.; Lamattina, L. *Plant Physiol.* **2002**, *128*, 790.
(911) Graziano, M.; Beligni, M-V.; Lamattina, L.; *Plant Physiol.* **2002**, *130*, 1852.
(912) Neill, S.J.; Desikan, R.; Clarke, A.; Hancock, J.T. *Plant Physiol.* **2002**, *128*, 13.
(913) Neill, S.J.; Desikan, R.; Clarke, A.; Hurst, R.D.; Hancock, J.T. *J. Exp. Bot.* **2002**, *53*, 1237.
(914) Rockel, P.; Strube, F.; Rockel, A.; Wildt, J.; Kaiser, W. M. *J. Exp. Bot.* **2002**, *53*, 103.
(915) Gould, K.S.; Lamotte, O.; Klinguer, A.; Pugin, A.; Wendehenne, D. *Free Rad. Res.* **2003**, *37*, 38.

(916) Hu, X.; Neill, S.J.; Cai, W.; Tang, Z. *Funct. Plant Biol.* **2003**, *30*, 901.

(917) Lamattina, L.; Garcia-Mata, C.; Graziano, M.; Pagnussat, G. *Annu. Rev. Plant Biol.* **2003**, *54*, 109.

(918) Neill, S.J.; Desikan, R.; Hancock, J.T. *New Phytologist* **2003**, *159*, 11.

(919) Desikan, R.; Cheung, M.K.; Bright, J.; Henson, D.; Hancock, J.T.; Neill, S. *J. Exp. Bot.* **2004**, *55*, 205.

(920) He, Y.; Tang, R.H.; Hao, Y.; Stevens, R.D.; Cook, C.W.; Ahn, S.M.; Jing, L.; Yang, Z.; Chen, L.; Guo, F.; Fiorani, F.; Jackson, R.B.; Crawford, N.M.; Pei, Z.M. *Science* **2004**, *305*, 1968.

(921) Huang, X.; Stettmaier, K.; Michel, C.; Hutzler, P.; Mueller, M.J.; Durner, J *Planta* **2004**, *218*, 938.

(922) Parani, M.; Rudrabhatla, S.; Myers, R.; Weirich, H.; Smith, B.; Leaman, D.W.; Goldman, S.L. *Plant Biotech. J.* **2004**, *2*, 359.

(923) Romero-Puertas, M.C.; Perazzolli, M.; Zago, E.D.; Delledonne, M. *Cell. Microbiol.* **2004**, *6*, 795.

(924) Wang, J.W.; Wu, J.Y. *Nitric Oxide* **2004**, *11*, 298.

(925) Wendehenne, D.; Durner, J.; Klessig, D.F. *Curr. Opin. Plant Biol.* **2004**, *7*, 449.

(926) Zhao, M.G.; Tian, Q.Y.; Zhang, W.H. *Plant Physiol.* **2007**, *144*, 206.

(927) Hong, J.K.; Yun, B.W.; Kang, J.G.; Raja, M.U.; Kwon, E.; Sorhagen, K.; Chu, C.; Wand, Y.; Loake, G.J. *J. Exp. Bot.* **2008**, *59*, 147.

(928) Neill, S.; Bright, J.; Desikan, R.; Hancock, J.; Harrison, J.; Wilson, I. *J. Exp. Bot.* **2008**, *59*, 25.

(929) Wilson, I.D.; Neill, S.J.; Hancock, J.T. *Plant Cell Environ.* **2008**, *31*, 622.

(930) Zhao, M.G.; Chen, L.; Zhang, L.L.; Zhang, W.H. *Plant Physiol.* **2009**, *151*, 755.

(931) Tun, N.N.; Santa-Catarina, C.; Begum, T.; Silveira, V.; Handro, W.; Floh, E.I.S.; Scherer, G.F.E. *Plant Cell Physiol.* **2006**, *47*, 346.

(932) Yamasaki, H.; Cohen, M.F. *Trends Plant Sci.* **2006**, *11*, 522.

(933) Rumer, S.; Gupta, K.J.; Kaiser, W.M. *J. Exp. Bot.* **2009**, *60*, 2065.

(934) Barroso, J.B.; Corpas, F.J.; Carreras, A.; Sandalio, L.M.; Valderrama, R.; Palma, J.M.; Lupianezi, J.A.; del Rio, L.A. *J. Biol. Chem.* **1999**, *274*, 36729.

(935) Del Rio, L.A.; Corpas, F.J.; Sandalio, L.M.; Palma, J.M.; Gomez, M.; Barroso, J.B. *J. Exp. Bot.* **2002**, *53*, 1255.

(936) Corpas F.J.; Barroso J.B.; Carreras A. *Plant Physiol.* **2004**, *136*, 2722.

(937) del Rio, L.A.; Corpas, F.J.; Barroso, J.B. *Phytochemistry* **2004**, *65*, 783.

(938) Corpas, F.J.; Barroso, J.B.; Carreras, A.; Valderrama, R.; Palma, J.M.; Leon, A.M.; Sandalio, L.M.; Rio L.A. *Planta* **2006**, *224*, 246.

(939) Guo, F.Q.; Crawford, N.M. *Plant Cell* **2005**, *17*, 3436.

(940) Gas, E.; Flores-Perez, U.; Sauret-Gueto, S.; Rodriguez-Concepcion, M. *Plant Cell* **2009**, *21*, 18.

(941) Del Rio, L.A. *Arch. Biochem. Biophys.* **2010**, *506*, 1.

(942) Chandok, M.R.; Ytterberg, A.J.; van Wijk, K.L.; Klessig, D.F. *Cell* **2003**, *113*, 469.

(943) Guo, F.Q.; Okamoto, M.; Crawford, M.J. *Science* **2003**, *302*, 100.

(944) Zemojtel, T.; Frohlich, A.; Palmieri, M.C.; Kolanczyk, M.; Mikula, I.; Wyrwicz, L.S.; Wanker, E.E.;

Mundlos, S.; Vingron, M.; Martasek, P.; Durner, J. *Trends Plant Sci.* **2006**, *11*, 524.

(945) Moreau, M.; Lindermayr, C.; Durner, J.; Klessig, D.F. *Physiol. Plant.* **2010**, *138*, 372.

(946) Derelle, E.; Ferraz, C.; Rombauts, S.; Rouzé, P.; Worden, A.Z.; Robbens, S.; Partensky, F.; Degroeve, S.; Echeynié, S.; Cooke, R.; Saeys, Y.; Wuyts, J.; Jabbari, K.; Bowler, C.; Panaud, O.; Piégu, B.; Ball, S.G.; Ral, J.P.; Bouget, F.Y.; Piganeau, G.; De Baets, B.; Picard, A.; Delseny, M.; Demaille, J.; Van de Peer, Y.; Moreau, M. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11647.

(947) Foresi, N.; Correa-Aragunde, N.; Parisi, G.; Calo, G.; Salerno, G.; Lamattina, L. *Plant Cell* **2010**, *22*, 3816.

(948) Trapido-Rosenthal, H.G.; Sharp, K.H.; Galloway, T.S.; Morall, C.E. *Am. Zoologist*. **2001**, *41*, 247.

(949) Trapido-Rosenthal, H.G.; Zielke, S.; Owen, R.; Buxton, L.; Boeing, B.; Bhagooli, R.; Archer, J. *Biol. Bull.* **2005**, *208*, 3.

(950) Kim, D.; Yamaguchi, K.; Oda, T. *J. Plankton Res.* **2006**, *28*, 613.

(951) Simontacchi, M.; Jasid, S.; Puntarulo, S. *Plant Sci.* **2004**, *167*, 839.

(952) Gupta, K.J.; Stoimenova, M.; Kaiser, W.M. *J. Exp. Bot.* **2005**, *56*, 2601.

(953) Solomonson, L.P.; Barber, M.J. *Annu. Rev. Plant Mol. Biol*. **1990**, *41*, 225.

(954) Crawford, N.M. *Plant Cell* **1995**, *7*, 859.

(955) Huber, S.C.; Bachmann, M.; Huber, J.L. *Trends Plant Sci.* **1996**, *1*, 432.

(956) Campbell, E.H. *Annu. Rev. Plant Mol. Biol.* **1999**, *50*, 277.

(957) Kaiser, W.M.; Weiner, H.; Huber, S.C. *Physiol. Plant* **1999**, *105*, 385.

(958) Stitt, M. *Curr. Opin. Plant Biol.* **1999**, *2*, 178.

(959) Campbell, W.H. *Cell. Mol. Life Sci.* **2001**, *58*, 194.

(960) Kelker, H.C.; Filner, P. *Biochim. Biophys. Acta* **1971**, *252*, 69.

(961) Dean, J.V.; Harper, J.E. *Plant Physiol*. **1988**, *88*, 389.

(962) Yamasaki, H.; Sakihama, Y.; Takahashi, S. *Trends Plant Sci.* **1999**, *4*, 128.

(963) Yamasaki, H.; Sakihama, Y. *FEBS Lett.* **2000**, *468*, 89.

(964) C-NaR is highly regulated by complex transcriptional, translational and posttranslational mechanisms. The posttranslational regulation involves the phosphorylation of a serine residue (in the linker region between the molybdenum and haem domains[965]) by protein kinases (including AMP-activated[966] and calcium-dependent kinases[967]). This phosphorylation creates a recognition site that recruits a specific regulatory protein (one member of the 14-3-3 family), whose binding effectively inhibits the enzyme[968,969]. Thus, mutation of the key serine residue to an aspartate results in a plant that has the C-NaR always active[970-972]. *In vivo*, this posttranslational regulation is essential to lower the C-NaR activity at night, when photosynthetically generated reducing equivalents are not available to reduce nitrite to ammonia (otherwise, the nocturnal nitrite levels would increase to dangerous concentrations)[973]. *In vitro*, the C-NaR inactivation occurs rapidly in darkness or when carbon dioxide is removed.

(965) Huber, J.L.; Huber, S.C.; Campbell, W.H.; Redinbaugh, M.G. *Arch. Biochem. Biophys.* **1992**, *296*, 58.

(966) Reaume, A.G.; Clark, S.H.; Chovnick, A. *Genetics* **1989**, *123*, 503.
(967) Douglas, P.; Moorhead, G.; Hong, Y.; Morrice, N.; MacKintosh, C. *Planta* **1996**, *206*, 435.
(968) Moorhead, G.; Douglas, P.; Morrice, N.; Scarabel, M.; Aitken, A.; MacKintosh, C. *Curr. Biol.* **1996**, *6*, 1104.
(969) Shen, W.; Huber, S.C. *Plant Cell Physiol.* **2006**, *47*, 764.
(970) Lillo, C.; Lea, U.S.; Leydecker, M.-T.; Meyer, C. *Plant J.* **2003**, *35*, 566.
(971) Lea, U.S.; Hoopen, F.; Provan, F.; Kaiser, W.M.; Meyer, C.; Lillo, C. *Planta* **2004**, *219*, 59.
(972) Lillo, C.; Meyer, C.; Lea, U.S.; Provan, F.; Oltedal, S *J. Exp. Bot.* **2004**, *55*, 1275.
(973) Sherameti, I.; Sopory, S.K.; Trebicka, A.; Pfannschmidt, T.; Oelmüller, R. *J. Biol. Chem.* **2002**, *277*, 46594.
(974) Morot-Gaudry-Talarmain, Y.; Rockel, P.; Moureaux, T.; Quilleré, I.; Leydecker, M.; Kaiser, W.; Morot-Gaudry, J. *Planta* **2002**, *215*, 708.
(975) Planchet, E.; Gupta, K.J.; Sonoda, M.; Kaiser, W.M. *Plant J.* **2005**, *41*, 732.
(976) Bright, J.; Desikan, R.; Hancock, J.T.; Weir, I.S.; Neill, S.J. *Plant J.* **2006,** *45*, 113.
(977) Yamamoto-Katou, A.; Katou, S.; Yoshioka, H.; Doke, N.; Kawakita, K. *Plant Cell Physiol.* **2006**, *47*, 726.
(978) Seligman, K.; Saviani, E.E.; Oliveira, H.C.; Pinto-Maglio, C.A.; Salgado, I. *Plant Cell Physiol* **2008**, *49*, 1112.
(979) Shi, F.M.; Li, Y.Z. *BMB Rep.* **2008**, *41*, 79.
(980) Ribeiro, D.M.; Desikan, R.; Bright, J.; Confraria, A.; Harrison, J.; Hancock, J.T.; Barros, R.S.; Neill, S.J.; Wilson, I.D. *Plant Cell Environ.* **2009**, *32*, 46.
(981) Oliveira, H.C.; Saviani, E.E.; Oliveira, J.F.P.; Salgado, I. *Tropical Plant Pathol.* **2010**, *35*, 104.
(982) Magalhaes, J.R.; Silva, F.L.I.M.; Salgado, I.; Ferrarese-Filho, O.; Rockel, P.; Kaiser, W.M. *Physiol. Mol. Biol. Plants.* **2002**, *8*, 11.
(983) Sakihama, Y.; Nakamura, S.; Yamasaki, H. *Plant Cell Physiol*. **2002**, *43*, 290.
(984) Liu, Y.; Wu, R.; Wan, Q.; Xie, G.; Bi, Y. *Plant Cell Physiol.* **2007**, *48*, 511.
(985) Sang, J.; Jiang, M.; Lin, F.; Xu, S.; Zhang, A.; Tan, M. *J. Integr. Plant Biol.* **2008**, *50*, 231.
(986) Srivastava, N.; Gonugunta, V.K.; Puli, M.R.; Raghavendra, A.S. *Planta* **2009**, *229*, 757.
(987) Wu, S.J.; Qi, J.L.; Zhang, W.J.; Liu, S.H.; Xiao, F.H.; Zhang, M.S.; Xu, G.H.; Zhao, W.G.; Shi, M.W.; Pang, Y.J.; Shen, H.G.; Yang, Y.H. *Plant Cell Physiol.* **2009**, *50*, 118.
(988) Harper, J.E. *Plant Physiol*. **1981**, *68*, 1488.
(989) Dean, J.V.; Harper, J.E. *Plant Physiol.* **1986**, *82*, 718.
(990) Wildt, J.; Kley, D.; Rockel, A.; Rockel, P.; Segschneider, H.J. *J. Geo. Res.* **1997**, *102*, 5919.
(991) Yamasaki, H. *Phil. Trans. R. Soc. Lond. B* **2000**, *355*, 1477.
(992) Kaiser, W.M.; Weiner, H.; Kandlbinder, A.; Tsai, C.B.; Rockel, P.; Sonoda, M.; Planchet, E. *J. Exp. Bot.* **2002**, *53*, 875.
(993) Dordas, C.; Hasinoff, B.B.; Rivoal, J.; Hill, R.D. *Planta* **2004**, *219*, 66.
(994) Meyer, C.; Lea, U.S.; Provan, F.; Kaiser, W.M.; Lillo, C. *Photosynth. Res.* **2005**, *83*, 181.
(995) Modolo, L.V.; Augusto, O.; Almeida, I.M.G.; Pinto-Maglio, C.A.F.; Oliveira, H.C.; Seligman, K.;

Salgado, L *Plant Sci.* **2006**, *171*, 34.

(996) Gupta, K.J.; Kaiser, W.M. *Plant Cell Physiol.* **2010**, *51*, 576.

(997) Lu, D.; Dong, J.; Jin, H.; Sun, L.; Xu, X.; Zhou, T.; Zhu, Y.; Xu, M. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1073.

(998) Galeeva, E.I.; Trifonova, T.V.; Ponomareva, A.A.; Viktorova, L.V.; Minibayeva, F.V. *Biochemistry (Mosc.)* **2012**, *77*, 404.

(999) Silaghi-Dumitrescu, R.; Mich, M.; Matyas, C.; Cooper, C.E. *Nitric Oxide* **2012**, *26*, 27.

(1000) Miller, A.J., Smith, S.J. *J. Exp. Bot.* **1996**, *47*, 843.

(1001) Kaise, W.N.; Huber, S.C. *J. Exp. Bot.* **2001**, *52*, 1981.

(1002) Like the ischaemic events of mammals (see section 3.1.1.), higher plants can also be subjected to a decrease of oxygen concentration. Roots can be subject to hypoxia or even anoxia after a strong rainfall, because the soil usually becomes flooded, for a short or long period, depending on its drainage capacity.[1003] Thus, hypoxia of root systems occurs frequently in nature[1004], affecting the majority of plants at some time during their life cycle[1005]. In addition, also the aerial photosynthetic tissues can be subjected to hypoxia, *e.g.*, upon stomatal closure triggered by an increase in temperature (inhibiting the photosynthetic activity).

As in other aerobic organisms, when the dioxygen concentration decreases, the plant mitochondrial oxidative phosphorylation is diminished/ceased (ATP decreases and NADH increases) and the glycolysis and fermentation are promoted to synthesise ATP and regenerate NAD^+, leading to the accumulation, in plants, of ethanol, lactate and alanine.[1006,1007] Subsequently, acidification of the cytoplasm occurs[1008,1009] and the photosynthetic activity is reduced[1010,1011]. However, the lactic fermentation is transient, as acidification of the cytoplasm inhibits the lactate production[1012] and stimulates the ethanol production[1012,1013]. The ethanolic fermentation, in its turn, enables, besides the generation of ATP, a more efficient (on a molar basis) regeneration of NAD^+ and consumption of protons.[1014,1015] In this way, in plants, the energy production can proceed during several hours of hypoxia without severe cytoplasmatic acidosis[1013,1016] -explaining (at least partially) why plants survive far longer under hypoxia than does any higher animal (that uses exclusively lactic fermentation).

(1003) Huang, B.; Johnson, J.W.; NeSmith, S.; Bridge, D.C. *Crop Sci.* **1994**, *34*, 1538.

(1004) Kennedy, R.A.; Rumpho, M.E.; Fox, T.C. *Plant Physiol.* **1992**, *100*, 1.

(1005) Jackson, M.B.; Herman, B.; Goodenough, A. *Plant Cell Environ.* **1982**, *5*, 163.

(1006) Ricard, B.; Couée, I.; Raymond, P.; Saglio, P.H.; Saint-Ges, V.; Pradet, A. *Plant Physiol. Biochem.* **1994**, *32*, 1.

(1007) Drew, M.C. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* **1997**, *48*, 223.

(1008) Roberts, J. K.; Callis, J.; Jardetzky, O.; Walbot, V.; and Freeling, M. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 6029.

(1009) Roberts, J.K.; Andrade, F.H.; and Anderson, I.C. *Plant Physiol.* **1985**, *77*, 492.

(1010) Huang, B.; Johnson, J.W.; NeSmith, S.; Bridge, D.C. *J. Exp. Bot.* **1994**, *45*, 193.

(1011) Liao, C.T.; Lin, C.H. *Plant Physiol. Biochem.* **1994**, *32*, 479.

(1012) Davies, D.D.; Grego, S.; Kenworthy, P. *Planta* **1974**, *118*, 297.

(1013) Roberts, J.K.M.; Callis, J.; Wemmer, D.; Walbot, V.; Jardetzky, O. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 3379.

(1014) Fan, T.W.-M.; Higashi, R.M.; Frenkiel, T.A.; Lane, A.N. *J. Exp. Bot.* **1997**, *48*, 1655.

(1015) Kato-Noguchi, H. *Physiol. Plant.* **2000**, *109*, 28.

(1016) Hochachka, P.W.; Mommsen, T.P. *Science* **1983**, *219*, 1391.

(1017) Ferrari, T.E.; Varner, J.E. *Plant Physiol.* **1971**, *47*, 790.

(1018) Garcia-Novo, F.; Crawford, R.M.M. *New Phytol.* **1973**, *72*, 1031.

(1019) Kaiser, W.M.; Brendle-Behnisch, E. *Planta* **1995**, *196*, 1.

(1020) Botrel, A.; Magne, C.; Kaiser, W.M. *Plant Physiol. Biochem.* **1996**, *34*, 645.

(1021) Botrel, A.; Kaiser, W.M. *Planta* **1997**, *201*, 496.

(1022) Kaiser, W.M.; Weiner, H.; Huber, S.C. *Physiol. Plant.* **1999**, *105*, 385.

(1023) Stohr, C.; Mack, G. *J. Exp. Bot.* **2001**, *52*, 1283.

(1024) Klok, E.J.; Wilson, I.W.; Wilson, D.; Chapman, S.C.; Ewing, R.M.; Somerville, S.C.; Peacock, W.J.; Dolferus, R.; Dennis. E.S. *Plant Cell* **2002**, 14:2481.

(1025) Stoimenova, M.; Libourel, I.G.L.; Ratcliff, R.G.; Kaiser, W.M. *Plant Soil* **2003**, *253*, 155.

(1026) Lager, I.D.A.; Andreasson, O.; Dunbar, T.L.; Andreasson, E.; Escobar, M.A.; Rasmusson, A.G. *Plant Cell Environ.* **2010**, *33*, 1513.

(1027) Kruger, N.J.; von Schaewen, A. *Curr. Opin. Plant Biol.* **2003**, *6*, 236.

(1028) Dry, I.; Wallace, W.; Nicholas, D.J.D. *Planta* **1981,** *152*, 234.

(1029) Wellbrun, A. R. *New Phytol.* **1990**, *115*, 395.

(1030) The inhibition of the photosynthetic electron flow causes the nitrite accumulation, because its transport to the chloroplasts depends on the pH gradient across the chloroplast envelope (build up by the photosynthetic activity); in addition, its reduction to ammonium is hindered in the absence of photosynthetically reduced ferredoxin.[954,1031]

(1031) Shingle, R.; Roh, M.H.; McCarty, R.E. *Plant Phyisiol.* **1996**, *112*, 1375.

(1032) Klepper, L.A. *Weed Sci.* **1975**, *23*, 188.

(1033) Klepper, L.A. *Weed Sci.* **1976**, *24*, 533.

(1034) Vaucheret, H.; Kronenberger, J.; Lepingle, A.; Vilaine, F.; Boutin, J.-P.; Caboche, M. *Plant J.* **1992**, *2*, 559.

(1035) Goshima, N.; Mukai, T.; Suemori, M.; Takahashi, M.; Caboche, M.; Morikawa, H. *Plant J.* **1999**, *19*, 75.

(1036) Neill, S.; Barros, R.; Bright, J.; Desikan, R.; Hancock, J.; Harrison, J.; Morris, P.; Ribeiro, D.; Wilson, I. *J. Exp. Bot.* **2008**, *59*, 165.

(1037) Hao, F.; Zhao, S.; Dong, H.; Zhang, H.; Sun, L.; Miao, C. *J. Integr. Plant Biol.* **2010**, *52*, 298.

(1038) Yamamoto, A.; Katou, S.; Yoshioka, H.; Doke, N.; Kawakita, K. *J. Gen. Plant Pathol.* **2003**, *69*: 218.

(1039) Santos-Filho, P.R.; Vitor, S.C.; Frungillo, L.; Saviani, E.E.; Oliveira, H,C.; Salgado, I. *Plant Cell Physiol.* **2012**, *53*, 1607.

(1040) Ruoff, P.; Lillo, C. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 1000.
(1041) Barber, M.J.; Kay, C.J. *Arch. Biochem. Biophys.* **1996**, *326*, 227.
(1042) Hesberg, C.; Haensch, R.; Mendel, R.R.; Bittner, F. J. Biol. Chem. **2004**, *279*, 13547.
(1043) Yesbergenova, Z.; Yang, G.; Oron, E.; Soffer, D.; Flur, R.; Sagi, M. *Plant J.* **2005**, *42*, 862.
(1044) Zarepour, M.; Kaspari, K.; Stagge, S.; Rethmeier, R.; Mendel, R.R.; Bittner, F. *Plant Mol. Biol.* **2010**, *72*, 301.
(1045) Datta, D.B.; Triplett, E.W.; Newcomb, E.H. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 4700.
(1046) Sandalio, L.M.; Fernandez, V.M.; Ruperez, F.L.; del Rio, L.A. *Plant Physiol.* **1988**, *87*, 1.
(1047) Corpas, F.J.; Colina, C.; Sanchez-Rasero, F.; Rio, L.A. *J. Plant Physiol.* **1997**, *151*, 246.
(1048) Corpas, F..J.; Palma, J.M.; Sandalio, L.M.; Valderrama, R.; Barroso, J.B.; del Rio, L.A. *J. Plant Physiol.* **2008**, *165*, 1319.
(1049) Reumann, S.; Ma, C.; Lemke, S.; Babujee, L. *Plant Physiol.* **2004**, *136*, 2587.
(1050) Montalbini, P. *J. Phytopathol.* **1992**, *134*, 218.
(1051) Montalbini, P. *Plant Sci.* **1992**, *87*, 225.
(1052) Silvestri, S.; Murphy, A.M.; Buonaurio, R.; Carr, J.P. *Virus Res.* **2008**, *137*, 257.
(1053) Montalbini, P.; Della-Torre, G. *Physiol. Mol. Plant Pathol.* **1996**, *48*,273.
(1054) Pastori, G.M.; Rio, L.A. *Plant Physiol.* **1997**, *113*, 411.
(1055) Wang, B.L.; Tang, X.Y.; Cheng, L.Y.; Zhang, A.Z.; Zhang, W.H.; Zhang, F.S.;Liu, J.Q.; Cao, Y.; Allan, D.L.; Vance, C.P.; Shen, J.B. *New Phytol.* **2010**, *187*, 1112.
(1056) Xiong, J.; Fu, G.; Yang, Y.; Zhu, C.; Tao, L. *J. Exp. Bot.* **2012**, *63*, 33.
(1057) Planchet, E.; Kaiser, W.M. *Plant Signal. Behav.* **2006**, *1*, 46.
(1058) Badwey, J.A.; Robinson, J.M.; Karnovsky, M.J.; Karnovsky, M.L. *J. Biol. Chem.* **1981**, *256*, 3479.
(1059) Zarepour, M.; Simon, K.; Wilch, M.; Nielander, U.; Koshiba, T.; Seo, M.; Lindel, T.; Bittner, F. *Plant Mol. Biol.* **2012**, *80*, 659.
(1060) Akaba, S.; Seo, M.; Dohmae, N.; Takio, K.; Sekimoto, H.; Kamiya, Y.; Furuya, N.; Komano, T.; Koshiba, T *J. Biochem.* **1999**, *126*, 395.
(1061) Koiwai, H.; Akaba, S.; Seo, M.; Komano, T.; Koshiba, T. *J. Biochem.* **2000**, *127*, 659.
(1062) Seo, M.; Koiwai, H.; Akaba, S.; Komano, T.; Oritani, T.; Kamiya, Y.; Koshiba, T. *Plant J.* **2000**, *23*, 481.
(1063) Koiwai, H.; Nakaminami, K.; Seo, M.; Mitsuhashi, W.; Toyomasu, T.; Koshiba, T. *Plant Physiol.* **2004**, *134*, 1697.
(1064) Seo, M.; Peeters, A.J.; Koiwai, H.; Oritani, T.; Marion-Poll, A.; Zeevaart, J.A.; Koornneef, M.; Kamiya, Y.; Koshiba, T. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 12908.
(1065) Seo, M.; Koshiba, T. *Trends Plant Sci.* **2002**, *7*, 41.
(1066) Mauch-Mani, B.; Mauch, F. *Curr. Opin. Plant Biol.* **2005**, *8*, 409.
(1067) Verslues, P.E.; Zhu, J.K. *Biochem. Soc. Trans.* **2005**, *33*, 375.
(1068) All the haemic proteins with the globin structure will be here identified by the family named "haemic globins", abbreviated as HG. To avoid ambiguities, the word "haemoglobin" (abbreviation Hb)

will be here used only for the well known blood oxygen transporter.

(1069) Bashford, D.; Chothia, C.; Lesk, A.M. *J. Mol. Biol.* **1987**, *196*, 199.

(1070) Although it is less familiar, plants also hold HG (or, as is usually found in literature, "haemoglobins"). Leghaemoglobin is a monomeric HG, present in the root nodules of leguminous plants and in a few other nitrogen-fixing species (in millimolar concentrations), where it facilitates the oxygen diffusion to the obligate aerobes involved in nitrogen fixation inside the nodule, at the same time as it maintains a low free oxygen concentration to, in this way, avoid the inhibition of the bacterial nitrogenase (the rate constants towards oxygen are k_{on}≈130μM^{-1}s^{-1} and k_{off}≈6s^{-1}; values that compare with k_{on}≈15μM^{-1}s^{-1} and k_{off}≈13s^{-1} for sperm whale Mb)[1071-1076] -leghaemoglobin is, thus, a vital component of the symbiotic nitrogen fixation machinery.

(1071) Wittenberg, J.B.; Appleby, C.A.; Wittenberg, B.A. *J. Biol. Chem.* **1972**, *247*, 527.

(1072) Appleby, C.; Tjepkema, J.; Trinick, M. *Science* **1983**, *220*, 951.

(1073) Appleby, C.A. *Annu. Rev. Plant Physiol.* **1984**, *35*, 443.

(1074) Hargrove, M.S.; Barry, J.K.; Brucker, E.A.; Berry, M.B.; Phillips, G.N.; Olson, J.S.; Arredondo-Peter, R.; Dean, J.M.; Klucas, R.V.; Sarath, G. *J. Mol. Biol.* **1997**, *266*, 1032.

(1075) Kundu, S.; Trent III, J.M.; Hargrove, M.S. *Trends Plant Sci.* **2003**, *8*, 387.

(1076) Ott, T.; van Dongen, J.T.; Gunther, C.; Krusell, L.; Desbrosses, G.; Vigeolas, H.; Bock, V.; Czechowski, T.; Geigenberger, P.; Udvardi, M.K. *Curr. Biol.* **2005**, *15*, 531.

(1077) The identification of leghaemoglobin triggered the search for HG in non-leguminous plants, what culminated with the discovery of new HG, named (not very originally) "non-symbiotic haemoglobins". NS-HG, contrary to leghaemoglobins, are believed to be present in the entire plant kingdom, in low concentrations, although with different expressions in different tissues and in response to different types of stress.[1075,1078-1087]

Plant HG can be divided in, at least, four groups[1087-1089]: the penta-coordinated leghaemoglobins and three classes of NS-HG, all showing some character of bis-histidinyl-hexa-coordination, via reversible intramolecular coordination. Class 1 NS-HG have the lowest affinity for the distal histidine coordination (K ≈1-2 [1087,1090,1091]) and display a low dioxygen dissociation rate constant and high dioxygen affinity (k_{on}≈60-70μM^{-1}s^{-1} and k_{off}≈0.03-0.2s^{-1} [1080,1081,1088,1092-1094], resulting in a global oxygen affinity (2-3nM) two orders of magnitude higher than the CcO one), evidence that they remain oxygenated at extremely low oxygen concentrations [1092,1094]). These characteristics suggest, as was described for Nb (section 3.1.1.2.(a)), that they do not meet the equilibrium and kinetic requirements for functioning in oxygen transport.[1075,1080,1087,1095,1096] Instead, it has been suggested that class 1 NS-HG are involved in NO scavenging and in the maintenance of the cellular redox status contributing to the cell survival under hypoxia (further discussed in the text; see also section 3.2.1.4. and its Fig. 4). In this respect, possible mechanisms of action of NS-HG involve sensing of oxygen and other gaseous ligands, NO scavenging and formation (further discussed in the text).[1095,1097] These proteins, expressed in low concentration (5-20μM in hypoxic tissues, two orders of magnitude lower than the leghaemoglobin[1098,1099]), are homodimers (≈35kDa)[1080,1093]; a conserved cysteine residue (located in

the same E helix that the distal histidine) seems to be involved in the dimer formation in barley[1093,1100], as well as in the increased rate of haem iron reduction (contributing to lower the molecule auto-oxidation rate)[1101]. Class 2 NS-HG have an higher affinity for the distal histidine coordination ($K \approx 90$ [1087,1091]), but display similar rate constants towards dioxygen ($k_{on} \approx 50\text{-}80 \mu M^{-1}s^{-1}$ and $k_{off} \approx 0.4\text{-}1 s^{-1}$ [1087,1088]), resulting in global oxygen affinity two orders of magnitude lower than class 1 proteins. Nevertheless, similar to class 1, overexpression of class 2 NS-HG also increases the cell survival during hypoxia.[1088] In contrast, the third class of NS-HG is down-regulated during hypoxia.[1102,1103] Class 3 NS-HG forms a transient hexa-coordinated structure after reduction and deoxygenation (being slowly re-converted to a penta-coordinated state) and shares a high sequence similarity (40-45%) with "truncated" bacterial HG.[1095,1102-1111] Nevertheless much less is known about the class 3 NS-HG, as well as about class 2, and both will not be discussed here, unless where explicitly mentioned.

(1078) Bogusz, D.; Appleby, C.A.; Landsmann, J.; Dennis, E.S.; Trinick, M.J.; Peacock, W.J. *Nature* **1988**, *331*, 178.

(1079) Taylor, E.R.; Nie, X.Z.; MacGregor, A.W.; Hill, R.D. *Plant Mol. Biol.* **1994**, *24*, 853.

(1080) Duff, S.; Wittenberg, J.; Hill, R. *J. Biol. Chem.* **1997**, *272*, 16746.

(1081) Trevaskis, B.; Watts, R.A.; Andersson, C.R.; Llewellyn, D.J.; Hargrove, M.S.; Olson, J.S.; Dennis, E.S.; Peacock, W.J. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12230.

(1082) Arredondo-Peter, R.; Hargrove, M. S.; Moran, J.F.; Sarath, G.; Klucas, R.V. *Plant Physiol.* **1998**, *118*, 1121.

(1083) Hunt, P.W.; Watts, R.A.; Trevaskis, B.; Llewellyn, D.J.; Burnell, J.; Dennis, E.S.; Peacock, W.J. *Plant Mol. Biol.* **2001**, *47*, 677.

(1084) Weiland, T.R.; Kundu, S.; Trent 3rd, J.T.; Hoy, J.A.; Hargrove, M.S. *J. Am. Chem. Soc.* **2004**, *126*, 11930.

(1085) Garrocho-Villegas, V.; Gopalasubramaniam, S.K.; Arredondo-Peter, R. *Gene* **2007**, *398*, 78.

(1086) Nadra, A.D.; Martí, M.A.; Pesce, A.; Bolognesi, M.; Estrin, D.A. *Proteins* **2008**, *71*, 695.

(1087) Smagghe, B.; Hoy, J.; Percifield, R.; Kundu, S.; Hargrove, M.; Sarath, G.; Hilbert, J.; Watts, R.; Dennis, E.; Peacock, W.; Dewilde, S.; Moens, L.; Blouin, G.; Olson, J.; Appleby, C. *Biopolymers* **2009**, *91*, 1083.

(1088) Kakar, S.; Hoffman, F.G.; Storz, J.F.; Fabian, M.; Hargrove, M.S. *Biophys. Chem.* **2010**, *152*, 1.

(1089)Trent III, J.T.; Hvitved, A.N.; Hargrove, M.S. *Biochemistry* **2001**, *40*, 6155.

(1090) Smagghe, B.; Sarath, G.; Ross, E.; Hilbert, J.; Hargrove, M. *Biochemistry* **2006**, *45*, 561.

(1091) Bruno, S.; Faggiano, S.; Spyrakis, F.; Mozzarelli, A.; Abbruzzetti, S.; Grandi, E.; Viappiani, C.; Feis, A.; Mackowiak, S.; Smulevich, G.; Cacciatori, E.; Dominici, P. *J. Am. Chem. Soc.* **2007**, *129*, 2880.

(1092) Arredondo-Peter, R.; Hargrove, M.; Sarath, G.; Moran, J.; Lohrman, J.; Olson, J.; Klucas, R. *Plant Physiol.* **1997**, *115*, 1259.

(1093) Hargrove, M.S.; Brucker, E.A.; Stec, B.; Sarath, G.; Arredondo-Peter, R.; Klucas, R.V.; Olson, J.S.; Phillips Jr, G.N.J. *Structure* **2000**, *8*, 1005.

(1094) Kundu, S.; Premer, S.A.; Hoy, J.A.; Trent III, J.T.; Hargrove, M.S. *Biophys. J.* **2003**, *84*, 3931.

(1095) Couture, M.; Das, T.K.; Lee, H. C.; Peisach, J.; Rousseau, D.L.; Wittenberg, B.A.; Wittenberg, J.B.; Guertin, M. *J. Biol. Chem.* **1999**, *274*, 6898.

(1096) Couture, M.; Das, T.; Savard, P.; Ouellet, Y.; Wittenberg, J.; Wittenberg, B.; Rousseau, D.; Guertin, M. *Eur. J. Biochem.* **2000**, *267*, 4770.

(1097) Sowa, A.W.; Duff, S.M.G.; Guy, M.G.; Hill, R.D. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10317.

(1098) Hill, R.D. *Can. J. Bot.* **1998**, *76*, 707.

(1099) Hebelstrup, K.H.; Igamberdiev, A.U.; Hill, R.D. *Gene* **2007**, *398*, 86.

(1100) Bykova, N.V.; Igamberdiev, A.U.; Ens, W.; Hill, R.D. *Biochem. Biophys. Res. Commun.* **2006**, *347*, 301.

(1101) Smagghe, B.J.; Trent III, J.T.; Hargrove, M.S. *PLoS One* **2008**, *3*, e2039.

(1102) Watts, R.; Hunt, P.; Hvitved, A.; Hargrove, M.; Peacock, W.; Dennis, E. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 10119.

(1103) Vieweg, M.; Hohnjec, N.; Küster, H. *Planta* **2005**, *220*, 757.

(1104) Iwaasa, H.; Takagi, T.; Shikama, K.; *J. Mol. Biol.* **1989**, *208*, 355.

(1105) Iwaasa, H.; Takagi, T.; Shikama, K.; *J. Biol. Chem.* **1990**, *265*, 8603.

(1106) Potts, M.; Angeloni, S.; Ebel, R.; Bassam, D. *Science* **1992**, *256*, 1690.

(1107) Takagi, T.; Iwaasa, H.; Yuasa, H.; Shikama, K.; Takemasa, T.; Watanabe, Y. *Biochim. Biophys. Acta* **1993**, *1173*, 75.

(1108) Yamauchi, K.; Tada, H.; Usuki, I. *Biochim. Biophys. Acta* **1995**, *1264*, 53.

(1109) Couture, M.; Yeh, S.; Wittenberg, B.; Wittenberg, J.; Ouellet, Y.; Rousseau, D.; Guertin, M. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11223.

(1110) Pesce, A.; Couture, M.; Dewilde, S.; Guertin, M.; Yamauchi, K.; Ascenzi, P.; Moens, L..; Bolognesi, M. *EMBO J.* **2000**, *19*, 2424.

(1111) Perazzolli, M.; Dominici, P.; Romero-Puertas, MC.; Zago, E.; Zeier, J.; Sonoda, M.; Lamb, C.; Delledonne, M. *Plant Cell* **2004**, *16*, 2785.

(1112) Nie, X.; R. Hill, R. *Plant Physiol.* **1997**, *114*, 835.

(1113) Hunt, P.W.; Klok, E.J.; Trevaskis, B.; Watts, R.A.; Ellis, M.H.; Peacock, W.J.; Dennis, E.S. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 17197.

(1114) Dordas, C.; Hasinoff, B.; Igamberdiev, A.; Manac'h, N.; Rivoal, J.; Hill, R. *Plant J.* **2003**, *35*, 763.

(1115) Dordas, C., Rivoal, J.; Hill, R.D. Ann. Bot. **2003**, *91*, 173.

(1116) Igamberdiev, A.U.; Hill, R.D. *J. Exp. Bot.* **2004**, *55*, 2473.

(1117) Sturms, R.; DiSpirito, A.A.; Hargrove, M.S. *Biochemistry* **2011**, *50*, 3873.

(1118) Trent III, J.T.; Watts, R.A.; Hargrove, M.S. *J. Biol. Chem.* **2001**, *276*, 30106.

(1119) Trent III, J.T.; Hargrove, M.S. *J. Biol. Chem.* **2002**, *277*, 19538.

(1120) Igamberdiev, A.U.; Seregelyes, C.; Manac'h, N.; Hill, R.D. *Planta* **2004**, *219*, 95.

(1121) Seregelyes, C.; Igamberdiev, A.U.; Maassen, A.; Hennig, J.; Dudits, D. *FEBS Lett.* **2004**, *571*, 61.

(1122) Delledonne, M. *Curr. Opin. Plant Biol.* **2005**, *8*, 390.

(1123) Igamberdiev, A.U.; Baron, K.; Mana'H-Little, N.; Stoimenova, M.; Hill, R.D. *Ann. Bot.* **2005**, *96*, 557.

(1124) Ohwaki, Y.; Kawagishi-Kobayashi, M.; Wakasa, K.; Fujihara, S.; Yonemama, T. *Plant Cell Physiol.* **2005**, *46*, 324.

(1125) Shimoda, Y.; Nagata, M.; Suzuki, A.; Abe, M.; Sato, S; Kato, T.; Tabata, S.; Higashi, S.; Uchiumi, T. *Plant Cell Physiol.* **2005**, *46*, 99.

(1126) Igamberdiev, A.U.; Bykova, N.V.; Hill, R.D. *Planta* **2006**, *223*, 1033.

(1127) Igamberdiev, A.U.; Stoimenova, M.; Seregelyes, C.; Hill, R.D. *Planta* **2006**, *223*, 1041.

(1128) Perazzolli, M..; Romero-Puertas, M.C.; Delledonne M. *J. Exp. Bot.* **2006**, *57*, 479.

(1129) Sasakura, F.; Uchiumi, T.; Shimoda, Y.; Suzuki, A.; Takenouchi, K.; Higashi, S.; Abe, M. *Mol. Plant Microbe Interact.* **2006**, *19*, 441.

(1130) Igamberdiev, A.U.; Bykova, N.V.; Shah, J.K.; Hill, R.D. *Physiol. Plant.* **2010**, *138*, 393.

(1131) Wang, R.; Guegler, K.; LaBrie, S.T.; Crawford, N.M. *Plant Cell* **2000**, *12*, 1491.

(1132) Stohr, C.; Strube, F.; Marx, G.; Ullrich, W.R.; Rockel, P. *Planta* **2001**, *212*, 835.

(1133) Meyer, C.; Stohr, C. *Photosynthetic nitrogen assimilation and associated carbon and respiratory metabolism*; Kluwer Academic Publishers: Dordecht, 2002; p49.

(1134) Stohr, C.; Stremlau, S. *J. Exp. Bot.* **2006**, *57*, 463.

(1135) Ward, M.R.; Tischner, R.; Huffaker, R.C. *Plant Physiol.* **1988**, *88*, 1144.

(1136) Ward, M.R.; Grimes, H.D.; Huffaker, R.C. *Planta* **1989**, *177*, 470.

(1137) Meyerhoff, P.A.; Fox, T.C.; Travis, R.L.; Huffaker, R.C. *Plant Physiol.* **1994**, *104*, 925.

(1138) Stohr, C.; Ullrich, W.R. *Planta* **1997**, *203*, 129.

(1139) Stohr, C. *Nitric oxide in plant growth, development and stress physiology*; Springer: Heidelberg, 2007; p15.

(1140) Moche, M.; Stremlau, S.; Hecht, L.; Göbel, C.; Feussner, I.; Stöhr, C. *Planta* **2010**, *231*, 425.

(1141) Malavolta, E *Plant Physiol.* **1954**, *29*, 98.

(1142) Trought, M.C.T.; Drew, M.C. *J. Exp. Bot.* **1981**, *32*, 509.

(1143) Prioul, J.L.; Guyot, C. *Physiol. Veg.* **1985**, *23*, 175.

(1144) Horchani, F.; Aschi-Smiti, S.; Brouquisse, R. *Acta Physiol. Plant* **2010**, *32*, 1113.

(1145) Sturms, R.; DiSpirito, A.A.; Fulton, D.B.; Hargrove, M.S. *Biochemistry* **2011**, *50*, 10829.

(1146) Colter, J.S.; Quastel, J.H. *Arch. Biochem.* **1950**, *27*, 368.

(1147) Bazylinski, D.A.; Arkowitz, R.A.; Hollocher, T.C. *Arch. Biochem. Biophys.* **1987**, *259*, 520.

(1148) Eick, M.; Stöhr, C. *Protoplasma* **2012**, *249*, 909.

(1149) Stohr, C.; Ullrich, W.R. *J. Exp. Bot.* **2002**, *53*, 2293.

(1150) Bethke, P.C.; Badger, M.R.; Jones, R.L. *Plant Cell* **2004**, *16*, 332.

(1151) Drozdowicz, Y.M.; Jones, R.L. *Plant Physiol.* **1995**, *108*, 769.

(1152) Yu, Q.; Tang, C.; Kuo, J. *Plant Soil* **2000**, *219*, 29.

(1153) Fasano, J.M.; Swanson, S.J.; Blancaflor, E.B.; Dowd, P.E.; Kao, T.H.; Gilroy, S. *Plant Cell* **2001**, *13*, 907.

(1154) Horemans, N.; Foyer, C.H.; Asard, H. *Trends Plant Sci.* **2000**, *5*, 263.

(1155) Modolo, L.V.; Augusto, O.; Almeida, I.M.G.; Magalhaes, J.R.; Salgado, I. *FEBS Lett.* **2005**, *579*, 3814.

(1156) Jasid, S.; Simontacchi, M.; Bartoli, C.G.; Puntarulo, S. *Plant Physiol.* **2006**, *142*, 1246.

(1157) del Rio, L. A. *Arch. Biochem. Biophys.* **2011**, *506*, 1.

(1158) Tewari, R.K.; Prommer, J.; Watanabe, M. *Plant Cell Rep.* **2013**, *32*, 31.

(1159) Roman, R.; Dunford, H.B. *Can. J. Chem.* **1973**, *51*, 588.

(1160) Patel, P.K.; Mondal, M.S.; Modi, S.; Behere, D.V. *Biochim. Biophys. Acta* **1997**, *1339*, 79.

(1161) Gebicka, L. *Acta Biochim. Pol.* **1999**, *46*, 919.

(1162) Lehnig, M. *Arch. Biochem. Biophys.* **2001**, *393*, 245.

(1163) Sakamoto, A.; Sakurao, S.H.; Fukunaga, K.; Matsubara, T.; Ueda-Hashimoto, M.; Tsukamoto, S.; Takahashi, M.; Morikawa, H. *FEBS Lett.* **2004**, *572*, 27.

(1164) Bloom, A.J.; Burger, M.; Asensio, J.S.R.; Cousins, A.B. *Science* **2010**, *328*, 899.

(1165) Bloom, A.J.; Caldwell, R.M.; Finazzo, J.; Warner, R.L.; Weissbart, J. *Plant Physiol.* **1989**, *91*, 352.

(1166) Smart, D.R.; Ritchie, K.; Bloom, A.J.; Bugbee, B.B. *Plant Cell Environ.* **1998**, *21*, 753.

(1167) Bloom, A.J.; Smart, D.R.; Nguyen, D.T.; Searles, P.S. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1730.

(1168) Searles, P.S.; Bloom, A.J. *Plant Cell Environ.* **2003**, *26*, 1247.

(1169) Rachmilevitch, S.; Cousins, A.B.; Bloom, A.J. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11506.

(1170) Bloom, A.J. *Ecology in Agriculture*; Academic Press: San Diego, 1997; p145.

(1171) Benamar, A.; Rolletschek, H.; Borisjuk, L.; Avelange-Macherel, M.-H.; Curien, G.; Mostefai, H.A.; Andriantsitohaina, R.; Macherel, D. *Biochim. Biophys. Acta* **2008**, *1777*, 1268.

(1172) Gupta, K.J.; Zabalza, A.; van Dongen, J.T. *Physiol. Plant.* **2009**, *137*, 383.

(1173) Zabalza, A.; van Dongen, J.T.; Froehlich, A.; Oliver, S.N.; Faix, B.; Gupta, K.J.; Schmalzlin, E.; Igal, M.; Orcaray, L.; Royuela, M.; Geigenberger, P. *Plant Physiol.* **2009**, *149*, 1087.

(1174) Buddha, M.R.; Tao, T.; Parry, R.J.; Crane, B.R. *J. Biol. Chem.* **2004**, *279*, 49567.

(1175) Kers, J.A.; Wach, M.J.; Krasnoff, S.B.; Widom, J.; Cameron, K.D.; Bukhalid, R.A.; Gibson, D.M.; Crane, B.R.; Loria, R. *Nature* **2004**, *429*, 79.

(1176) Wach, M.J.; Kers, J.A.; Krasnoff, S.B.; Loria, R.; Gibson DM. *Nitric Oxide* **2005**, *12*, 46.

(1177) Johnson, E.G.; Sparks, J.P.; Dzikovski, B.; Crane, B.R.; Gibson, D.M.; Loria, R. *Chem. Biol.* **2008**, *15*, 43.

(1178) Patel, B.A.; Moreau, M.; Widom, J.; Chen, H.; Yin, L.; Hua, Y.; Crane, B.R. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 18183.

(1179) Marshall, H.E.; Merchant, K.; Stamler, J.S. *FASEB J.* **2000**, *14*, 1889.

(1180) Nakano, M.M. *J. Bacteriol.* **2002**, *184*, 1783.

(1181) Mukhopadhyay, P.; Zheng, M.; Bedzyk, L.A.; LaRossa, R.A.; Storz, G. *Proc. Natl. Acad. Sci.*

USA **2004**, *101*, 745.

(1182) Gusarov, I.; Nudler, E. *Proc. Natl. Acad. Sci, USA* **2005**, *102*, 13855.

(1183) Hochgrafe F.; Wolf C.; Fuchs S.; Liebeke M.; Lalk M.; Engelmann, S.; Hecker, M. *J. Bacteriol.* **2008**, *190*, 4997.

(1184) Shatalin K.; Gusarov I.; Avetissova E.; Shatalina Y.; McQuade LE.; Lippard, S.J.; Nudler, E. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 1009.

(1185) Gusarov, I.; Shatalin, K.; Starodubtseva, M.; Nudler, E. *Science* **2009**, *325*, 1380.

(1186) Chen, Y.; Rosazza, J.P. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 1251.

(1187) Chen, Y.J.; Rosazza, J.P.N. *J. Bacteriol.* **1995**, *177*, 5122.

(1188) Stachura, J.; Konturek, J.W.; Karczewska, A.; Domschke, W.; Popiela, T.; Konturek, S.J. *J. Physiol. Pharmacol.* **1996**, *47*, 131.

(1189) Choi, W.S.; Chang, M.S.; Han, J.W.; Hong, S.Y.; Lee, H.W. *Biochem. Biophys. Res. Commun.* **1997**, *237*, 554.

(1190) Adak, S.; Aulak, K.; Stuehr, D.J. *J. Biol. Chem.* **2002**, *277*, 16167.

(1191) Adak, S.; Bilwes, A.M.; Panda, K.; Hosfield, D.; Aulak, K.S.; McDonald, J.F.; Tainer, J.A.; Getzoff, E.D.; Crane, B.R.; Stuehr, D.J. *Proc. Natl Acad. Sci. USA* **2002**, *99*, 107.

(1192) Bird, L.E.; Ren, J.; Zhang, J.; Foxwell, N.; Hawkins, A.R.; Charles, I.G.; Stammers, D.K. *Structure* **2002**, *10*, 168.

(1193) Pant, K.; Bilwes, A.M.; Adak, S.; Stuehr, D.J.; Crane, B.R. *Biochemistry* **2002**, *41*, 11071.

(1194) Sudhamsu, J.; Crane, B.R. *J. Biol. Chem.* **2006**, *281*, 9623.

(1195) Crane, B.R. *Biochem. Soc. Trans.* **2008**, *36*, 1149.

(1196) Gusarov, I.; Starodubtseva, M.; Wang, Z.; McQuade, L.; Lippard, S.J.; Stuehr, D.J.; Nudler, E. *J. Biol. Chem.* **2008**, *283*, 13140.

(1197) Wang, Z.Q.; Lawson, R.J.; Buddha, M.R.; Wei, C.C.; Crane, B.R.; Munro, A.W.; Stuehr, D.J. *J. Biol. Chem.* **2007**, *282*, 2196.

(1198) Schneiker, S.; Perlova, O.; Kaiser, O.; Gerth, K.; Alici, A.; Altmeyer, M.O.; Bartels, D.; Bekel, T.; Beyer, S.; Bode, E.; Bode, H.B.; Bolten, C.J.; Choudhuri, J.V.; Doss, S.; Elnakady, Y.A.; Frank, B.; Gaigalat, L.; Goesmann, A.; Groeger, C.; Gross, F.; Jelsbak, L.; Jelsbak, L.; Kalinowski, J.; Kegler, C.; Knauber, T.; Konietzny, S.; Kopp, M.; Krause, L.; Krug, D.; Linke, B.; Mahmud, T.; Martinez-Arias, R.; McHardy, A.C.; Merai, M.; Meyer, F.; Mormann, S.; Muñoz-Dorado, J.; Perez, J.; Pradella, S.; Rachid, S.; Raddatz, G.; Rosenau, F.; Rückert, C.; Sasse, F.; Scharfe, M.; Schuster, S.C.; Suen, G.; Treuner-Lange, A.; Velicer, G.J.; Vorhölter, F.J.; Weissman, K.J.; Welch, R.D.; Wenzel, S.C.; Whitworth, D.E.; Wilhelm, S.; Wittmann, C.; Blöcker, H.; Pühler, A.; Müller, R. *Nat. Biotechnol.* **2007**, *25.*, 1281.

(1199) Agapie, T.; Suseno, S.; Woodward, J.J.; Stoll, S.; Britt, R.D.; Marletta, M.A. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 16221.

(1200) Reece, S.Y.; Woodward, J,J.; Marletta M.A. *Biochemistry* **2009**, *48*, 5483.

(1201) Sudhamsu, J.; Crane, B.R. Trends Microbiol. **2009**, *17*, 212.

(1202) Smith, M.S. *Appl. Environ. Microbiol.* **1983**, *45*, 1545.

(1203) Calmels, S.; Ohshima, H.; Bartsch, H. *J. Gen. Microbiol.* **1988**, *134*, 221.

(1204) Ji, X.B.; Hollocher, T.C. *Appl. Environ. Microbiol.* **1988**, *54*, 1791.
(1205) Ji, X.B.; Hollocher, T.C. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 106.
(1206) Metheringham, R.; Cole, J.A. *Microbiology* **1997**, *143*, 2647.
(1207) Corker, H.; Poole, R.K. *J. Biol. Chem.* **2003**, *278*, 31584.
(1208) Tang, X.; Chen, J.; Wang, W.-H.; Liu, T.-W.; Zhang, J.; Gao, Y.H.; Pei, Z.-M.; Zheng, H.-L. *Environ. Pollut.* **2011**, *159*, 3784.
(1209) Weiss, B. J. Bacteriol. **2006**, *188*, 829.
(1210) Coleman, K.J.; Cornish-Bowden, A.; Cole, J.A. *Biochem. J.* **1978**, *175*, 483.
(1211) Vine, C.E.; Cole, J.A. *Biochem. Soc. Trans.* **2011**, *39*, 213.
(1212) Vine, C.E.; Cole, J.A. *FEMS Microbiol. Lett.* **2011**, *325*, 99.
(1213) Smith, M.S.; Zlmmerman, K. *Soil Sci. Soc. Am. J.* **1981**, *45*, 865.
(1214) Bleakley, B.H.; Tiedje, J.M. *Appl. Environ. Microbiol.* **1982**, *44*, 1342.
(1215) Smith, M.S. *Appl. Environ. Microbiol.* **1982**, *43*, 854.
(1216) Satoh, T.; Hom, S.S.; Shanmugam, K.T. *J. Bacteriol.* **1983**, *155*, 454.
(1217) Ralt, D.; Wishnok, J.S.; Fitts, R.; Tannenbaum, S.R. *J. Bacteriol.* **1988**, *170*, 359.
(1218) Gilberthorpe, N.J.; Poole, R.K. *J. Biol. Chem.* **2008**, *283*, 11146.
(1219) Vine, C.E.; Purewal, S.K.; Cole, J.A. *FEMS Microbiol. Lett.* **2011**, *325*, 108.
(1220) Rowley, G.; Hensen, D.; Felgate, H.; Arkenberg, A.; Appia-Ayme, C.; Prior, K.; Harrington, C.; Field, S.J.; Butt, J.N.; Baggs, E.; Richardson, D.J. *Biochem. J.* **2012**, *441*, 755.
(1221) The diversion of up to 20% of nitrate, in *S. enterica*, results in the formation of a huge concentration of NO, which is then converted into nitrous oxide and released (this bacterium can not further reduce the nitrous oxide). Given the cytotoxicity of NO, why is the bacterium producing it in such amounts? Is it to generate nitrous oxide? With what purposes? As previously pointed out[1220], the physiological relevance of the nitrous oxide production by pathogenic bacteria (that have truncated denitrification pathways) must be further investigated.
(1222) Couture, M.; Das, T., Savard, P.; Ouellet, Y.; Wittenberg, J.; Wittenberg, B.; Rousseau, D.; Guertin, M. *Eur. J. Biochem.* **2000**, *267*, 4770.
(1223) Scott, N.; Lecomte, J. *Protein Sci.* **2000**, *9*, 587.
(1224) Hvitved, A.N.; Trent 3rd, J.T.; Premer, S.A.; Hargrove, M.S. *J. Biol. Chem.* **2001**, *276*, 34714.
(1225) Vu, B..; Jones, A.; Lecomte, J. *J. Am. Chem. Soc.* **2002**, *124*, 8544.
(1226) Hoy, J.; Kundu, S.; Trent, J.T.; Ramaswamy, S.; Hargrove, M. *J. Biol. Chem.* **2004**, *279*, 16535.
(1227) Forrester, M.T.; Foster, M.W. *Free Radic. Biol. Med.* **2012**, *52*, 1620.
(1228) Gardner, P.R.; Gardner, A.M.; Martin, L.A.; Salzman, A.L. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10378.
(1229) Scott, N.L.; Xu, Y.; Shen, G.; Vuletich, D.A.; Falzone, C.J.; Li, Z.; Ludwig, M.; Pond, M.P.; Preimesberger, M.R.; Bryant, D.A.; Lecomte, J.T. Biochemistry **2010**, *49*, 7000.
(1230) Moura, J.J.G.; Xavier, A.V.; Bruschi, M.; Le Gall, J.; Hall, D.O.; Cammack, R. *Biochem. Biophys. Res. Commun.* **1976**, *72*, 782.

(1231) Barata, B.A.; LeGall, J.; Moura, J.J. *Biochemistry* **1993**, *32*, 11559.
(1232) Krippahl, L., Palma, N.; Moura, I.; Moura, J.J.G. *Eur. J. Inorg. Chem.* **2006**, *19*, 3835.
(1233) Kaim, W.; Schwederski, B. *Bioinorganic chemistry: inorganic elements in the chemistry of life*; Wiley: Chichester, 1996.
(1234) Poulos, T.L. *J. Biol. Inorg. Chem.* **1996**, *1*, 356.
(1235) Sigfridsson, E.; Olsson, M.H.M.; Ryde, U. *J. Phys. Chem. B* **2001**, *105*, 5546.
(1236) Moura, I.; Pauleta, S.R.; Moura, J.J.G. *J. Biol. Inorg. Chem.* **2008**, *13*, 1185.
(1237) Dias, J.M.; Alves, T.; Bonifacio, C.; Pereira, A.S.; Trincao, J.; Bourgeois, D.; Moura, I.; Romao, M.J. *Structure* **2004**, *12*, 961.
(1238) Echalier, A.; Goodhew, C.F.; Pettigrew, G.W.; Fulop, V. *Structure* **2006**, *14*, 107.
(1239) Echalier, A.; Brittain, T.; Wright, J.; Boycheva, S.; Mortuza, G.B.; Fulop, V.; Watmough, N.J. *Biochemistry* **2008**, *47*, 1947.
(1240) Li, H. *Handbook of metalloproteins*; Wiley: Chichester, 2001; p 486.
(1241) Rosenfeld, A. *Handbook of metalloproteins*; Wiley: Chichester, 2001; p 285.
(1242) Gajhede, M. *Handbook of metalloproteins*; Wiley: Chichester, 2001; p 195.
(1243) Mate, M.J.; Bravo, J.; Fita, I.; Murshudov, G.; Melik-Adamyan, W.; Loewen, P.C. *Handbook of metalloproteins*; Wiley: Chichester, 2001; p 486.
(1244) Malkin, R.; Malmstrom, B. G. *Adv. Enzymol.* **1970**, *33*, 177.
(1245) Vanngard, T. *Biological Applications of Electron Spin Resonance*; Wiley-Interscience: New York, 1972; p 411.
(1246) Reinhammar, B. *Copper Proteins and Copper Enzymes* (Vol. III); CRC Press: Boca Raton, 1984; p 1.
(1247) Solomon, E. I.; Baldwin, M. J.; Lowery, M. D. *Chem. Rev.* **1992**, *92*, 521.
(1248) Allendorf, M. D.; Spira, D. J.; Solomon, E. I. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3063.
(1249) Spira-Solomon, D. J.; Allendorf, M. D.; Solomon, E. I. *J. Am. Chem. Soc.* **1986**, *108*, 5318.
(1250) Messerschmidt, A.; Ladenstein, R.; Huber, R.; Bolognesi, M.; Avigliano, L.; Petruzzelli, R.; Rossi, A.; Finazzi-Agro, A. *J. Mol. Biol.* **1992**, *224*, 179.
(1251) Antholine, W. E.; Kastrau, D. H. W.; Steffens, G. C. M.; Buse, G.; Zumft, W. G.; Kroneck, P. M. H. *Eur. J. Biochem.* **1992**, *209*, 875.
(1252) Kroneck, P. M. H.; Antholine, W. E.; Koteich, H.; Kastrau, D. H. W.; Neese, F.; Zumft, W. G. *Bioinorganic Chemistry of Copper*; Chapman & Hall: New York, 1993.
(1253) Tsukihara, T.; Aoyama, H.; Yamashita, E.; Tomizaki, T.; Yamaguchi, H.; Shinwaza-Itoh, K.; Nakashima, R.; Yaono, R.; Yoshikawa, S. *Science* **1995**, *269*, 1069.
(1254) Iwata, S.; Ostermeier, C.; Ludwig, B.; Michel, H. *Nature* **1995**, *376*, 660.
(1255) Tsukihara, T.; Aoyama, H.; Yamashita, E.; Tomizaki, T.; Yamaguchi, H.; Shinwaza-Itoh, K.; Nakashima, R.; Yaono, R.; Yoshikawa, S. *Science* **1996**, *272*, 1136.
(1256) Malmstrom, B. G. *Chem. Rev.* **1990**, *90*, 1247.
(1257) Farrar, J. A.; Thomson, A. J.; Cheesman, M. R.; Dooley, D. M.; Zumft, W. G. *FEBS Lett.* **1991**, *294*, 11.

(1258) Brown, K.; Tegoni, M.; Prudêncio, M.; Pereira A.S.; Besson, S.; Moura, J.J.; Moura, I.; Cambillau, C. *Nat. Struct. Biol*. **2000**, *7*, 191.

(1259) Brown, K.; Djinovic-Carugo, K.; Haltia, T.; Cabrito, I.; Saraste, M.; Moura, J.J.; Moura, I.; Tegoni, M.; Cambillau, C. *J. Biol. Chem.* **2000**, *275*, 41133.

(1260) Pomowski. A.; Zumft, W.G.; Kroneck, P.M.; Einsle, O. *Nature* **2011**,*477*, 234.

(1261) Hille, R. *Trends Biochem. Sci.* **2002**, *27*, 360.

(1262) Brondino, C.D.; Rivas M.G.; Romão, M.J.; Moura, J.J.G.; Moura, I. *Acc. Chem. Res.* **2006**, *39*, 788.

(1263) Schwarz, G.; Mendel, R.R.; Ribbe, M.W. *Nature* **2009**, *460*, 839.

(1264) Hille, R.; Mendel, R. *Coord. Chem. Rev.* **2011**, *255*, 991.

(1265) Mendel, R.; Kruse, T. *Biochim. Biophys. Acta* **2012**, *1823*, 1568.

(1266) Hille, R. *Dalton Trans.* **2013**, *42*, 3029.

(1267) George, G.N.; Pickering, I.J.; Yu, E.Y.; Prince, R.C.; Bursakov, S.A; Gavel, O.Y.; Moura, I.; Moura, J.J.G. *J. Am. Chem. Soc.* **2000**, *122*, 8321.

(1268) Bursakov, S.A.; Gavel, O.Y.; Di Rocco, G.; Lampreia, J.; Calvete, J.; Pereira, A.S.; Moura, J.J.; Moura, I. *J. Inorg. Biochem.* **2004**, *98*, 833.

(1269) Rivas, M.G.; Carepo, M.S.; Mota, C.S.; Korbas, M.; Durand, M.C.; Lopes, A.T.; Brondino, C.D.; Pereira, A.S.; George, G.N.; Dolla, A.; Moura, J.J.; Moura, I. *Biochemistry* **2009**, *48*, 873.

(1270) Havemeyer, A.; Bittner, F.; Wollers, S.; Mendel, R.; Kunze, T.; Clement, B. *J. Biol. Chem.* **2006**, *281*, 34796.

(1271) Klein, J.M.; Busch, J.D.; Potting, C.; Baker, M.J.; Langer, T.; Schwarz, G. *J. Biol. Chem.* **2012**, *287*, 42795.

(1272) Plitzko, B.; Ott, G.; Reichmann, D.; Henderson, C.J.; Wolf, C.R.; Mendel, R.; Bittner, F.; Clement, B.; Havemeyer, A. *J. Biol. Chem.* **2013**, *288*, 20228.

(1273) The term *promiscuity* will be here used to describe ***all*** the reactions that are not the function *commonly attributed* to the enzyme (*i.e.*, the well known function, described on textbooks). The definition proposed by other authors (*e.g.*, in [740]), that distinguishes between promiscuity, broad-specificity and substrate ambiguity, or between function for which an enzyme evolved and non-physiological functions, will not be here used.

(1274) Summers, D.P.; Chang, S. *Nature* **1993**, *365*, 630.

(1275) NrfA after the name of the encoding gene *nrf*: *nitrite reduction with formate*.

(1276) Einsle, O.; Stach, P.; Messerschmidt, A.; Simon, J.; Kroger, A.; Huber, R.; Kroneck, P. M. H. *J. Biol. Chem.* **2000**, *275*, 39608.

(1277) Simon, J.; Gross, R.; Einsle, O.; Kroneck, P.M.H.; Kroger, A.; Klimmek, O. *Mol. Microbiol.* **2000**, *35*, 686.

(1278) Almeida, M.G.; Macieira, S.; Gonçalves, L.; Huber, R.; Cunha, C.A.; Romão, M.J.; Costa, C.; Lampreia, J.; Moura, J.J.G.; Moura, I. *Eur. J. Biochem.* **2003**, *270*, 3904.

(1279) Rodrigues, M. L.; Oliveira, T. F.; Pereira, I. A. C.; Archer, M. *EMBO J.* **2006**, *25*, 5951.

(1280) Todorovic, S.; Rodrigues, M.L.; Matos, D.; Pereira, I.A.C. *J. Phys. Chem. B* **2012**, *116*, 5637.

(1281) Simon, J.; Pisa, R.; Stein, T.; Eichler, R.; Klimmek, O.; Gross, R. *Eur. J. Biochem.* **2001**, *268*, 5776.

(1282) Kern, M.; Einsle, O.; Simon, J. *Biochem. J.* ***2008***, *414*, 73.

(1283) Simon, J.; Kern, M. *Biochem. Soc. Trans.* **2008**, *36*, 1011.

(1284) Berks, B.C.; Ferguson, S.J.; Moir, J.W.B.; Richardson, D.J. *Biochim. Biophys. Acta* **1995**, *1232*, 97.

(1285) Bamford, V.A.; Angove, H.C.; Seward, H.E.; Thomson, A.J.; Cole, J.C.; Butt, J.N.; Hemmings, A.M.; Richardson, D.J. *Biochemistry* **2002**, *41*, 2921.

(1286) Clarke, T.; Cole, J. A.; Richardson, D. J.; Hemmings, A. M. *Biochem. J.* **2007**, *406*, 19.

(1287) Lockwood, C.; Butt, J.N.; Clarke, T.A.; Richardson, D.J. *Biochem. Soc. trans.* **2011**, *39*, 263.

(1288) Einsle, O.; Messerschmidt, A.; Stach, P.; Bourenkov, G.P.; Bartunik, H.D.; Huber, R.; Kroneck, P.M. *Nature* **1999**, *400*, 476.

(1289) Lampreia, J.; Moura, J.J.G.; Moura, I.; Romão, M.J. *Acta Crystallogr D* **2000**, *56*, 215.

(1290) Cunha, C.A.; Macieira, S.; Dias, J.M.; Almeida, G.; Goncalves, L.L.; Costa, C.; Lampreia, J.; Huber, R.; Moura, J.J.G.; Moura, I.; Romão, M.J. *J. Biol. Chem.* **2003**, *278*, 17455.

(1291) Pereira, I.A.; LeGall. J.; Xavier, A.V.; Teixeira, M. *Biochim. Biophys. Acta* **2000**, *1481*, 119.

(1292) Rodrigues, M.L.; Oliveira, T.; Matias, P.M.; Martins, C.; Valente, F.M.; Pereira, I.A.; Archer, M. Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun. **2006**, *62*, 565.

(1293) Youngblut, M.; Judd, E.T.; Srajer, V.; Sayyed, B.;Goelzer, T.; Elliott, S.J.; Schmidt, M.; Pacheco, A.A. *J. Biol. Inorg. Chem.* **2012**, *17*, 647.

(1294) Burlat, B.; Gwyer, J.D.; Poock, S.; Clarke, T.; Cole, J.A.; Hemmings, A.M.; Cheesman, M.R.; Butt, J.N.; Richardson, D.J. *Biochem. Soc. Trans.* **2005**, *33*, 137.

(1295) Costa, C.; Moura, J.J.G.; Moura, I.; Liu, M.Y.; Peck, H.D.; LeGall, J.; Wang, Y.; Huynh, B.H. *J. Biol. Chem.* **1990**, *265*,14382.

(1296) Costa, C.; Moura, J.J.G.; Moura, I.; Wang, Y.; Huynh, B.H. *J. Biol. Chem.* **1996**, *271*, 23191.

(1297) Stach, P.; Einsle, O.; Schumacher, W.; Kurun, E.; Kroneck, P.M.H. *J. Inorg. Biochem.* **2000**, *79*, 381.

(1298) Clarke, T.A.; Kemp, G.L.; Van Wonderen, J.H.; Doyle, R.A.S.; Cole, J.A.; Tovell, N.; Cheesman, M.R.; Butt, J.N.; Richardson, D.J.; Hemmings A.H. *Biochemistry* **2008**, *47*, 3789.

(1299) Bykov, D.; Neese, F. *J. Biol. Inorg. Chem.* **2012**, *17*, 741.

(1300) Tikhonova, T. V.; Slutsky, A.; Antipov, A. N.; Boyko, K. M.; Polyakov, K. M.; Sorokin, D. Y.; Zvyagilskaya, R.A.; Popov, V.O. *Biochim. Biophys. Acta* **2006**, *1764*, 715.

(1301) Polyakov, K.M.; Boyko, K. M.; Tikhonova, T.V.; Slutsky, A.; Antipov, A.N.; Zvyagilskaya, R.A.; Popov, A.N.; Bourenkov, G.P.; Lamzin, V.S.; Popov, V.O. *J. Mol. Biol.* **2009**, *389*, 846.

(1302) Tikhonova, T. V.; Slutskaya, E. S.; Filimonenkov, A. A.; Boyko, K. M.; Kleimenov, S. Y.; Konarev, P. V.; Polyakov, K.M.; Svergun, D.I.; Trofimov, A.A.; Khomenkov, V.G.; Zvyagilskaya, R.A.; Popov, V.O. *Biochemistry (Moscow)* **2008**, *73*, 164.

(1303) Tikhonova, T.; Tikhonov, A.; Trofimov, A.; Polyakov, K.; Boyko, K.; Cherkashin, E.; Rakitina, T.; Sorokin, D.; Popov, V. *FEBS J.* **2012**, *279*, 4052.

(1304) Kajie, S.; Anraku, Y. *Eur. J. Biochem.* **1986**, *154*, 457.

(1305) Costa, C.; Macedo, A.; Moura, I.; Moura, J.J.G.; Le Gall, J.; Berlier, Y.; Liu, M.Y.; Payne, W.J. *FEBS Lett.* **1990**, *276*, 67.

(1306) Rudolf, M.; Einsle, O.; Neese, F.; Kroneck, P.M.H. *Biochem. Soc. Trans.* **2002**, *30*, 649.

(1307) van Wonderen, J.H.; Burlat, B.; Richardson, D.J; Cheesman, M.R; Butt, J.N. *J. Biol. Chem.* **2008**, *283*, 9587.

(1308) Silveira, C.M.; Besson, S.; Moura, I.; Moura, J.J.G.; Almeida, M.G. *Bioinorg. Chem. Appl.* **2010** (http://dx.doi.org/10.1155/2010/634597).

(1309) Payne, W.J.; Grant, M.A.; Shapleigh, J.; Hoffman, P. *J. Bacteriol.* **1982**, *152*, 915.

(1310) Poock, S. R.; Leach, E. R.; Moir, J. W.; Cole, J. A.; Richardson, D. J. *J. Biol. Chem.* **2002**, *277*, 23664.

(1311) In this context, it is noteworthy that *W. succinogenes* have no a catalase or HG. To survive when under stress induced by those reactive oxygen and nitrogen species, the *W. succinogenes* relies on CcNiR, cytochrome *c* peroxidase (another periplasmatic multi-*c*-haems-containing protein) and on the cytoplasmatic flavodiiron protein.[1312] So, after all the proteins described in sections 3., CcNiR (including the octa-haemic enzyme[1300]) is another haemic protein with a "parallel" activity on the hydrogen peroxide and NO metabolisms.

(1312) Kern, M.; Volz, J.; Simon, J. *Environ. Microbiol.* **2011**, *13*, 2478.

(1313) Poole, R.K. *Biochem. Soc. Trans.* **2005**, *33*, 176.

(1314) Mills, P.C.; Rowley, G.; Spiro, S.; Hinton, J.C.D., Richardson, D.J. *Microbiology* **2008**, *154*, 1218.

(1315) Vine, C.E.; Cole, J.A. *Biochem. Soc. Trans.* **2011**, *39*, 213.

(1316) Simon, J.; Kern, M.; Hermann, B.; Einsle, O.; Butt, J. N. *Biochem. Soc. Trans.* **2011**, *39*, 1864.

(1317) Liu, M.C.; Peck Jr., H. D. *J. Biol. Chem.* **1981**, *256*, 13159.

(1318) Pereira, I. C.; Abreu, I. A.; Xavier, A. V.; LeGall, J.; Teixeira, M. *Biochem. Biophys. Res. Commun.* **1996**, *224*, 611.

(1319) Clarke, T. A.; Hemmings, A. M.; Burlat, B.; Butt, J. N.; Cole, J. A.; Richardson, D. J. *Biochem. Soc. Trans.* **2006**, *34*, 143.

(1320) Lukat, P.; Rudolf, M.; Stach, P.; Messerschmidt, A.; Kroneck, P. M. H.; Simon, J.; Einsle, O. *Biochemistry* **2008**, *47*, 2080.

(1321) Klotz, M.G.; Schmid, M.C.; Strous, M.; op den Camp, H.J.M; Jetten, M.S.M.; Hooper, A.B. *Environ. Microbiol.* **2008**, *10*, 3150.

(1322) Kemp, G.L.; Clarke, T-H.; Marritt, S.J.; Lockwood, C.; Poock, S.R; Hemmings, A.M.; Richardson, D.J; Cheesman, M.R.; Butt, J.N. *Biochem. J.* **2010**, *431*, 73.

(1323) Eaves, D.J.; Grove, J.; Staudenmann, W.; James, P.; Poole, R.K.; White, S.A.; Griffiths, I.; Cole, J.A. *Mol. Microbiol.* **1998**, *28*, 205.

(1324) Pisa, R.; Stein, T.; Eichler, R.; Gross, R.; Simon, J. *Mol. Microbiol.* **2002**, *43*, 763.

(1325) Hartshorne, S.; Richardson, D.J.; Simon, J. *Biochem. Soc. Trans.* **2006**, *34*, 146.

(1326) Ferguson, S.J.; Stevens, J.M.; Allen, J.W.A.; Robertson, I.B. *Biochim. Biophys. Acta* **2008**, *1777*, 980.

(1327) Einsle, O.; Messerschmidt, A.; Huber, R.; Kroneck, P.M.H.; Neese, F. *J. Am. Chem. Soc.* **2002**, *124*, 11737.

(1328) Bykov, D.; Neese, F. *J. Biol.Inorg. Chem.* **2011**, *16*, 417.

(1329) Ranghino, G.; Scorza, E.; Sjogren, T.; Williams, P. A.; Ricci, M.; Hajdu, J. *Biochemistry* **2000**, *39*, 10958.

(1330) Nasri, H.; Ellison, M.K.; Krebs, C.; Huynh, B.H.; Scheidt, W.R. *J. Am. Chem. Soc.* **2000**, *122*, 10795.

(1331) Nasri, H.; Ellison, M.K.; Shang, M.; Schulz, C.E.; Scheidt, W.R. *Inorg. Chem.* **2004**, *43*, 2932.

(1332) Martins, G.; Rodrigues, L.; Cunha, F.M.; Matos, D.; Hildebrandt, P.; Murgida, D.H.; Pereira, I.A.C.; Todorovic, S. *J Phys Chem B* **2010**, *114*, 5563.

(1333) Einsle, O. *Methods Enzymol.* **2011**, *496*, 399.

(1334) Nasri, H.; Wang, Y.; Huynh, B.H.; Walker, F.A.; Scheidt, W.R.; *Inorg. Chem.* **1991**, *30*, 1483.

(1335) Wyllie, G.R.A.; Sheidt W.R. *Chem. Rev.* **2002**, *102*, 1067.

(1336) Markley, J.L. *Acc. Chem. Res.* **1975**, *8*, 70.

(1337) Almeida, M.G.; Silveira, C.M.; Guigliarelli, B.; Bertrand, P.; Moura, J.J.G.; Moura, I.; Leger, C. *FEBS Lett* **2007**, *581*, 284.

(1338) Because the charge distributions in metal-NO adducts are frequently ambiguous, the Enemark and Feltham notation is adopted, where the superscript indicates the number of metal *d* electrons plus the number of NO π-anti-bonding electrons.[1339]

(1339) Enemark, J. H.; Feltham, R. D. *Coord. Chem. Rev.* **1974**, *13*, 339.

(1340) Averill, B.A. Chem. Rev. **1996**, *96*, 2951.

(1341) Wasser, I.M.; de Vries, S.; Moenne-Loccoz, P.; Schroder. I.; Karlin, K. D. Chem. Rev. **2002**, *102*, 1201.

(1342) Roncaroli, F.; Videla, M.; Slep, L.D.; Olabe, J.A. *Coord. Chem. Rev.* **2007**, *251*, 1903.

(1343) Goodrich, L.E.; Paulat, F.; Praneeth, V.K.K.; Lehnert, N. *Inorg. Chem.* **2010**, *49*, 6293.

(1344) As described in section 2.1., both ferredoxin- and NAD(P)H-dependent enzymes hold a sirohaem, where the nitrite reduction takes place, and an Fe/S. The NAD(P)H-dependent enzymes contain, in addition, a FAD domain, bound to an extended N-terminus, that is involved on the NAD(P)H binding and oxidation. This review will focus only the ferredoxin-dependent nitrite reductases.

(1345) Swamy, U.; Wang, M.; Tripathy, J.N; Kim, S.K.; Hirasawa, M.; Knaff, D.B.; Allen, J. P. *Biochemistry* **2005**, *44*, 16054.

(1346) Flores, E.; Frias, J.E.; Rubio, L.M.; Herrero, A. *Photosyn. Res.* **2005**, *83*, 117.

(1347) Sekine, K.; Sakakibara, Y.; Hase, T.; Sato, N. *Biochem. J.* **2009**, *423*, 91.

(1348) Hirasawa, M.; Tripathy, J.N.; Sommer, F.; Somasundaram, R.; Chung, J.S.; Nestander, M.; Kruthiventi, M.; Zabet-Moghaddam, M.; Johnson, M.K.; Merchant,S.S.; Allen, J.P.; Knaff, D.B.

Photosynth. Res. **2010**, *103*, 67.

(1349) Imamura, S.; Terashita, M.; Ohnuma, M.; Maruyama, S.; Minoda, A.; Weber, A.P.M.; Inouye, T.; Sekine, Y.; Fujita, Y.; Omata, T.; Tanaka, K. *Plant Cell Physiol.* **2010**, *51*, 707.

(1350) Nakano, S.; Takahashi, M.; Sakamoto, A.; Morikawa, H.; Katayanagi, K. *Proteins* **2012**, *80*, 2035.

(1351) Nakano, S.; Takahashi, M.; Sakamoto, A.; Morikawa, H.; Katayanagi, K. *Chem. Biodivers.* **2012**, *9*, 1989.

(1352) Nakano, S.; Takahashi, M.; Sakamoto, A.; Morikawa, H.; Katayanagi, K. *Protein Sci.* **2012**, *21*, 383.

(1353) Gates, A.J.; Luque-Almagro, V.M.; Goddard, A.D.; Ferguson, S.J.; Roldán, M.D.; Richardson, D.J. *Biochem. J.* **2011**, *435*, 743.

(1354) Harborne, N.R.; Griffiths, L.; Busby, S.J.; Cole, J.A. *Mol. Microbiol.* **1992**, *6*, 2805.

(1355) Kato, C.; Takahashi, M.; Sakamoto, A.; Morikawa. H. *J. Exp. Bot.* **2004**, *55*, 1761.

(1356) Knaff, D. B. *Oxygenic Photosynthesis: The Light Reactions*; Kluwer Publishers: Dordrecht, 1996; p 333.

(1357) Hirasawa, M.; Tollin, G.; Salamon, Z.; Knaff, D.B. *Biochem. Biophys. Acta* **1994**, *1185*, 336.

(1358) Hase, T.; Schurmann, P.; Knaff, D.B. Photosystem 1; Springer: Dordrecht, 2006; p 477.

(1359) Murphy, M.J.; Siegel, L.M.; Tove, S.R.; Kamin, H. *Proc. Nat. Acad. Sci. USA* **1974**, *71*, 612.

(1360) Setif, P.; Hirasawa, M.; Cassan, N.; Lagoutte, B.; Tripathy, J.N.; Knaff, D.B. *Biochemistry* **2009**, *48*, 2828.

(1361) Kuznetsova, S.; Knaff, D.B.; Hirasawa, M.; Setif, P.; Mattioli, T.A. *Biochemistry* **2004**, *43*, 10765.

(1362) Crane, B.R.; Siegel, L.M.; Getzoff, E.D. *Science* **1995**, *270*, 59.

(1363) Schnell, R.; Sandalova, T.; Hellman, U.; Lindqvist, Y.; Schneider, G. *J. Biol. Chem.* **2005**, *280*, 27319.

(1364) Schiffer, A.; Parey, K.; Warkentin, E.; Diederichs, K.; Huber. H.; Stetter, K.O.; Kroneck, P.M.; Ermler, U. *J. Mol. Biol.* **2008**, *379*, 1063.

(1365) Oliveira, T.F.; Vonrhein, C.; Matias, P.M.; Venceslau, S.S.; Pereira, I.A.; Archer, M. T. *J. Biol. Chem.* **2008**, *283*, 34141.

(1366) Hsieh, Y.C.; Liu, M.Y.; Wang, V.C.; Chiang, Y.L.; Liu, E.H.; Wu, W.G.; Chan, S.I.; Chen, C.J. *Mol. Microbiol.* **2010**, *78*, 1101.

(1367) Oliveira, T.F.; Franklin, E.; Afonso, J.P.; Khan, A.R.; Oldham, N.J.; Pereira, I.A.; Archer, M. *Front. Microbiol.* **2011**, *2*, 71.

(1368) Hirasawa, M.; Knaff, D. B. *Biochim. Biophys. Acta* **1985**, *830*, 173.

(1369) Mikami, B.; Ida, S. *J. Biochem.* **1989**, *105*, 47.

(1370) Hijcklesby, D.P.; Hewitt, E.J. *Biochem. J.* **1970**, *119*, 615.

(1371) Hewitt, E.J. *Annu. Rev. Plant Physiol.* **1975**, *26*, 73.

(1372) Vega, J.; Kamin H. *J. Biol. Chem.* **1977**, *252*, 896.

(1373) Krueger, R.J.; Siegel, L.M. *Biochemistry* **1982**, *21*, 2892.

(1374) Crane, B. R.; Getzoff, E. D. *Curr. Opin. Struct. Biol.* **1996**, *6*, 744.
(1375) Parey, K.; Warkentin, E.; Kroneck, P.M.; Ermler. U. *Biochemistry* **2010**, *49*, 8912.
(1376) Lancaster, J. R.; Vega, J. M.; Kamin, H.; Orme-Johnson, N. R.; Orme-Johnson, W. H.; Krueger, R. J.; and Siegel, L. M. *J. Biol. Chem.* **1979**, *254*, 1268.
(1377) Ondrias, M. R.; Carson, S. D.; Hirasawa, M.; Knaff, D. B. *Biochim. Biophys. Acta* **1985**, *830*, 159.
(1378) Hirasawa, M.; Shaw, R. W.; Palmer, G.; Knaff, D. B. *J. Biol. Chem.* **1987**, *262*, 12428.
(1379) Day, E.P.; Peterson, J.; Bonvoisin, J.J.; Young, L.J.; Wilkerson, J.O.; Siegel, L.M. *Biochemistry* **1988**, *27*, 2126.
(1380) Young, L.J.; Siegel, L.M. *Biochemistry* **1988**, *27*, 2790.
(1381) Kuznetsova, S.; Knaff, D.B.; Hirasawa, M.; Lagoutte, B.; Setif, P. *Biochemistry* **2004**, *43*, 510.
(1382) Aparicio, P.J.; Knaff, D.B.; Malkin, R. *Arch. Biochem. Biophys.* **1975**, *169*, 102.
(1383) Cammack, R.; Hucklesby, D.P.; Hewitt, E.J. *Biochem. J.* **1978**, *171*, 519.
(1384) Warren, M.J.; Raux, E.; Schubert, H.L.; Escalante-Semerena, J.C. *Nat. Prod. Rep.* **2002** *19*, 390.
(1385) Raux, E.; Leech, H.K.; Beck, R.; Schubert, H.L.; Santander, P.J.; Roessner, C.A.; Scott, A.I.; Martens, J.H.; Jahn, D.; Thermes, C.; Rambach, A.; Warren, M.J. *Biochem. J.* **2003**, *370*, 505.
(1386) Layer, G.; Reichelt, J.; Jahn, D.; Heinz, D.W. *Protein Sci.* **2010** *19*, 1137.
(1387) Tripathy, B.C.; Sherameti, I.; Oelmüller, R. *Plant Signal. Behav.* **2010**, *5*, 14.
(1388) Tanaka, R.; Tanaka, A. Annu. Rev. Plant Biol. **2007**, *58*, 321.
(1389) Frankenberg, N.; Moser, J.; Jahn, D. *Appl Microbiol Biotechnol.* **2003**, *63*, 115.
(1390) Knaff, D. B., Hirasawa M. *Biochim. Biophys. Acta* **1991**, *1056*, 93.
(1391) Hirasawa, M.; Tripathy, J.N.; Somasundaram, R.; Johnson, M.K.; Bhalla, M.; Allen, J.P.; Knaff, D.B. *Mol. Plant* **2009**, *2*, 407.
(1392) Hendrich, M. P.; Upadhyay, A. K.; Riga, J.; Arciero, D. M.; Hooper, A. B. *Biochemistry* **2002**, *41*, 4603.
(1393) Mowat, C.G.; Rothery, E.; Miles, C.S.; McIver, L.; Doherty, M.K.; Drewette, K.; Taylor, P.; Walkinshaw, M.D.; Chapman, S.K.; Reid, G.A. *Nat. Struct. Mol. Biol.* **2004**, *11*, 1023.
(1394) Atkinson, S.J.; Mowat, C.G.; Reid, G.A.; Chapman, S.K. *FEBS Lett.* **2007**, *581*, 3805.
(1395) Kostera, J.; Youngblut, M.D.; Slosarczyk, J.M.; Pacheco, A.A. *J. Biol. Inorg. Chem.* **2008**, *13*, 1073.
(1396) Fernandez, M. L.; Estrin, D. A.; Bari, S. E. *J. Inorg. Biochem.* **2008**, *102*, 1523.
(1397) Kostera, J.; McGarry, J.; Pacheco, A.A. *Biochemistry* **2010**, *49*, 8546.
(1398) Arciero, D. M.; Hooper, A. B. *J. Biol. Chem.* **1993** *268*, 14645.
(1399) Igarashi, N.; Moriyama, H.; Fujiwara, T.; Fukumori, Y.; Tanaka, N. *Nat. Struct. Biol.* **1997**, *4*, 276.
(1400) Kurnikov, I. V.; Ratner, M. A.; Pacheco, A. A. *Biochemistry* **2005**, *44*, 1856.
(1401) Pearson, A.R.; Elmore, B.O.; Yang, C.; Ferrara, J.D.; Hooper, A.B.; Wilmot, C.M. *Biochemistry* **2007**, *46*, 8340.

(1402) Bergmann, D. J.; Hooper, A. B.; Klotz, M. G. *Appl. Environ. Microbiol.* **2005**, *71*, 5371.

(1403) Kartal, B.; Maalcke, W.J.; de Almeida, N.M.; Cirpus, I.; Gloerich, J.; Geerts, W.; Op den Camp, H.J.M.; Harhangi, H.R.; Janssen-Megens, E.M.; Francoijs, K.J.; Stunnenberg, H.G.; Keltjens, J.T.; Jetten, M.S.M.; Strous, M. *Nature* **2011**, *479*, 127.

(1404) Nurizzo, D.; Silvestrini, M.C.; Mathieu, M.; Cutruzzolà F.; Bourgeois, D.; Fülöp, V.; Hajdu, J.; Brunori, M.; Tegoni, M.; Cambillau, C. *Structure* **1997**, *5*, 1157.

(1405) Nurizzo, D.; Cutruzzolà, F.; Arese, M.; Bourgeois, D.; Brunori, M.; Cambillau, C.; Tegoni, M. *Biochemistry* **1998**, *37*, 13987.

(1406) Cutruzzolà, F.; Brown, K.; Wilson, E.K.; Bellelli, A.; Arese, M.; Tegoni, M.; Cambillau, C.; Brunori, M. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 2232.

(1407) Sun, W.; Arese, M.; Brunori, M.; Nurizzo, D.; Brown, K.; Cambillau, C.; Tegoni, M.; Cutruzzolà, F.
Biochem. Biophys. Res. Commun. **2002**, *291*, 1.

(1408) Fulop, V.; Moir, J.W.B.; Ferguson, S.J.; Hajdu, J. *Cell* **1995**, *81*, 369.

(1409) Baker, S.C.; Saunders, N.F.W.; Willis, A.C.; Ferguson, S.J.; Hajdu, J.; Fulop, V. *J. Mol. Biol.* **1997**, *269*, 440.

(1410) Cheesman, M.R.; Ferguson, S.J.; Moir, J.W.B.; Richardson, D.J.; Zumft, W.G.; Thomson, A.J. *Biochemistry* **1997**, *36*, 16267.

(1411) Williams, P.A.; Fülöp, V.; Garman, E.F.; Saunders, N.F.; Ferguson, S.J.; Hajdu, J. *Nature* **1997**, *389*, 406.

(1412) Jafferji, A.; Allen, J.W.A.; Ferguson, S.J.; Fülöp, V. *J. Biol. Chem.* **2000**, *275*, 25089.

(1413) Silvestrini, M.C.; Tordi, M.G.; Musci, G.; Brunori, M. *J. Biol. Chem.* **1990**, *265*, 11783.

(1414) Silvestrini, M.C.; Cutruzzolà, F.; D'Alessandro, R.; Brunori, M.; Fochesato, N.; Zennaro, E. *Biochem. J.* **1992**, *285*, 661.

(1415) Williams, P.A.; Fülöp, V.; Leung, Y.C.; Chan, C.; Moir, J.W.B.; Howlett, G.; Ferguson, S.J.; Radford, S.E.; Hajdu, J. *Nat. Struct. Biol.* **1995**, *2*, 975.

(1416) Fülöp, V.; Watmough, N.J.; Ferguson, S.J. Adv. Inorg. Chem. **2001**, *51*, 163.

(1417) Richter, C.D.; Allen, J.W.A.; Higham, C.W.; Koppenhofer, A.; Zajicek, R.S.; Watmough, N.J.; Ferguson, S.J. *J. Biol. Chem.* **2002**, *277*, 3093.

(1418) Pearson, I.V.; Page, M.D.; van Spanning, R.J.M.; Ferguson, S.J. *J. Bacteriol.* **2003**, *185*, 6308.

(1419) Brown, K.; Roig-Zamboni, V.; Cutruzzolà, F.; Arese, M.; Sun, W.; Brunori, M.; Cambillau, C.; Tegoni, M. *J. Mol. Biol.* **2001**, *312*, 541.

(1420) Rinaldo, S.; Arcovito, A.; Brunori, M.; Cutruzzolà, F. *J. Biol. Chem.* **2007**, *282*, 14761.

(1421) Nurizzo, D.; Cutruzzolà, F.; Arese, M.; Bourgeois, D.; Brunori, M.; Cambillau, C.; Tegoni, M. *J. Biol. Chem.* **1999**, *274*, 14997.

(1422) Allen, J.W.A.; Cheesman, M.R.; Higham, C.W.; Ferguson, S.J.; Watmough, N.J. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 674.

(1423) Sjogren, T.; Hajdu, J. *J. Biol. Chem.* **2001**, *276*, 29450.

(1424) Besides the conversion of the active site into an "open" penta-coordinated haem, also the

increase in the reduction potential of the *c* haem[1425], associated with a methioninyl-histidinyl-coordination, should contribute to the activation of the enzyme. Note that a methionine residue is more effective than an histidine at stabilising the reduced *c* haem iron, what makes the reduction potential of a methioninyl-histidinyl-coordinated *c* haem more positive than that of a bis-histidinyl counterpart.[1426,1427]

(1425) Zajicek, R.S.; Cartron, M.L.; Ferguson, S.J. *Biochemistry* **2006**, *45*, 11208.

(1426) Musveteau, I.; Dolla, A.; Guerlesquin, F.; Payan, F.; Czjzek, M.; Haser, R.; Bianco, P.; Haladjian, J.; Rappgiles, B. J.; Wall, J. D.; Voordouw, G.; Bruschi, M. *J. Biol. Chem.* **1992**, *267*, 16851.

(1427) Koppenhofer, A.; Turner, K. L.; Allen, J. W.; Chapman, S. K.; Ferguson, S. J. *Biochemistry* **2000**, *39*, 4243.

(1428) Allen, J.W.A.; Watmough, N.J.; Ferguson, S.J. *Nat. Struct. Biol.* **2000**, *7*, 885.

(1429) Gordon, E.H.J.; Sjogren, T.; Lofqvist, M.; Richter, C.D.; Allen, J.W.A.; Higham, C.W.; Hajdu, J.; Fulop, V.; Ferguson, S.J. *J.Biol. Chem.* **2003**, *278*, 11773.

(1430) Cutruzzolà, F.; Arese, M.; Grasso, S.; Bellelli, A.; Brunori, M. *FEBS Lett.* **1997**, *412*, 365.

(1431) Radoul, M.; Centola, F.; Rinaldo, S.; Cutruzzolà, F.; Pecht, I. Goldfarb, D. *Inorg. Chem.* **2009**, *48*, 3913.

(1432) Timkovich, R.; Dhesi, R.; Martinkus, K.J.; Robinson, M.K.; Rea, T.M. *Arch. Biochem. Biophys.* **1982**, *215*, 47.

(1433) Ranghino, G.; Scorza, E.; Sjogren, T.; Williams, P.A.; Ricci, M.; Hajdu, J. *Biochemistry* **2000**, *39*, 10958.

(1434) Radoul, M.; Bykov, D.; Rinaldo, S.; Cutruzzolà, F.; Neese, F.; Goldfarb, D. *J. Am. Chem. Soc.* **2011**, *133*, 3043.

(1435) Nitrite, with its π-anti-bonding LUMO, is a π-acceptor ligand, a feature that would promote its binding to a reduced iron porphyrin rather than to a ferric iron species and, in fact, the ferric d_1 haem affinity for nitrite is low.[1436] This iron-nitrite back-bonding would strengthen the Fe-N bond and contribute to weaken the N-O bond, as was discussed for CcNiR. However, the two electron withdrawing carbonyl groups and inversion of the iron *d* orbitals energy levels of the d_1 haem may not favour the nitrite binding as much as the *c* haem does. Nevertheless, as will be discussed, nitrite is able to displace the NO bound to the ferrous enzyme[1437], what, once more, indicates that the ferrous d_1 haem has a high affinity for nitrite.

(1436) Timkovich, R.; Cork, M.S. *Biochemistry* **1982**, *21*, 3794.

(1437) Rinaldo, S.; Brunori, M.; Cutruzzolà, F. *Biochem. Biophys. Res. Commun*. **2007**, *363*, 662.

(1438) George, S.J.; Allen, J.W.A.; Ferguson, S.J.; Thorneley, R.N.F. *J. Biol. Chem.* **2000**, *275*, 33231.

(1439) Sam, K.A.; Strampraad, M.J.F.; de Vries, S.; Ferguson, S.J. *J. Biol. Chem.* **2008**, *283*, 27403.

(1440) Rinaldo, S.; Sam, K.A.; Castiglione, N.; Stelitano, V.; Arcovito, A.; Brunori, M.; Allen, J.W.A.; Ferguson, S.T.; Cutruzzolà, F. *Biochem. J.* **2011**, *435*, 217.

(1441) Kobayashi, K.; Koppenhofer, A.; Ferguson, S.J.; Watmough, N.J.; Tagawa, S. *Biochemistry* **2001**, *40*, 8542.

(1442) Sam, K.A.; Fairhurst, S.A.; Thorneley, R.N.F.; Allen, J.W.A.; Ferguson, S.J. *J. Biol. Chem.* **2008**, *283*, 12555.

(1443) Sam, K.A.; Tolland, J.D.; Fairhurst, S.A.; Higham, C.W.; Lowe, D.J.; Thorneley, R.N.F.; Allen, J.W.A; Ferguson, S.J. *Biochem. Biophys. Res. Commun.* **2008**, *371*, 719.

(1444) Farver, O.; Brunori M.; Cutruzzolà, F.; Rinaldo, S.; Wherland, S.; Pecht, I. *Biophys. J.* **2009**, *96*, 2849.

(1445) It should be noted that the NO association rate constants for Cd_1NiR (k_{on}≈3-4x10^8 $M^{-1}s^{-1}$ [1420,1437,1440]) are similar to the ones for other haemic proteins (≈10^7-$10^8M^{-1}s^{-1}$; see references on the text above). However, due to the k_{off} values, the affinity of Cd_1NiR to NO is considerably lower.

(1446) Das, T.K.; Wilson, E.K.; Cutruzzolà, F.; Brunori, M.; Rousseau, D.L. *Biochemistry* **2001**, *40*, 10774.

(1447) Lopes, H.; Besson, S.; Moura, I.; Moura, J.J.G. *J. Biol. Inorg. Chem.* **2001**, *6*, 55.

(1448) Note that this hydrogen bonds network, observed in frozen solutions[1434], is different from the one observed in the crystal structure[1404-1406], where the two conserved histidines were found within hydrogen bond distance to the NO oxygen atom (3.4Å and 2.6 Å to His_{327} and His_{369}, respectively) and the Tyr_{10} was shifted away (with its HO- group at 4.9 and 4.1Å from the NO nitrogen and oxygen atoms, respectively).

(1449) It should be remembered that Cd_1NiR is a key enzyme in denitrification (for organism not expressing the CuNiR), AnAmmOX, "denitrification/intra-aerobic methane oxidation" and probably other not yet identified pathways (as discussed in sections 2.).

(1450) Balia, S.; Lawrence, A.D.; Lobo, S.A.; Saraiva, L.M.; Golding, B.T.; Palmer, D.J.; Howard, M.J.; Ferguson, S.J.; Warren, M.J. *Proc Natl Acad Sci USA* **2011**, *108*, 18260.

(1451) Kukimoto, M.; Nishiyama, M.; Murphy, M.E.P.; Turley, S.; Adman, E.T. Horinouchi, S.; Beppu, T. *Biochemistry* **1994**, *33*, 5246.

(1452) Murphy, M.E.P.; Turley, S.; Kukimoto, M.; Nishiyama, M.; Horinouchi, S.; Sasaki, H.; Tanokura, M.; Adman, E.T. *Biochemistry* **1995**, *34*, 12107.

(1453) Murphy, M.E.P.; Turley, S.; Adman, E.T. *J.Biol. Chem.* **1997**, *272*, 28455.

(1454) Boulanger, M.J.; Kukimoto, M.; Nishiyama, M.; Horinouchi, S.; Murphy, M.E.P. *J. Biol. Chem.* **2000**, *275*, 23957.

(1455) Godden, J.W.; Turley, S.; Teller, D.C.; Adman, E.T.; Liu, M.Y.; Payne, W.J.; LeGall, J. *Science* **1991**, *253*, 438.

(1456) Adman, E.T.; Godden, J.W.; Turley, S. *J. Biol. Chem.* **1995**, *270*, 27458.

(1457) Antonyuk, S.V.; Strange, R.W.; Sawers, G.; Eady, R.R.; Hasnain, S.S. *Proc. Nat. Acad. Sci. USA* **2005**, *102*, 12041.

(1458) Strange, R.W.; Dodd, F.E.; Abraham, Z.H.; Grossmann, J.G.; Brüser, T.; Eady, R.R.; Smith, B.E.; Hasnain, S.S. *Nat. Struct. Biol.* **1995**, *2*, 287.

(1459) Dodd, F.E.; Beeumen, J.V.; Eady, R.R. Hasnain, S.S. *J. Mol. Biol.* **1998**, *282*, 369.

(1460) Inoue, T.; Gotowda, M.; Deligeer; Kataoka, K.; Yamaguchi, K.; Suzuki, S.; Watanabe, H.; Gohow, M.; Kai, Y. *J. Biochem.* **1998**, *124*, 876.

(1461) Strange, R.W.; Murphy, L.M.; Dodd, F.E.; Abraham, Z.H.L.; Eady, R.R.; Smith, B.E.; Hasnain, S.S. *J. Mol. Biol.* **1999**, *287*, 1001.

(1462) Ellis, M.J.; Prudêcncio, M.; Dodd, F.E.; Strange, R.W.; Sawers, G.; Eady, R.R.; Hasnain, S.S. *J. Mol. Biol*. **2002**, *316*, 51.

(1463) Ellis, M.J.; Dodd, F.E.; Sawers, G.; Eady, R.R.; Hasnain, S.S. *J. Mol. Biol.* **2003**, *328*, 429.

(1464) van Wonderen, J.H.; Kostrz, D.N.; Dennison, C.; MacMillan,F. *Angew. Chem. Int. Ed.* **2013**, *52*, 1990.

(1465) Jacobson, F.; Pistorius, A.; Farkas, D.; De Grip, W. Hansson, O.; Sjolin, L.; Neutze, R. *J. Biol. Chem.* **2007**, *282*, 6347.

(1466) Suzuki,S.; Kohzuma, T.; Yamaguchi, K.; Nakamura, N.; Shidara, S.; Kobayashi, K.; Tagawa, S. *J. Am. Chem. Soc.* **1994**, *116*, 11154.

(1467) Kobayashi, K.; Tagawa, S.; Deligeer; Suzuki, S. *J. Biochem.* **1999**, *126*, 408.

(1468) Prudêncio, M; Eady, R.R., Sawers, G. *Biochem. J.* **2001**, *353*, 259.

(1469) Boulanger, M.J.; Murphy, M.E.P. *Protein Sci.* **2003**, *12*, 248.

(1470) Ferroni, F.M.; Guerrero, S.A.; Rizzi, A.C.; Brondino, C.D. *J. Inorg. Biochem.* **2012**, *114*, 8.

(1471) LaCroix, L.B.; Shadle, S.E.; Wang, Y.; Averill, B.A.; Hedman, B.; Hodgson, K.O.; Solomon, E.I. *J. Am.Chem. Soc.* **1996**, *118*, 7755.

(1472) Kukimoto, M.; Nishiyama, M.; Ohnuki, T.; Turley, S.; Adman, E.T.; Horinouchi, S.; Beppu, T. *Protein Eng.* **1995**, *8*, 153.

(1473) Kukimoto, M.; Nishiyama, M; Tanokura, M.; Adman, E.T.; Horinouchi, S. *J. Biol. Chem.* **1996**, *271*, 13680.

(1474) Nojiri, M.; Xie, Y.; Inoue, T.; Yamamoto, T.; Matsumura, H.; Kataoka, K.; Deligeer; Yamaguchi, K.; Kai, Y.; Suzuki, S. *Proc Natl Acad Sci USA* **2007**, *104*, 4315.

(1475) Yamaguchi, K.; Kataoka, K.; Kobayashi, M.; Itoh, M.; Fukui, A.; Suzuki, S. *Biochemistry* **2004**, *43*, 14180.

(1476) Suzuki, S.; Kohzuma, T.; Shidara, S.; Ohki, K.; Aida, T. *Inorg. Chim. Acta* **1993** *208*, 107.

(1477) Deligeer; Fukunaga, R.; Kataoka, K.; Yamaguchi, K.; Kobayashi, K.; Tagawa, S.; Suzuki. S. *J. Inorg. Biochem.* **2002**, *91*, 132.

(1478) Yamaguchi, K.; Kobayashi, M.; Kataoka, K.; Suzuki, S. *Biochem. Biophys. Res. Commun.* **2003**, *300*, 36.

(1479) Ellis, M.J.; Grossmann, J.G.; Eady, R.R.; Hasnain, S.S. *J. Biol. Inorg. Chem.* **2007**, *12*, 1119.

(1480) Antonyuk, S.V., Han, C.; Eady, R.R.; Hasnain, S.S. *Nature* **2013**, *496*, 123.

(1481) Tsuda, A.; Ishikawa, R.; Koteishi, H.; Tange, K.; Fukuda, Y.; Kobayashi, K.; Inoue, T.; Nojiri, M. *J. Biochem.* **2013**, *154*, 51.

(1482) Bertini, I.; Cavallaro, G.; Rosato, A. *Chem. Rev.* **2006**, *106*, 90.

(1483) Han, C.; Wright, G.S.A.; Fisher, K.; Rigby, S.E.J.; Eady, R.R.; Hasnain, S.S. *Biochem. J.* **2012**, *444*, 219.

(1484) Boulanger, M.J.; Murphy, M.E.P. *J. Mol. Biol.* **2002**, *315*, 1111.

(1485) Ku, S.C.; Schulz, B.L.; Power, P.M.; Jennings, M.P. *Biochem. Biophys. Res. Commun.* **2009**,

378, 84.

(1486) Kakutani, T.; Watanabe, H.; Arima, K.; Beppu, T. *J. Biochem.* **1981**, *89*, 463.

(1487) Boulanger, M.J.; Murphy, M.E. *Biochemistry* **2001**, *40*, 9132.

(1488) Barrett, M.L.; Harris, R.L.; Antonyuk, S.; Hough, M.A.; Ellis, M.J.; Sawers, G.; Eady, R.R.; Hasnain, S.S. *Biochemistry* **2004**, *43*, 16311.

(1489) Tocheva, E.I.; Rosell, F.I.; Mauk, A.G.; Murphy, M.E. *Science* **2004**, *304*, 867.

(1490) Sundararajan, M.; Hillier, I.H.; Burton, N.A. *J. Phys. Chem. B* **2007**, *111*, 5511.

(1491) Tocheva, E.I.; Rosell, F.I.; Mauk, A.G.; Murphy, M.E.P. *Biochemistry* **2007**, *46*, 12366.

(1492) Ghosh, S.; Dey, A.; Sun, Y.; Scholes, C.P.; Solomon, E.I. *J. Am. Chem. Soc.* **2009**, *131*, 277.

(1493) Leferink, N.G.G.; Han, C.; Antonyuk, S.V.; Heyes, D.J.; Rigby, S.E.J.; Hough, M.A.; Eady, R.R.; Scrutton,N.S.; Hasnain, S.S. *Biochemistry* **2011**, *50*, 4121.

(1494) Halfen J.A.; Tolman, W.B. *J. Am. Chem. Soc.* **1994**, *116*, 5475.

(1495) Halfen, J.A.; Mahapatra, S.; Wilkinson, E.C.; Gengenbach, A.J.; Young, V.G.; Que, L.; Tolman, W.B. *J. Am. Chem. Soc.* **1996**, *118*, 763.

(1496) Kujime, M.; Izumi, C.; Tomura, M.; Hada, M.; Fujii, H. J. Am. Chem. Soc., **2008**, *130*, 6088.

(1497) Hsu, S.C.N.; Chang, Y.L.; Chuang, W.J.; Chen, H.Y.; Lin, I.J.; Chiang, M.Y.; Kao, C.L.; Chen, H.Y. *Inorg. Chem.* **2012**, *51*, 9297.

(1498) Chen, C.-S.; Yeh, W.-Y. *Chem. Commun.* **2010**, *46*, 3098.

(1499) Chuang, W.-J.; Lin, I. J.; Chen, H.-Y.; Chang, Y.-L.; Hsu, S. C. N. *Inorg. Chem.* **2010**, *49*, 5377.

(1500) Kumar, M.; Dixon, N.A.; Merkle, A.C.; Zeller, M.; Lehnert, N.; Papish, E.T. *Inorg. Chem.* **2012**, *51*, 7004.

(1501) Zhao, Y.; Lukoyanov, D.A.; Toropov, Y. V.; Wu, K.; Shapleigh, J.P.; Scholes, C.P. *Biochemistry* **2002**, *41*, 7464.

(1502) Tocheva, E.I.; Eltis, L.D.; Murphy, M.E.P. *Biochemistry* **2008**, *47*, 4452.

(1503) Merkle, A.C.; Lehnert, N. *DaltonTrans.* **2012**, *41*, 3355.

(1504) Hulse, C.L.; Averill, B.A.; Tiedje, J.M. *J. Am. Chem. Soc.* **1989**, *111*, 2322.

(1505) Abraham, Z.H.L.; Smith, B.E.; Howes, B.D.; Lowe, D.J.; Eady, R.R. *Biochem. J.* **1997**, *324*, 511.

(1506) Kataoka, K.; Furusawa, H.; Takagi, K.; Yamaguchi, K.; Suzuki, S. *J. Biochem.* **2000**, *127*, 345.

(1507) Wijma, H.J.; Jeuken, L.J.C.; Verbeet, M.P.; Armstrong, F.A.; Canters, G.W. *J. Biol. Chem.* **2006**, *281*, 16340.

(1508) De Marothy, S.A.; Blomberg, M.R.A.; Siegbahn, P.E.M. *J. Comput. Chem*. **2007**, *28*, 528.

(1509) Ghosh, S.; Dey, A.; Usov, O.M.; Sun, Y.; Grigoryants, V.M.; Scholes, C.P.; Solomon, E.I. *J. Am. Chem. Soc.* **2007**, *129*, 10310.

(1510) Wijma, H.J.; Jeuken, L.J.C.; Ph. Verbeet,M.; Armstrong, F.A.; Canters, G.W. *J. Am. Chem. Soc.* **2007**, *129*, 8557.

(1511) Hough, M.A.; Antonyuk, S.V.; Strange, R.W.; Eady, R.R.; Hasnain, S.S. *J. Mol. Biol.* **2008**, *378*, 353.

(1512) Hough, M.A.; Eady, R.R.; Hasnain, S.S. *Biochemistry* **2008**, *47*, 13547.

(1513) Kuznetsova, S.; Zauner, G.; Aartsma, T.J.; Engelkamp, H.; Hatzakis, N.; Rowan, A.E.; Nolte, R.J.M.; Christianen, P,C.M.; Canters, G.W. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 3250.

(1514) Brenner, S.; Heyes, D.J.; Hay, S.; Hough, M.A.; Eady, R.R.; Hasnain, S.S.; Scrutton, N.S. *J. Biol. Chem.* **2009**, *284*, 25973.

(1515) Goldsmith, R.H.; Tabares, L.C.; Kostrz, D.; Dennison, C.; Aartsma, T.J.; Canters, G.W.; Moerner, W.E. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17269.

(1516) Krzeminski, L.; Ndamba, L.; Canters, G.W.; Aartsma, T.J.; Evans, S.D.; Jeuken, L.J.C. *J. Am. Chem. Soc.* **2011**, *133*, 15085.

(1517) Tabares, L.C.; Kostrz, D.; Elmalk, A.; Andreoni, A.; Dennison, C.; Aartsma, T.J.; Canters, G.W. *Chemistry* **2011**, *17*, 12015.

(1518) Suzuki, S.; Deligeer; Yamaguchi, K.; Kataoka, K.; Kobayashi, K.; Tagawa, S.; Kohzuma, T.; Shidara, S.; Iwasaki, H. *J. Biol. Inorg. Chem.* **1997**, *2*, 265.

(1519) Farver, O., Eady, R.R., Abraham, Z.H. and Pecht, I. *FEBS Lett.* **1998**, *436*, 239.

(1520) Olesen, K.; Veselov, A.; Zhao, Y., Wang, Y.; Danner, B.; Scholes, C.P.; Shapleigh, J.P. *Biochemistry* **1998**, *37*, 6086.

(1521) Leferink, N.G.H.; Eady, R.R.; Hasnain, S.S.; Scrutton, N.S. *FEBS J.* **2012**,*279*, 2174.

(1522) Leferink, N.G.H.; Pudney, C.R.; Brenner, S.; Heyes, D.J.; Eady, R.R.; Hasnain, S.S.; Hay, S.; Rigby, S. E.J.; Scrutton, N.S. *FEBS Lett.* **2012**, *586*, 578.

(1523) Iwasaki, H.; Matsubara, T. *J. Biochem.* **1972**, *71*, 645.

(1524) Kakutani, T.; Watanabe, H.; Arima, K.; Beppu, T. *J. Biochem.* **1981**, *89*, 453.

(1525) Note that the copper HOMO is a d_{x2-y2} orbital and the nitrite LUMO is a π-anti-bonding orbital, while the σ-anti-bonding orbital is quite high in energy.

(1526) Merkle, A.C.; Lehnert, N. *Inorg. Chem.* **2009**, *48*, 11504.

(1527) Santos-Silva, T.; Ferroni, F.; Thapper, A.; Marangon, J.; Gonzàlez, P.J.; Rizzi, A.C.; Moura, I.; Moura, J.J.G.; Romão, M.J.; Brondino, C.D. *J. Am. Chem. Soc.* **2009**, *131*, 7990.

(1528) Romão, M.J.; Archer, M.; Moura, I.; Moura, J.J.G.; LeGall, J.; Engh, R.; Schneider, M.; Hof, p.; Huber, R. *Science* **1995**, *270*, 1170.

(1529) Huber, R.; Hof, P.; Duarte, R.O.; Moura, J.J.G.; Moura, I., Liu, M-Y.; Legall, J.; Hille, R.; Archer, M.; Romão, M.J. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8846.

(1530) Rebelo, J.M.; Dias, J.M.; Huber, R.; Moura, J.J.G.; Romão, M.J. *J. Biol. Inorg. Chem.* **2001**, *6*, 791.

(1531) Enroth, C.; Eger, B.T.; Okamoto, K.; Nishino, T.; Nishino, T.; Pai, E.F. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10723.

(1532) Mahro, M.; Coelho, C.; Trincão, J.; Rodrigues, D.; Terao, M.; Garattini, E.; Saggu, M.; Lendzian, F.; Hildebrandt, P.; Romão, M.J.; Leimkühler, S. *Drug Metab. Dispos.* **2011**, *39*, 1939.

(1533) Coelho, C.; Mahro, M.; Trincão, J.; Carvalho, A.T.P.; Ramos, M.J.; Terao, M.; Garattini, E.; Leimkühler, S.; Romão, M.J. *J. Biol. Chem.* **2012**, *287*, 40690.

(1534) Okamoto, K.; Matsumoto, K.; Hille, R.; Eger, B.T.; Pai, E.F.; Nishino, T. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7931.

(1535) Doonan, C.J.; Stockert, A., Hille, R.; George, G.N. *J. Am. Chem. Soc.* **2005**, *127*, 4518.

(1536) Sticht, H.; Rosch, P. *Prog. Biophys. Mol. Biol.* **1998**, *70*, 95.

(1537) Grazina, R.; Pauleta, S.R.; Moura, J.J.G.; Moura, I. *Bioinorganic Fundamentals and Applications: Metals in Natural Living Systems and Metals in Toxicology and Medicine*; Elsevier: 2013; p103.

(1538) It should be noted that mammalian XO and XD are two forms of the same protein (same gene product), even though, historically, they had been studied as distinct enzymes (XO was mostly studied from bovine milk[1539-1544], while XD was obtained from turkey or chicken[1545-1547], but also from bacteria[1548]). Noteworthy, the mammalian enzyme can be purified in both forms; depending on the experimental conditions (presence/absence of proteases inhibitors and thiol reducing reagents), the rat liver or bovine milk enzymes can be purified in the XD, reversible XO or irreversible XO forms.[1549-1559]

(1539) Massey, V.; Brumby, P. E.; Komai, H. *J. Biol. Chem.* **1969**, *244*, 1682.

(1540) Fridovich, I. *J. Biol. Chem.* **1970**, *245*, 4053.

(1541) Hart, L. I.; McGartoll, M. A.; Chapman, H. R.; Bray, R. C. *Biochem. J.* **1970**, *116*, 851.

(1542) Olson, J.S.; Ballow, D. P.; Palmer, G.; Massey, V. *J. Biol. Chem.* **1974**, *249*, 4350.

(1543) Olson, J.S.; Ballou, D.P.; Palmer, G.; Massey, V. *J. Biol. Chem.* **1974**, *249*, 4363.

(1544) Massey, V.; Harris, C.M. *Biochem. Soc. Trans.* **1997**, *25*, 750.

(1545) Rajagopalan, K. V.; Handler, P. *J. Biol. Chem.* **1967**, *242*, 4097.

(1546) Barber, M. J.; Bray, R. C.; Lowe, D. J.; Coughlan, M. P. *Biochem. J.* **1976**, *153*, 297.

(1547) Barber, M. J.; Bray, R. C.; Cammack, R.; Coughlan, M. P. *Biochem. J.* **1977**, *163*, 279.

(1548) Truglio, J.J; Theis, K.; Leimkühler, S., Rappa, R.; Rajagopalan, K.V.; Kisker, C. *Structure* **2002**, *10*, 115.

(1549) Della Corte, E.; Stirpe, F. *Biochem. J.* **1968**, *108*, 349.

(1550) Stirpe, F.; Della Corte E. *J. Biol. Chem.* **1969**, *244*, 3855.

(1551) Della Corte, E.; Stirpe, F. *Biochem. J.* **1972**, *126*, 739.

(1552) Battelli, M. G.; Lorenzoni, E.; Stripe, F. *Biochem. J.* **1973**, *131*, 191.

(1553) Waud, W. R.; Rajagopalan, K. V. *Arch. Biochem. Biophys.* **1976**, *172*, 354.

(1554) Waud, W. R.; Rajagopalan, K. V. *Arch. Biochem. Biophys.* **1976**, *172*, 365.

(1555) Nakamura, M.; Yamazaki, I. *J. Biochem.* **1982**, *92*, 1279.

(1556) Amaya, Y.; Yamazaki, K.; Sato, M.; Noda, K.; Nishino, T. *J. Biol. Chem.* **1990**, *265*, 14170.

(1557) Hunt, J.; Massey, V. *J. Biol. Chem.* **1992**, *267*, 21479.

(1558) Nishino, T.; Nishino, T. *J. Biol. Chem.* **1997**, *272*, 29859.

(1559) Maia, L.; Mira, L. *Arch. Biochem. Biophys.* **2002**, *400*, 48.

(1560) Saito, T.; Nishino, T. *J. Biol. Chem.* **1989**, *264*, 10015.

(1561) Harris, C. M.; Massey, V. *J. Biol. Chem.* **1997**, *272*, 8370.

(1562) Kaminski, Z. W.; Jezewska, M. M. *Biochem. J.* **1982**, *207*, 341.

(1563) Kuwabara, Y. Nishino, T.; Okamoto, K.; Matsumura, T.; Eger, B.T.; Pai, E.F.; Nishino, T. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 8170.

(1564) Nishino, T.; Okamoto, K.; Kawaguchi, Y.; Hori, H.; Matsumura, T.; Eger, B.T.; Pai, E.F.; Nishino, T. *J. Biol. Chem.* **2005**, *280*, 24888.

(1565) Tsujii, A.; Nishino, T.; *Nucleosides Nucleotides Nucleic Acids* **2008**, *27*, 881.

(1566) Ishikita, H.; Eger, B.T., Okamoto, K.; Nishino, T.; Pai, E.F. *J. Am. Chem. Soc.* **2012**, *134*, 999.

(1567) Stone, J.R.; Yang, S. *Antioxid. Redox Signal.* **2006**, *8*, 243.

(1568) Veal, E.A.; Day, A.M.; Morgan, B.A. *Mol. Cell* **2007**, *26*, 1.

(1569) Hearse, D. J.; Manning, A. S.; Downey, J. M.; Yellon, D. M. *Acta Physiol. Scand.* **1986**, *548*, 65.

(1570) Granger, D. N.; Hollwarth, M. E.; Parks, D. A. *Acta Physiol. Scand.* **1986**, *548*, 47.

(1571) Nishino, T. *J. Biochem.* **1994**, *116*, 1.

(1572) Zhang, Z.; Blake, D. R.; Stevens, C. R.; Kanczler, J. M.; Winyard, P. G.; Symons, M. C.; Benboubetra, M.; Harrison, R. *Free Radic. Res.* **1998**, *28*, 151.

(1573) Morpeth, F. F. *Biochim. Biophys. Acta* **1983**, *744*, 328.

(1574) Janssen, M.; Tavenier, M.; Koster, J. F.; de Jong, J. W. *Biochim. Biophys. Acta* **1993**, *1156*, 307.

(1575) Krenitsky, T. A. *Biochem. Pharmacol.* **1978**, *27*, 2763.

(1576) Fabre, G.; Seither, R.; Goldman, D. *Biochem. Pharmacol.* **1986**, *35*, 1325.

(1577) Kitamura, S.; Sugihara, K.; Ohta, S. *Drug Metab. Pharmacokinet.* **2006**, *21*, 83.

(1578) Ali, H.I.; Fujita, T.; Akaho, E.; Nagamatsu, T. *J Comput. Aided Mol. Des.* **2010**, *24*, 57.

(1579) Lespade, L.; Bercion, S. *J. Phys. Chem. B* **2010**, *114*, 921.

(1580) Rashidi, M.R.; Nazemiyeh, H. *Expert Opin. Drug Metab. Toxicol.* **2010**, *6*, 133.

(1581) Benedetti M.S. *Ann. Pharm. Fr.* **2011**, *69*, 45.

(1582) Kumar, R., Darpan; Sharma, S.; Singh, R. *Expert Opin. Ther. Pat.* **2011**, 211071.

(1583) Burgrnayer, S.J.N.; Stlefel, E.I. *J. Chem. Educ.* **1985**, *62*, 943.

(1584) Harlan, E.E.; Berg, J.M.; Holm, R.H. J. Am. Chem. Soc. **1986**, *108*, 6992.

(1585) Unciuleac, M.; Warkentin, E.; Page, C.C.; Boll, M.; Ermler, U. *Structure* **2004**, *12*, 2249.

(1586) Boll, M. *Biochim. Biophys. Acta* **2005**, *1707*, 34.

(1587) Johannes, J.; Unciuleac, M.; Friedrich, T.; Warkentin, E.; Ermler, U.; Boll, M. *Biochemistry*, **2008**, *47*, 4964.

(1588) Maia, L.; Moura, J.J.G. unpublished results.

(1589) Silvestrini, M. C.; Colosimo, A.; Brunori, M.; Walsh, T. A.; Barber, D.; Greenwood, C. *Biochem. J.* **1979**, *183*, 701.

(1590) Maia, L.; Moura, J.J.G. personal communication.

(1591) George, G.N.; Bray, R.C. *Biochemistry* **1988**, *27*, 3603.

(1592) Wilson, G.L.; Greenwood, R.J.; Pilbrow, J.R.; Spence, J.T.; Wedd, A.G. *J. Am. Chem. Soc.* **1991**, *113*, 6803.

(1593) Doonan, C.J.; Rubie, N.D.; Peariso, K.; Harris, H.H.; Knottenbelt, S.Z.; George, G.N.; Young,

C.C.; Kirk, M.L. *J. Am. Chem. Soc.* **2008**, *130*, 55.

(1594) Stiefel, E.I. *Proc. Nat. Acad. Sci. USA* **1973**, *70*, 988.

(1595) Rajapakshe, A.; Snyder, R.A.; Astashkin, A.V.; Bernardson, P., Evans, D.J.; Young, C.G.; Evans, D.H.; Enemark, J.H. *Inorg. Chim. Acta* **2009**, *362*, 4603.

(1596) Kalthoff, H.; Fehr, S.; Sundermeyer, H.; Renwrantz, L.; Bock, E. *Curr. Microbiol.* **1979**, *2*, 375.

(1597) Ehrich, S.; Behrens, D.; Lebedeva, E.; Ludwig, W.; Bock, E. *Arch. Microbiol.* **1995**, *164*, 16.

(1598) Spieck, E.; Muller, S.; Engel, A.; Mandelkow, E.; Patel, H.; Bock, E. *J. Structural Biol.* **1996**, *117*, 117.

(1599) Cobley, J. G. *Biochem. J.* **1976**, *156*, 493.

(1600) van de Vossenberg, J.; Woebken, D.; Maalcke, W.J.; Wessels, H.J.; Dutilh, B.E.; Kartal, B.; Janssen-Megens, E.M.; Roeselers, G.; Yan, J.; Speth, D.; Gloerich, J.; Geerts, W.; van der Biezen, E.; Pluk, W.; Francoijs, K.J.; Russ, L.; Lam, P.; Malfatti, S.A.; Tringe, S.G.; Haaijer, S.C.; Op den Camp, H.J.; Stunnenberg, H.G.; Amann, R.; Kuypers, M.M.; Jetten, M.S. *Environ. Microbiol.* **2013**, *15*, 1275.

(1601) Sundermeyer-Klinger, H.; Meyer, W.; Warninghoff, B.; Bock, E. *Arch. Microbiol.* **1984**, *140*, 153.

(1602) Watson, S.W.; Book, E.; Valois, F.W.; Waterbury, J.B.; Schlosser, U. *Arch. Microbiol.* **1986**, *144*, 1.

(1603) Yamanaka, T.; Fukumori, Y. *FEMS Microbiol. Rev.* **1988**, *54*, 259.

(1604) Spieck, E.; Ehrich, S.; Aamand, J.; Bock, E. *Arch. Microbiol.* **1998**, *169*, 225.

(1605) Alawi, M., Lipski, A., Sanders, T., Pfeiffer, E. M., and Spieck, E. *ISME J.* **2007**, *1*, 256.

(1606) Lücker, S.; Wagner, M.; Maixner, F.; Pelletier, E.; Koch, H., Vacherie, H.; Rattei, T.;Damsté, J.S.S.; Spieck, E.; Le Paslier, D.; Daims, H. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 13479.

(1607) Lücker, S., Nowka, B.; Rattei, T.; Spieck, E.; Daims, H. *Front. Microbiol.* **2013**, *4*, 27.

(1608) The existence of these two types of enzyme is, probably, the response to different environmental pressures. *Nitrospira*, *Nitrospina* and "*Candidatus* Nitrotoga" bacteria (with periplasm-faced enzyme) grow only under nitrite limitation (0.29–2.9 mM), while *Nitrobacter* and *Nitrococcus* (with citoplasm-faced enzyme) are adapted to high nitrite concentrations (up to 29mM).[102] Therefore, it can be speculated that a periplasm-faced MoNiOR would allow the bacteria to grow with a low nitrite concentrations and, at the same time, would be responsible for the inhibitory effects of high nitrite concentration on the growth of *Nitrospira*, *Nitrospina*, and Nitrotoga. On the other hand, a cytoplasm-faced enzyme would depend on a nitrite/nitrate antiport system and would necessitate/tolerate an higher nitrite concentration.[1606,1609]

(1609) Starkenburg, S. R.; Larimer, F. W.; Stein, L. Y.; Klotz, M. G.; Chain, P. S.; Sayavedra-Soto, L. A.; Poret-Peterson, A. T.; Gentry, M. E., Arp, D. J., Ward, B., Bottomley, P. J. *Appl. Environ. Microbiol.* **2008**, *74*, 2852.

(1610) Poly, F.; Wertz, S.; Brothier, E.; Degrange, V. *FEMS Microbiol. Ecol.* **2008**, *63*, 132.

(1611) Starkenburg, S.R.; Chain, P.S.; Sayavedra-Soto, L.A.; Hauser, L., Land, M.L.; Larimer, F.W.; Malfatti, S.A.; Klotz, M.G.; Bottomley, P.J.; Arp, D.J.; Hickey, W.J. *Appl. Environ. Microbiol.* ***2006***, *72*, 2050.

(1612) Sorokin, D.Y.; Lucker, S., Vejmelkova, D.; Kostrikina, N.A.; Kleerebezem, R., Rijpstra, W.I.C.; Damste, J.S.S.; Le Paslier, D.; Muyzer, G.; Wagner, M.; van Loosdrecht, M.C.M.; Daims, H. *ISME J.* **2012**, *6*, 2245.

(1613) Prokaryotes use nitrate for assimilatory and dissimilatory processes (see section 2.) and, for those purposes, hold three types of NaR[1614-1617]: (i) "respiratory" membrane-bound cytoplasm-faced NaR (associated with the generation of a proton motive force across the cytoplasmatic membrane), (ii) periplasmatic NaR (generation of a proton motive force or as an electron sink to eliminate excess of reducing equivalents) and (iii) cytoplasmatic assimilatory NaR (nitrogen assimilation). All the prokaryotic NaR are molybdoenzymes of the dimethylsulfoxide reductase family (see section 4.; Fig. 6-c) and catalyse the two electrons reduction of nitrate to nitrite at their molybdenum centre ($NO_3^- + 2e^- + 2H^+ \longrightarrow NO_2^- + H_2O$). Apart from the four sulfur atoms from the two pyranopterin molecules (Fig. 6-c), the molybdenum atom is coordinated by: (i) in "respiratory" membrane-bound NaR, by two oxygen atoms (both from an aspartate residue or one from a terminal oxo group plus another one from an aspartate residue[1618]), (ii) in periplasmatic NaR, by two sulfur atoms (from a terminal sulfo group and another from a cysteine residue or one terminal hydroxo group plus a sulfur atom from a cysteine residue[1619-1623], and (iii) in cytoplasmatic assimilatory NaR, probably by a sulfur atom from a cysteine residue (although clear structural insight awaits further investigation)[1624].

It should be noted that, in spite of catalysing the same reaction, the eukaryotic assimilatory cytoplasmatic NaR (C-NaR, mentioned in the 3.2.1.1. section) is distinct from any type of prokaryotic NaR. Belonging to the sulfite oxidase family, its molybdenum atom (responsible for the nitrate reduction) is coordinated by only one pyranopterin molecule (two sulfur atoms), plus one sulfur atom from a cysteine residue, one oxo and one hydroxo groups (Fig. 6-c).

(1614) González, P.G.; Correia,C.; Moura, I.; Brondino, C.D.; Moura, J.J.G, *J. Inorg. Biochem.* **2006**, *100*, 1015.

(1615) Dobbek, H. *Coord. Chem. Rev.* **2011**, *255*, 1104.

(1616) Pushie, M.J.; George, G.N. *Coord. Chem. Rev.* **2011**, *255*, 1055.

(1617) Grimaldi, S.; Schoepp-Cothenet, B.; Ceccaldi, P.; Guigliarelli, B.; Magalon, A. *Biochim. Biophys. Acta* **2013**, *1827*, 1048.

(1618) Jormakka, M.; Richardson, D.; Byrne, B.; Iwata, S. *Structure* **2004**, *12*, 95.

(1619) Arnoux, P.; Sabaty, M.; Alric, J.; Frangioni, B.; Guigliarelli, B.; Adriano, J.-M.; Pignol, D. *Nat. Struct. Biol.* **2003**, *10*, 928.

(1620) Coelho, C.; Gonzalez, P.J.; Trincão, J.; Carvalho, A.L.; Najmudin, S.; Hettman, T.; Dieckman, S.; Moura, J.J.G., Moura, I.; Romão, M.J. *Acta Cryst. Sect. F* **2007**, *63*, 516.

(1621) Jepson, B.J.N.; Mohan, S.; Clarke, T.A.; Gates, A.J.; Cole, J.A.; Butler, C.S.; Butt, J.N.; Hemmings, A.M.; Richardson, D.J.; *J. Biol. Chem.* **2007**, *282*, 6425.

(1622) Najmudin, S.; Gonzalez, P.J.; Trincão, J.; Coelho, C.; Mukhopadhyay, A.; Romão, C.C.; Moura, I.; Moura, J.J.G.; Brondino, C.D.; Romão, M.J. *J. Biol. Inorg. Chem.* **2008**, *13*, 737.

(1623) Cerqueira, M.; Gonzalez, P.J.; Brondino, C.D.; Romão, M.J.; Romão, C.C.; Moura, I.; Moura, J.J.G.; *J. Comput. Chem*. **2009**, *30*, 2466.

(1624) Jepson, B.J.N.; Anderson, L.J.; Rubio, L.M.; Taylor, C.J.; Butler, C.S.; Flores, E., Herrero, A.; Butt, J.N.; Richardson, D.J. *J. Biol. Chem.* **2004**, *279*, 32212.

(1625) The "respiratory" NaR are membrane-bound cytoplasm-faced molybdoenzymes and, as the name indicates, are used by the organisms to generate a proton motive force across the cytoplasmatic membrane.1614,1618,1626 They are also called NaRGHI, because they are the product of the *narG*, *H* and *I* genes. These enzymes of the dimethylsulfoxide reductase family are heterotrimers, comprising: (i) a cytolasmatic nitrate-reducing NaRG subunit (≈125kDa) that holds one molybdenum centre (with the molybdenum atom coordinated as described in[1613]; the active site) and one [4Fe-4S] centre; (ii) an electron-transfer NaRH subunit (≈60kDa) that holds one [3Fe-4S] and three [4Fe-4S] centres; (iii) and a membrane-bound quinol-oxidising NaRI subunit (≈22kDa) that holds two *b*-type haems. Interestingly, and in harmony with the MoNiOR dual localisation, it was recently concluded that there is evidence for the occurrence of both archaeal and bacterial periplasm-faced NaRGHI-type nitrate reductases (*i.e.*, NaRGHI enzymes with the active site on the outside of the cytoplasmatic membrane).[1627] This exciting hypothesis, however, waits for experimental confirmation.

(1626) Bertero, M.G.; Rothery, R.A.; Palak, M.; Hou, C.; Lim, D.; Blasco, F., Weiner, J.H.; Strynadka, N.C.J. *Nat. Struct. Biol.* **2003**, *10*, 681.

(1627) Martinez-Espinosa, R.M.; Dridge, E.J.; Bonete, M.J.; Butt, J.N.; Butler, C.S.; Sargent, F., Richardson, D.J. *FEMS Microbiol. Lett.* **2007**, *276*, 129.

(1628) Kroneck, P. M. H.; Abt, J. A. *Met. Ions Biol. Syst.* **2002**;*39*, 369.

(1629) Bock, E.; Koops, H.-P.; Moller, U.C.; Rudert, M. *Arch. Microbiol.* **1990**, *153*, 105.

(1630) Meincke, M; Bock, E.; Kastrau, D.; Kroneck, P.M.H. *Arch. Microbiol.* **1992**, *158*, 127.

(1631) Krüger, B., Meyer, O.; Nagel, M.; Andreesen, J.R.; Meincke, M.; Bock, E.; Blümle, S.; Zumft, W.G. *FEMS Microbiol. Lett.* **1987**, *48*, 225.

(1632) Aleem, M.I.H.; Hoch, G.E.; Varner, J.E. *Proc. Natl. Acad. Sci. USA* **1965**, *54*, 869.

(1633) Kumar,S.; Nicholas, D.J.D.; Williams, E.H. *FEBS Lett.* **1983**, *152*, 71.

(1634) DiSpirito, A.A.; Hooper, A.B. *J. Biol. Chem.* **1986**, *261*, 10534.

(1635) Friedman, S.H.; Massefski, W.; Hollocher, T.C. *J. Biol. Chem.* **1986**, *261*, 10538.

(1636) Freitag, A.; Rudert, M.; Bock, E. *FEMS Microbiol. Lett.* **1987**, *48*, 105.

(1637) Bock, E., Wilderer, P.A.; Freitag, A. *Water Res.* **1988**, *22*, 245.

(1638) Freitag, A.; Bock, E. *FEMS Microbiol. Lett.* **1990**, *66*, 157.

(1639) There is at least one important exception that does not involve an oxygen atom transfer: the formate (HCO_2^-) oxidation to produce carbon dioxide (CO_2) catalysed by formate dehydrogenase.

(1640) Copley, S.D. *Bioessays* **2012**, *34*, 578.

CAPTIONS FOR FIGURES

Fig. 1: Nitrogen biochemical cycle.

Dinitrogen fixation, yellow arrow; assimilatory ammonification, orange arrows; "organic nitrogen pool", pink arrows; denitrification, blue arrows; dissimilatory nitrate reduction to ammonium (DNRA), green arrows; nitrification, black arrows; anaerobic ammonium oxidation (AnAmmOx), gray arrows; "denitrification/intra-aerobic methane oxidation", violet arrows.

Fig. 2: Nitrite in signalling pathways.

See text for details and abbreviations.

Fig. 3: *In vitro* haemoglobin/myoglobin reactivity towards nitrite, nitric oxide and related compounds.

The green numbers (inside the circles) refer to the reaction numbering in the text. (*) Reaction 14 initiates an autocatalytic radical chain reaction responsible for nitrite oxidation (see section 3.1.2.1. for details).

Fig. 4: Suggested cycle for the regeneration of $NAD(P)^+$ during hypoxia - potential *new* role for nitrite.

Figure modified from[1116].

Fig. 5: Chemical *view* of the nitrite handling in the nitrogen biochemical cycle and signalling pathways.

The pathways represented in figure 1 and 2 are here depicted as a whole series of nitrogen compounds, with oxidations states ranging from 5+ (nitrate) to 3- (ammonium), to emphasise the

redox chemistry involved in each step.

Signalling pathways, red arrows; dinitrogen fixation, yelow arrow; assimilatory ammonification, orange arrows; "organic nitrogen pool", pink arrows; denitrification, blue arrows; dissimilatory nitrate reduction to ammonium (DNRA), green arrows; nitrification, black arrows; anaerobic ammonium oxidation (AnAmmOx), gray arrows; "denitrification/intra-aerobic methane oxidation", violet arrows. The dotted lines represent reactions that do not involve nitrite handling.

Fig. 6: Structures relevant to nitrite handling.

(a) Haem structures. The structure of the porphyrin ring determines the haem classification as a *b*, *c*, d_1, sirohaem, P_{460}, among others types here not shown.

(b) Structure of type 1 copper centre. The T1 single copper is coordinated by two histidines and one cysteine residue, in a trigonal planar geometry, and by a variable axial ligand (A), while the T2 copper centre is coordinated in a similar geometry by nitrogen and/or oxygen atoms-containing ligands, but with no sulfur atom coordination.

(c) Molybdenum centres structures. (i) Structure of the pyranopterin cofactor. The cofactor is a pyranopterin-dithiolate moiety, which forms a five-membered ene-1,2-dithiolate chelate ring with the molybdenum atom; in eukaryotes, the cofactor is found in the simplest monophosphate form (R is an hydrogen atom), while in prokaryotes is found esterificated with several nucleotides (R can be one cytidine monophosphate, guanosine monophosphate or adenosine monophosphate). **(ii)** Structures of the molybdenum centres of the three families of molybdoenzymes; for simplicity, only the dithiolate moiety of the pyranopterin cofactor is represented. The xanthine oxidase family enzymes hold a L-Mo=X(–OH/OH_2)(=O) core, where L stands for the pyranopterin cofactor and X represents terminal =O, =S, =Se or –S–Cu–S–cysteine residue; the sulfite oxidase family has a L-Mo–S–cysteine residue (–OH/OH_2)(=O) core; the dimethylsulfoxide reductase family comprises a L_2-Mo-X(-Y) core, where X and Y represent terminal =O, –OH, =S, and –SH groups and/or oxygen, sulfur or selenium atoms from cysteine, selenocysteine, serine or aspartate residue side chains.

Fig. 7: Nitrite binding modes.

(a) Possible binding modes of nitrite to the metal atom (Me). Nitrite can bind the metal atom through the nitrogen atom, the so-called "nitro" mode; via one of the oxygen atoms, in a *trans* or *cis* conformation of the "nitrito" mode; or through both oxygen atoms, in a "bidentate nitrito" mode.

(b) Binding modes for each product of nitrite reaction expected *a priori* ; schematic representation (it is not intended to be a mechanistic representation). **(i)** To form ammonium, it would be expected that only the "nitro" binding mode is productive, in order to "remove" both oxygen atoms and "add" several protons without releasing intermediates. **(ii) - (v)** To synthesise NO, all the binding modes seem feasible, although the simplest possible mechanism would be the (ii), via a "nitrito" binding mode, involving only one N-O bond cleavage with "automatic" release of NO, remaining the abstracted oxygen atom bound to the metal, probably as a water molecule. **(vi)** To produce nitrate is necessary to "add" one oxygen atom and the simplest mechanism would be with a "nitro" binding mode to an oxo group of the metal (which, in the course of the reaction, would be "added" to the product molecule).

Fig. 8: Cytochrome *c*-containing nitrite reductase - penta-haemic enzyme.

(a) Three-dimensional structure view of the *Wolinella succinogenes* CcNiR homodimer (α helices and β sheets are shown in pink and yellow, respectively). The protein folds into one compact domain, with α-helices as the predominant secondary structural motif, ranging from short helical turns to four long helices at the C-terminal end of the peptide chain. **(b)** Haems arrangement in the same orientation as in (a). The five haems are numbered according to their attachment to the protein chain (shown on the monomer on the left); haem #1 is the catalytic center. The distances (Fe-to-Fe) between adjacent centres (expressed in Å) are shown on the monomer on the right. **(c)** Catalytic haem with the nitrite molecule bounded (haem is represented in dark red and the calcium atom in green). The structures shown in (a) and (b) are based on the PDB file 1FS7 [1276] (the images were obtained from www.rcsb.org/pdb/explore/jmol); the structure shown in (c) is based on the PDB file 2E80 [1327] (the image was produced with Accelrys DS Visualizer v2.0.1.7347, Accelrys Software Inc.).

Fig. 9: Mechanism of nitrite reduction to ammonium catalysed by cytochrome *c*-containing nitrite reductase.

See text for details. (*) When all the reducing substrate or nitrite is consumed, the enzyme returns to the oxidised resting state.

Fig. 10: Sirohaem-containing nitrite reductase.

(a) Three-dimensional structure view of the *Spinacia oleracea* CSNiR monomer (α helices and β sheets are shown in pink and yellow, respectively). The protein folds into three domains, in a fairly compact structure. The sirohaem and [4Fe-4S] centre are localised at the interface of the three domains, in a wide (>8Å) tunnel. **(b)** Sirohaem and [4Fe-4S] centre arrangement in the same orientation as in (a). The distance (sirohaem)Fe-to-(Fe/S)Fe of 4.6 Å is indicated. **(c)** Catalytic sirohaem (haem is represented in dark red). The structures shown are based on the PDB file 2AKJ [1345] (the images in (a) and (b) were obtained from www.rcsb.org/pdb/explore/jmol); the image in (c) was produced with Accelrys DS Visualizer v2.0.1.7347, Accelrys Software Inc.).

Fig. 11: Cytochrome d_1-containing nitrite reductase.

(a) Three-dimensional structure view of oxidised *Pseudomonas aeruginosa* Cd_1NiR homodimer (α helices and β sheets are shown in pink and yellow, respectively). The protein folds into two domains: (i) one typical bacterial α-helical cytochrome *c* N-terminal domain (on top), containing one *c* haem, and (ii) one eight-bladed β-propeller C-terminal domain (on bottom), comprising one d_1 haem. The N-terminal Ala_7-Pro_{29} loop is "swapped" between the two monomers; as a result, the Tyr_{10} of one monomer is found close to the d_1 haem of the *partner* monomer (*i.e.*, the Tyr_{10} of monomer 'A' is hydrogen-bonded to the hydroxyl group that coordinates the d_1 haem of monomer 'B'). **(b)** Haems arrangement in the same orientation as in (a), *c* haem on top and d_1 haem on bottom. The distance between the *c* and d_1 haem within a monomer (Fe-to-Fe of 19.7Å; edge-to-edge of 9.0 Å) is shown on the monomer on the right. **(c)** Catalytic haem with a hydroxyl

moiety bounded (haem is represented in dark red). The structures shown in (a) and (b) are based on the PDB file 1NIR [1404] (the images were obtained from www.rcsb.org/pdb/explore/jmol); the structure shown in (c) is based on the same PDB file (the image was produced with Accelrys DS Visualizer v2.0.1.7347, Accelrys Software Inc.).

Fig. 12: Mechanism of nitrite reduction to NO catalysed by cytochrome d_1-containing nitrite reductase.

See text for details. The *c* haem is represented as a blue box. The nitrite reduction reaction, taking place at the d_1 haem, is explicitly represented (except in 'a', where the d_1 haem is represented as a black box). The fast NO release is triggered by the electron transfer from *c* to d_1 haem, but the present data does not allow to discriminate if the NO is released from the "mixed-valence" protein (g→i), or from the fully reduced enzyme (h→c). (*) In the absence of reducing substrate or nitrite, the enzyme is trapped in a "dead-end" species (g).

Fig. 13: Copper-containing nitrite reductase.

(a) Three-dimensional structure view of the *Achromobacter cycloclastes* CuNiR homotrimer and monomer (on the left and right, respectively; α helices and β sheets are shown in pink and yellow, respectively). Each monomer folds into two eight-stranded β-barrel domains (domain I and II), stacked on each other, with one long and two short α-helical regions. The three monomers are tightly associated around a central channel of 5-6Å, with domains I positioned at the corners of the trimer and domains II forming the core of the molecule. Each monomer contains two different copper centres, T1 and T2. The T1 centre is located within domain I, buried 7Å beneath the protein surface. The T2 is found bound at the interface of adjacent monomers, in a cleft formed by apposition of domain II of one monomer and domain I of the adjacent monomer, at the bottom of a ≈12Å deep solvent channel. **(b)** Copper centres arrangement in the same orientation as in the homotrimer in (a); the three T1 and three T2 centres are identified. The distance (T1)Cu-to-(T2)Cu of 12.5 Å is indicated in one of the three "catalytic pairs". **(c)** T1 and T2 copper centres. The T2 copper centre (the active

site) is coordinated by three histidines: His_{100} and His_{135}, from domain I of monomer 'A', and His_{306}, from domain II of the adjacent monomer 'B'. The structures shown in (a) and (b) are based on the PDB file 2BW4 [1457] (the images were obtained from www.rcsb.org/pdb/explore/jmol); the structure shown in (c) is based on the PDB file 1NIA [1456] (the image was produced with Accelrys DS Visualizer v2.0.1.7347, Accelrys Software Inc.).

Fig. 14: Mechanism of nitrite reduction to NO catalysed by copper-containing nitrite reductase.

See text for details.

Fig. 15: Molybdenum-containing mammalian xanthine oxidase.

(a) Three-dimensional structure view of the bovine milk XO homodimer (α helices and β sheets are shown in pink and yellow, respectively). **(b)** Arrangement of the four redox centres shown in the same orientation as in (a). The four redox centres are identified on the monomer on the left (molybdenum, Fe/SI, Fe/SII and FAD centres) and the distances between adjacent centres (expressed in Å) are shown on the monomer on the right. **(c)** Molybdenum catalytic centre (pyranopterin cofactor is represented in dark red). The structures shown are based on the PDB file 1FO4 [1531] (the images in (a) and (b) were obtained from www.rcsb.org/pdb/explore/jmol); the image in (c) was produced with Accelrys DS Visualizer v2.0.1.7347, Accelrys Software Inc.).

Fig. 16: Mechanism of nitrite reduction to NO catalysed by xanthine oxidase.

See text for details.

FIGURES

Fig. 1: Nitrogen biochemical cycle.

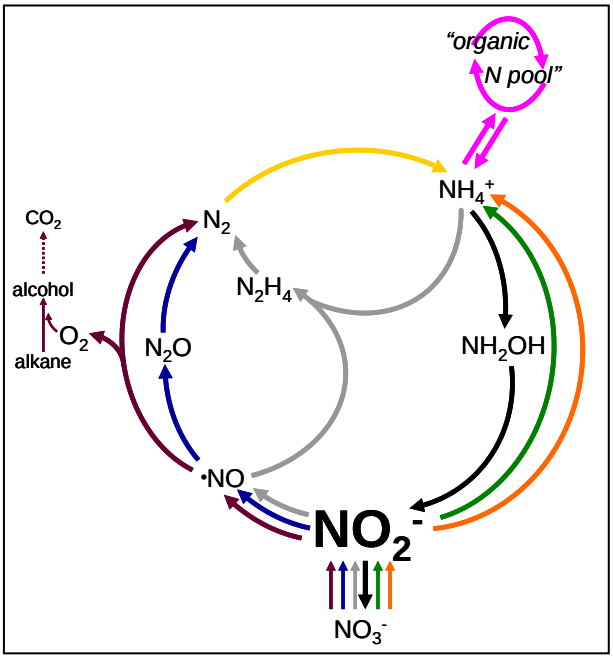


Fig. 2: Nitrite in signalling pathways.

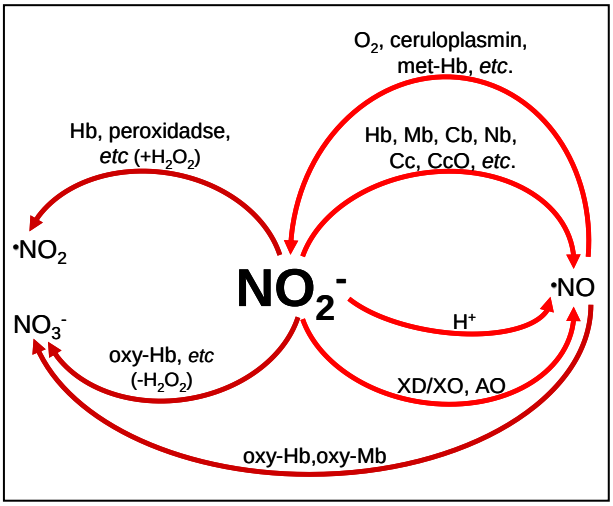


Fig. 3: *In vitro* haemoglobin/myoglobin reactivity towards nitrite, nitric oxide and related compounds.

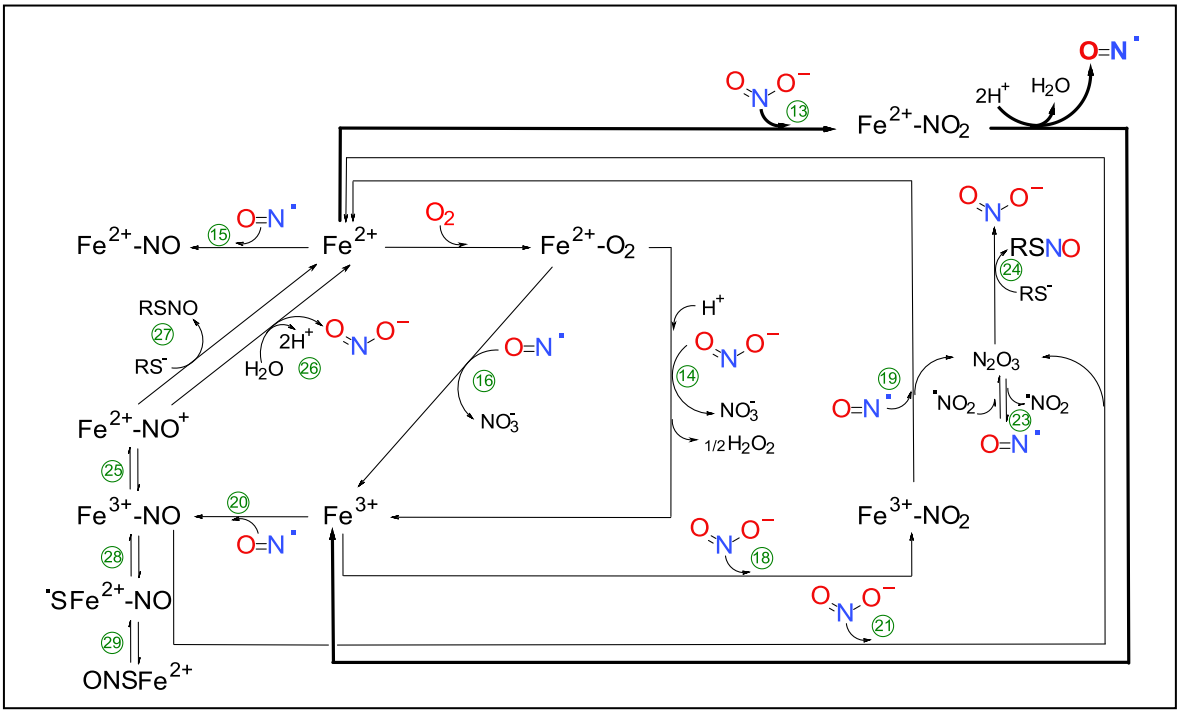


Fig. 4: Suggested cycle for the regeneration of NAD(P)$^+$ during hypoxia - potential *new* role for nitrite.

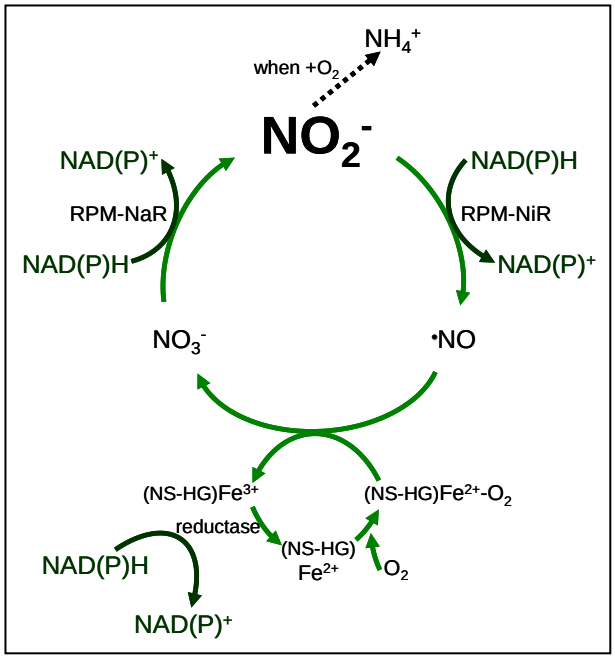


Fig. 5: Chemical *view* of the nitrite handling in the nitrogen biochemical cycle and signalling pathways.

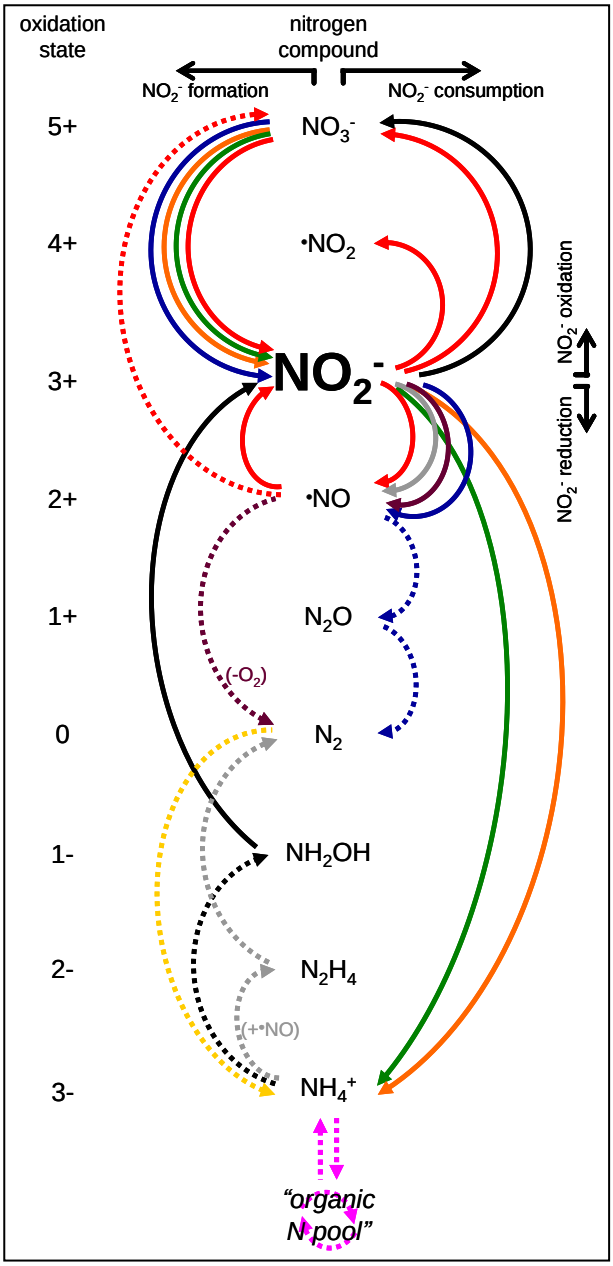


Fig. 6: Structures relevant to nitrite handling.

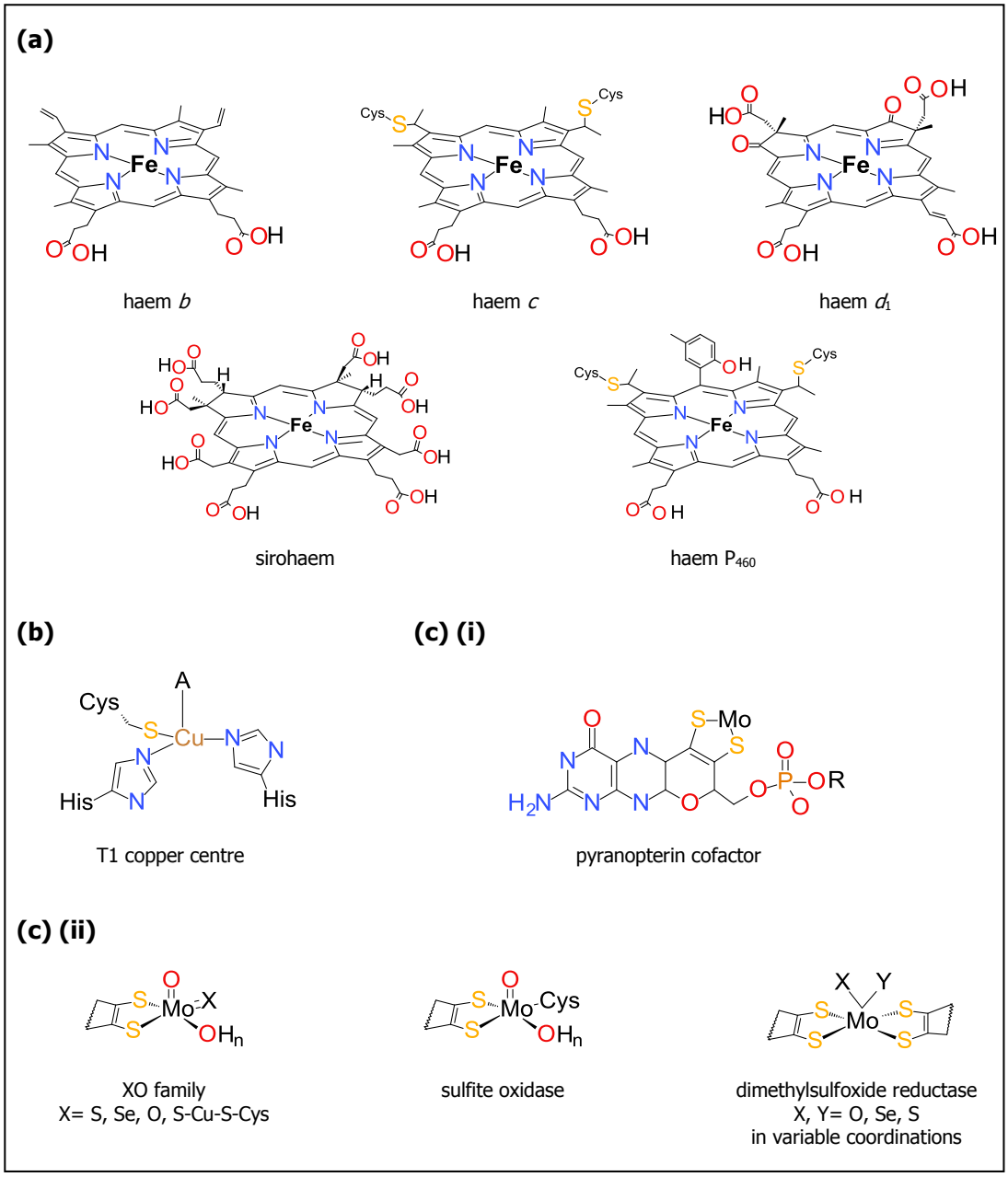


Fig. 7: Nitrite binding modes.

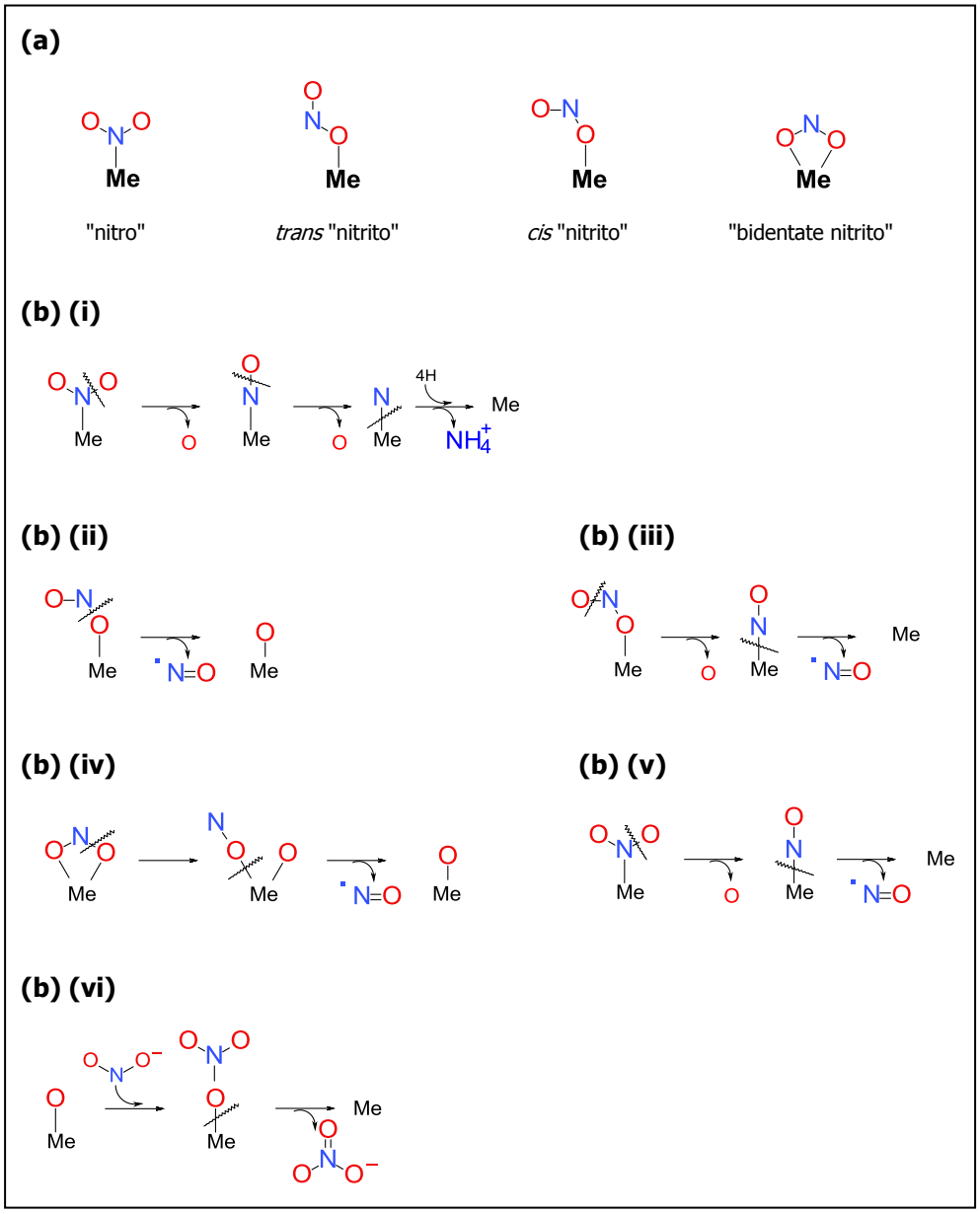


Fig. 8: Cytochrome *c*-containing nitrite reductase - penta-haemic enzyme.

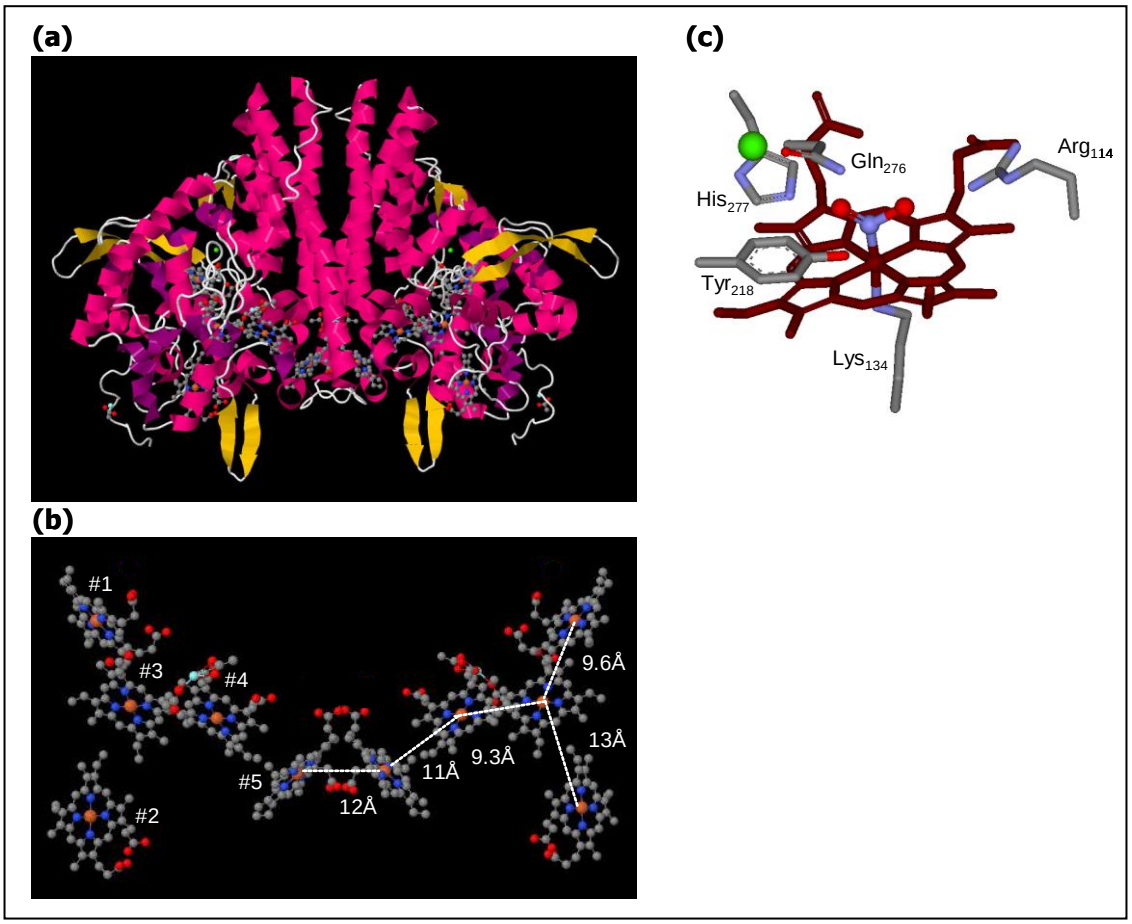


Fig. 9: Mechanism of nitrite reduction to ammonium catalysed by cytochrome *c*-containing nitrite reductase.

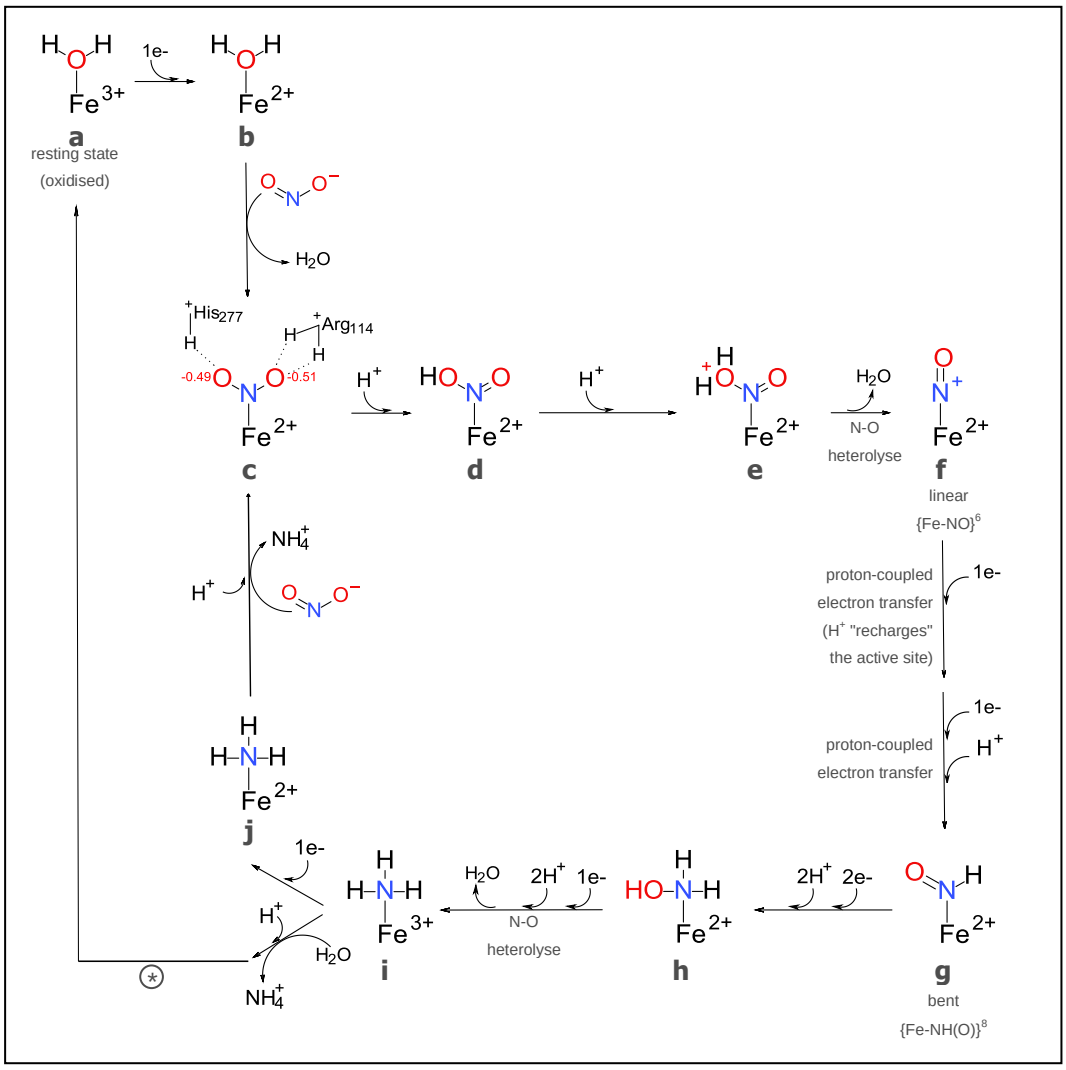


Fig. 10: Sirohaem-containing nitrite reductase.

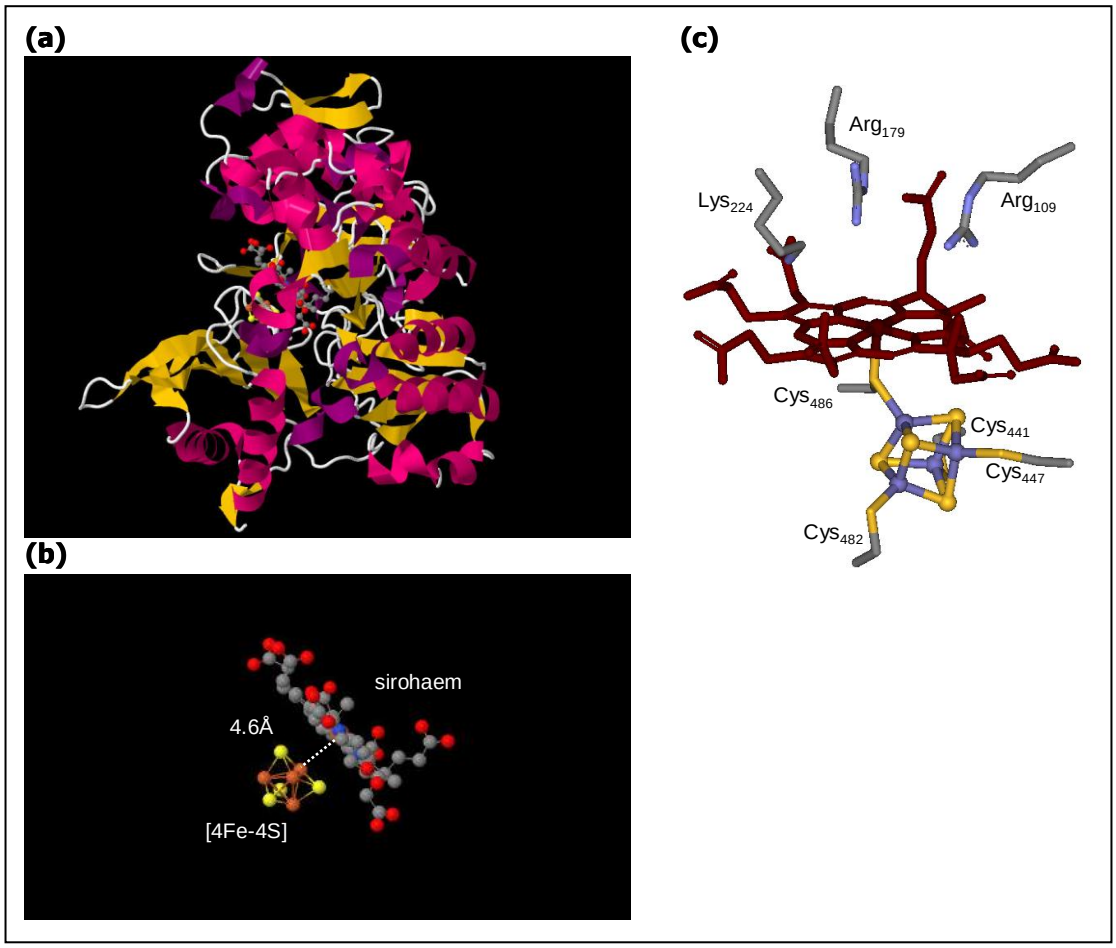


Fig. 11: Cytochrome d_1-containing nitrite reductase.

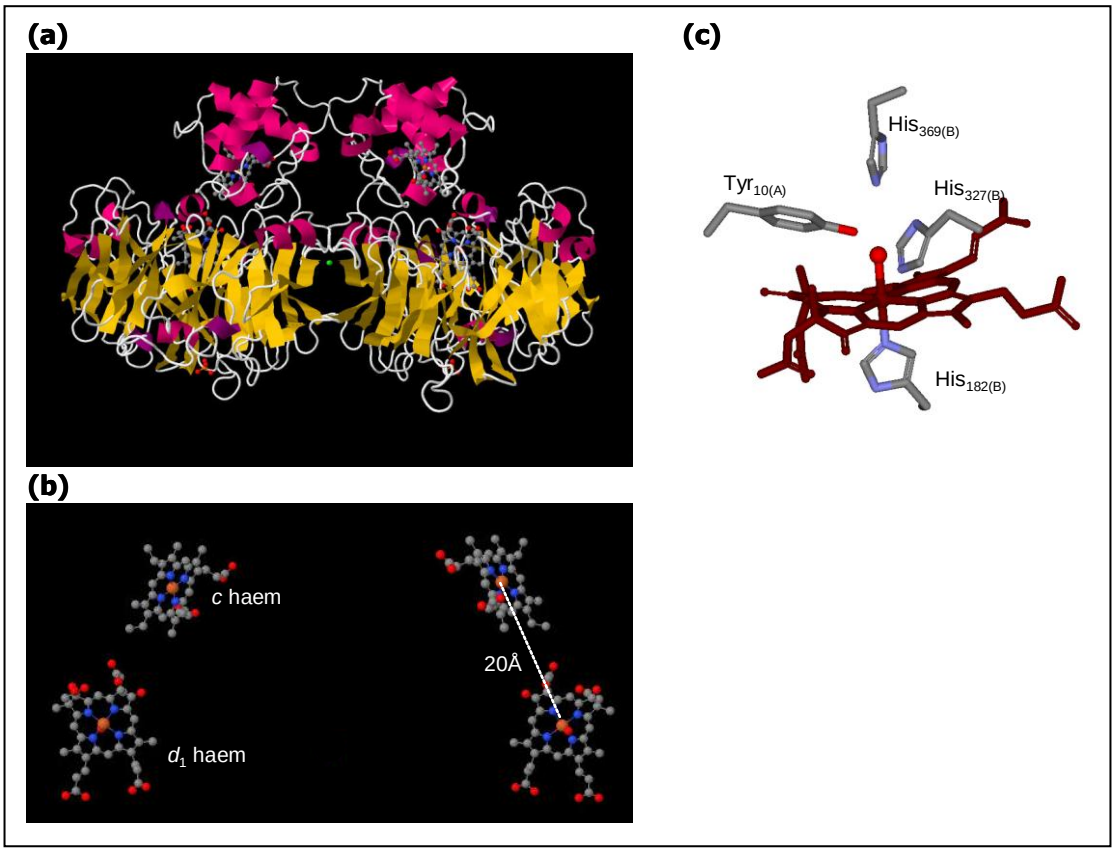


Fig. 12: Mechanism of nitrite reduction to NO catalysed by cytochrome d_1-containing nitrite reductase.

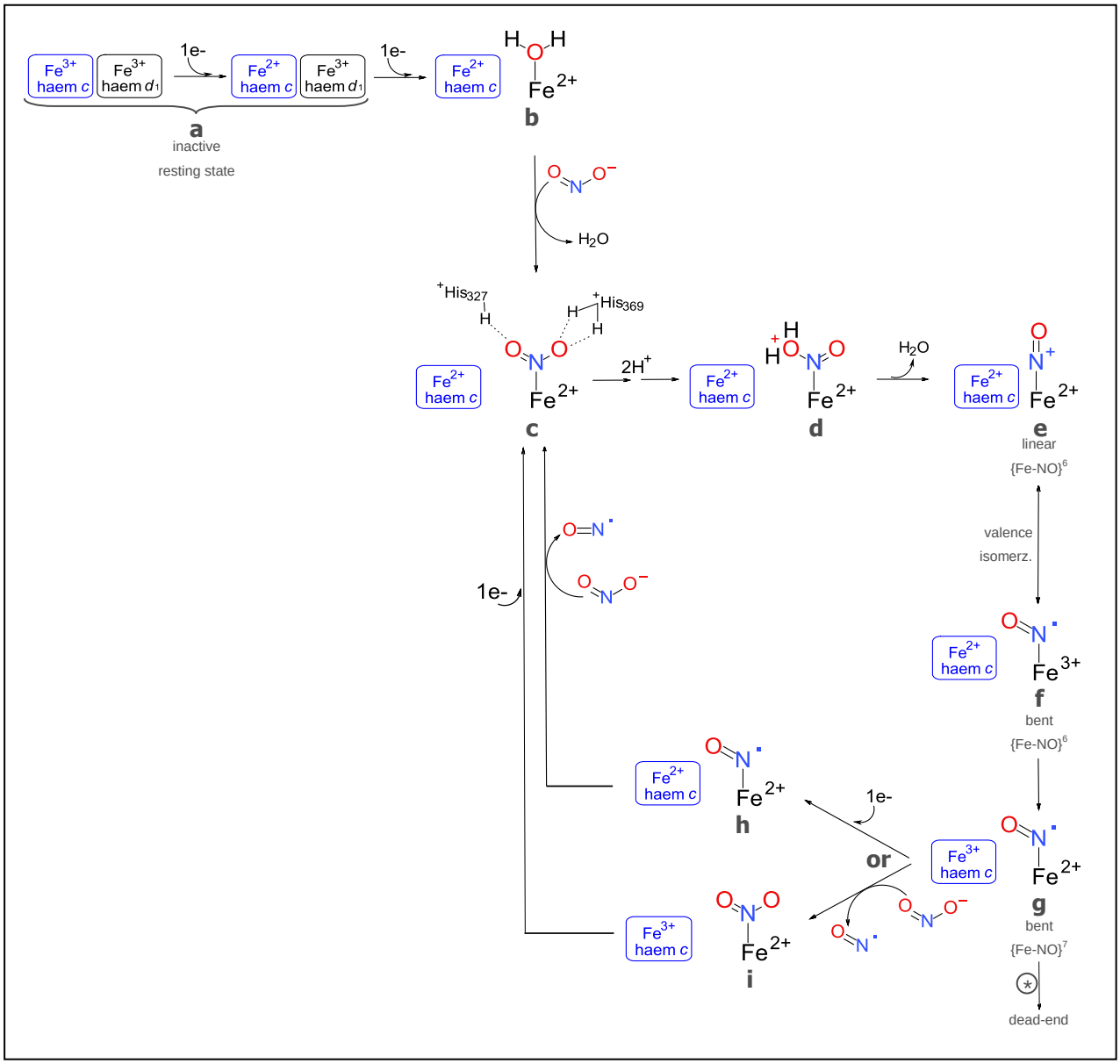


Fig. 13: Copper-containing nitrite reductase.

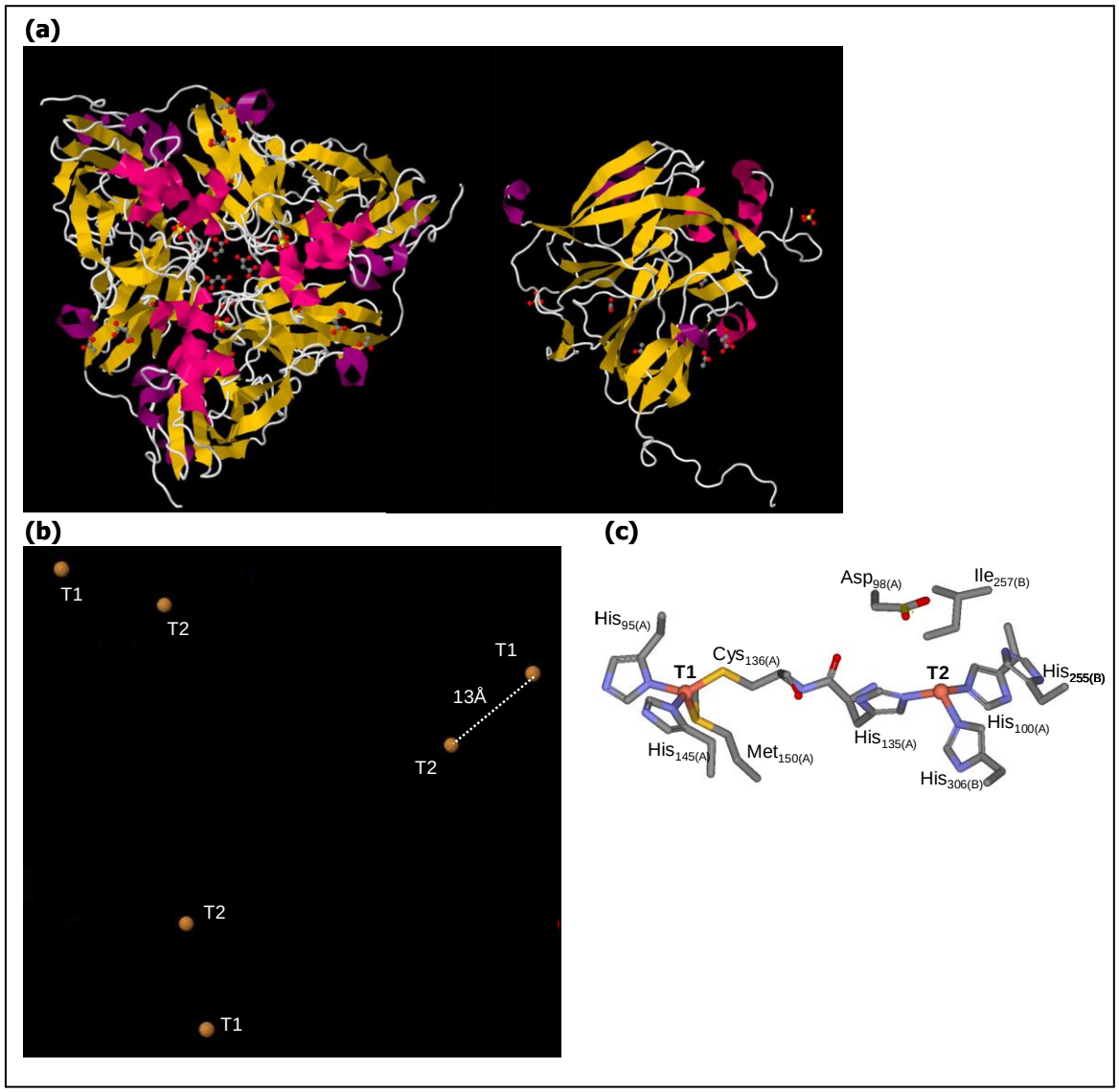


Fig. 14: Mechanism of nitrite reduction to NO catalysed by copper-containing nitrite reductase.

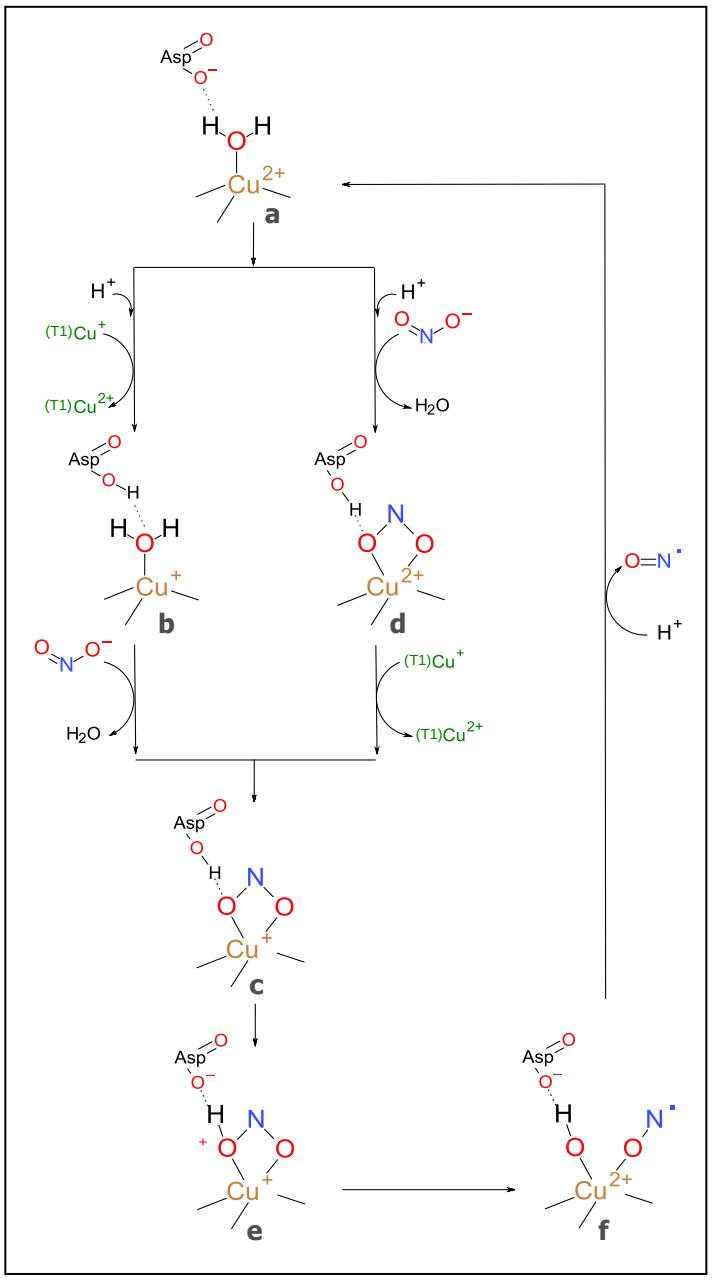


Fig. 15: Molybdenum-containing mammalian xanthine oxidase.

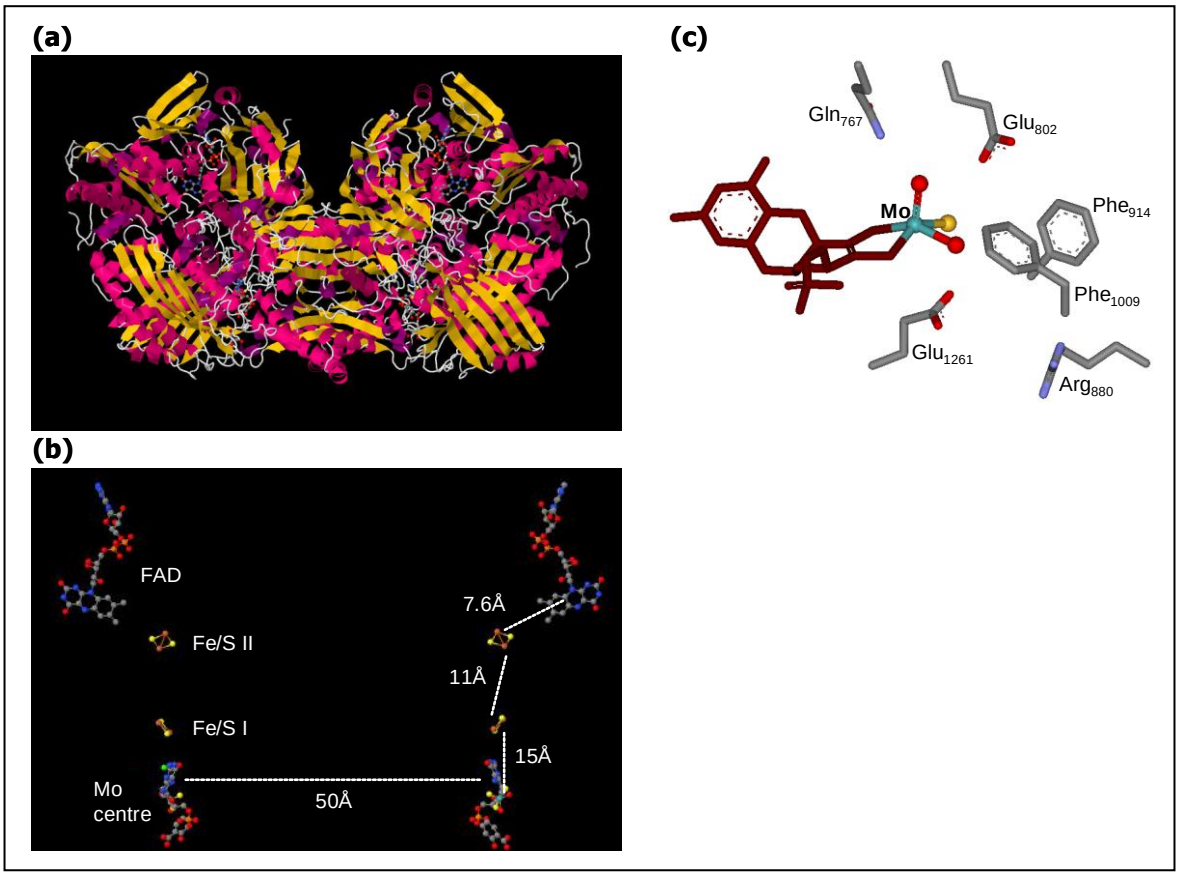


Fig. 16: Mechanism of nitrite reduction to NO catalysed by xanthine oxidase.