

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/6394844>

# Accommodation of NO in the active site of mammalian and bacterial cytochrome c oxidase aa<sub>3</sub>

ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · JUNE 2007

Impact Factor: 4.66 · DOI: 10.1016/j.bbabo.2007.03.001 · Source: PubMed

CITATIONS

7

READS

14

## 4 AUTHORS:



**Eric Pilet**

Pierre and Marie Curie University - Paris 6

18 PUBLICATIONS 253 CITATIONS

SEE PROFILE



**Wolfgang Nitschke**

French National Centre for Scientific Resea...

152 PUBLICATIONS 3,806 CITATIONS

SEE PROFILE



**Ursula Liebl**

MINES ParisTech

84 PUBLICATIONS 1,809 CITATIONS

SEE PROFILE



**Marten H Vos**

École Polytechnique

142 PUBLICATIONS 3,665 CITATIONS

SEE PROFILE

# Accommodation of NO in the active site of mammalian and bacterial cytochrome *c* oxidase *aa*<sub>3</sub>

Eric Pilet<sup>a,b,1</sup>, Wolfgang Nitschke<sup>c</sup>, Ursula Liebl<sup>a,b</sup>, Marten H. Vos<sup>a,b,\*</sup>

<sup>a</sup> Laboratoire d'Optique et Biosciences, CNRS, Ecole Polytechnique, F-91128 Palaiseau Cedex, France

<sup>b</sup> INSERM U696, F-91128 Palaiseau, France

<sup>c</sup> Laboratoire de Bioénergétique et Ingénierie des Protéines UPR 9036, Institut de Biologie Structurale et Microbiologie, CNRS, 31, Chemin Joseph Aiguier, 13402 Marseille, Cedex 20, France

Received 11 January 2007; received in revised form 23 February 2007; accepted 1 March 2007

Available online 12 March 2007

## Abstract

Following different reports on the stoichiometry and configuration of NO binding to mammalian and bacterial reduced cytochrome *c* oxidase *aa*<sub>3</sub> (CcO), we investigated NO binding and dynamics in the active site of beef heart CcO as a function of NO concentration, using ultrafast transient absorption and EPR spectroscopy. We find that in the physiological range only one NO molecule binds to heme *a*<sub>3</sub>, and time-resolved experiments indicate that even transient binding to Cu<sub>B</sub> does not occur. Only at very high (~2 mM) concentrations a second NO is accommodated in the active site, although in a different configuration than previously observed for CcO from *Paracoccus denitrificans* [E. Pilet, W. Nitschke, F. Rappaport, T. Soulimane, J.-C. Lambry, U. Liebl and M.H. Vos. *Biochemistry* 43 (2004) 14118–14127], where we proposed that a second NO does bind to Cu<sub>B</sub>. In addition, in the bacterial enzyme two NO molecules can bind already at NO concentrations of ~1 μM. The unexpected differences highlighted in this study may relate to differences in the physiological relevance of the CcO–NO interactions in both species.

© 2007 Elsevier B.V. All rights reserved.

**Keywords:** Cytochrome *c* oxidase; Nitric oxide; Femtosecond spectroscopy; Electron paramagnetic resonance

## 1. Introduction

During the last decade, many groups have shown that nitric oxide (NO) is implicated in respiratory regulation by reversibly inhibiting cytochrome *c* oxidase *aa*<sub>3</sub> *in vivo* [1–4]. Nanomolar concentrations of NO have been found to inhibit cytochrome oxidase reversibly and competitively with molecular oxygen (O<sub>2</sub>). This has immediately raised questions about a potential physiological role of this inhibitory effect in the control of cell respiration, and about possible pathological implications. One key question is the nature of this inhibition.

The active site of cytochrome *c* oxidase (CcO) comprises a heme (heme *a*<sub>3</sub>) and a nearby (~5 Å) copper atom (Cu<sub>B</sub>),

coordinated by 3 histidine residues. In principle both heme *a*<sub>3</sub> and Cu<sub>B</sub> can bind external ligands. While it had been known for many years that NO binds to reduced heme *a*<sub>3</sub> [5], more recently it has been demonstrated that NO interacts with *aa*<sub>3</sub> oxidase in different redox states, and in particular with Cu<sub>B</sub> in the oxidized complex, drawing a more complex picture of the CcO:NO interaction [6,7]. Based upon the homology between CcO and nitric oxide reductase (an enzyme requiring two NO molecules for its function [8–10]) and the known interaction between NO and Cu<sub>B</sub><sup>2+</sup> [11], it appears that two NO molecules can be present simultaneously in the active site. For reduced CcO, this was corroborated by infrared absorption [12] and EPR [13] studies on the mammalian enzyme (CcO<sub>m</sub>) at high NO concentration, and time-resolved visible absorption and EPR measurements on bacterial *aa*<sub>3</sub> oxidases at physiological and high NO concentration [14,15]. In contrast, NO-uptake titrations [16–18] indicate that only one NO molecule can be bound in the active site of mammalian CcO, and the involvement of only one NO molecule in its inhibition has

\* Corresponding author. Laboratoire d'Optique et Biosciences, CNRS, Ecole Polytechnique, F-91128 Palaiseau Cedex, France. Tel.: +33 169085066; fax: +33 169085084.

E-mail address: [Marten.Vos@polytechnique.edu](mailto:Marten.Vos@polytechnique.edu) (M.H. Vos).

<sup>1</sup> Present address: LCBM/iRTSV/CEA, 38054 Grenoble cedex 09, France.

been demonstrated [4]. In order to further address this discrepancy, we investigated the effect of NO concentration on  $CcO_m$  by time resolved absorption and EPR spectroscopy.

Cytochrome *c* oxidase  $aa_3$  from *Paracoccus denitrificans* ( $CcO_{Pd}$ ) is one of the closest bacterial homologues to mammalian oxidase [19]. Thus, results obtained with  $CcO_{Pd}$  have generally been extended to the mammalian enzyme complex, in particular those of mutagenesis experiments on proton pathways to and out of the active site, although some differences have been implied [20]. In the present study, we provide evidence that both  $aa_3$  oxidases react differently with NO at physiological (micro-/nanomolar) NO concentrations:  $CcO_{Pd}$  is able to bind two NO molecules in its active site, whereas  $CcO_m$  apparently can accommodate only one NO molecule.

## 2. Materials and methods

Native four-subunit  $CcO$   $aa_3$  from wild-type *P. denitrificans* was purified as described [15] from cells grown at low manganese concentrations [21] and resuspended in 50 mM Tris/HCl, pH 8.0, 100 mM NaCl with 0.03%  $\beta$ -dodecylmaltoside (DM). The mammalian oxidase was purified from beef heart mitochondria following van Buuren's method [22] with minor modifications as described in [23].  $CcO_m$  was finally solubilized in 0.1% DM and 50 mM Tris/HCl, pH 7.4. The enzyme concentrations used were 35  $\mu$ M for the femtosecond absorption experiments and 50  $\mu$ M for the EPR experiments. The samples were extensively degassed in a gastight vial using a home-built stainless steel gas train and reduced using 10 mM sodium ascorbate and (in some experiments) 10  $\mu$ M ruthenium hexamine. The samples (volumes 80  $\mu$ L for the optical experiments and 250  $\mu$ L for the EPR experiments) were transferred into a degassed gastight optical cell (Hellma, 117.007 QS, optical pathlength of 1 mm) or a degassed EPR tube sealed with a rubber septum. The indicated stoichiometries refer to the ratios of NO and enzyme (in nanomoles) present in the sample cell. For NO: enzyme stoichiometries  $\gg 1$ , the NO concentration in the liquid phase is indicated (1 atm. NO in the gas phase corresponds to 2 mM in solution). The sample was nitrosylated either by addition of defined volumes of 1% NO gas (in argon) using gastight microliter syringes as described in [14], or by equilibration with 1% or 100% NO gas, and was allowed to equilibrate for 30 min.

Static absorption spectra were recorded using a Shimadzu 1601 UV-vis spectrophotometer. Femtosecond spectroscopy using 55 fs pump pulses centered at 590 nm and white-light continuum probe pulses was performed as described elsewhere [14,24,25], with a 1-m delay line. All visible absorption spectroscopy was performed at 20 °C.

EPR spectra were taken on a Bruker ElexSys X-band spectrometer fitted with an Oxford Instrument liquid helium cryostat and temperature control system. The redox and ligation state of the sample in the EPR tube was monitored spectrophotometrically immediately before freezing in a Kontron Uvikon 922 UV-vis spectrophotometer equipped with an integrating sphere.

## 3. Results

### 3.1. Static absorption spectra

The static spectra of fully reduced unliganded and NO-liganded  $CcO_m$  are shown in Fig. 1. The spectrum of the NO-liganded form is very similar to that previously described [13] showing a double peak in the Soret region at 428 and 444 nm, corresponding to nitrosylated heme  $a_3$ , and heme  $a$  (that does not bind external ligands), respectively, and a main peak in the  $\alpha$ -band region at 602 nm. The static reduced and reduced nitrosylated (NO-saturated) spectra of the mammalian (Fig. 1A) and bacterial (Fig. 1B, [14]) enzymes are practically identical.

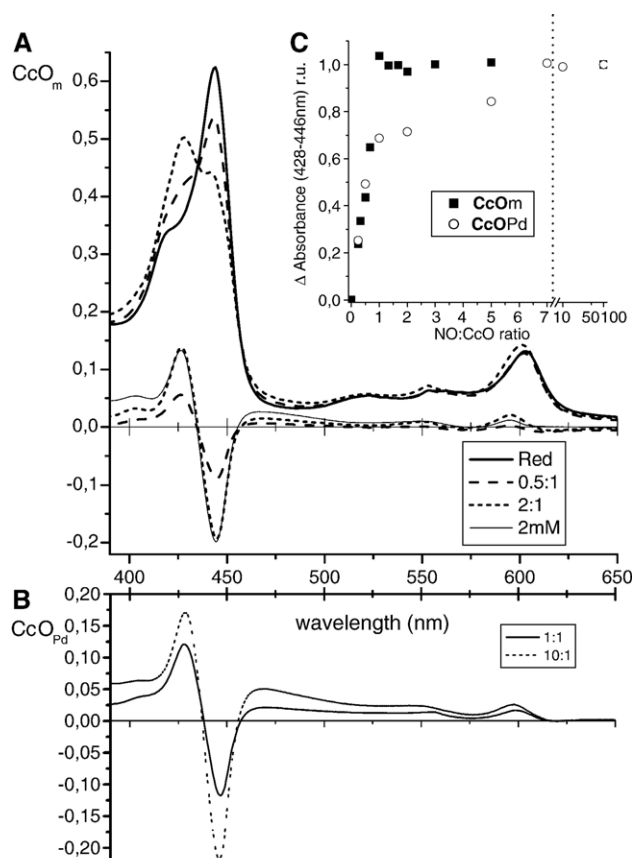


Fig. 1. Static absolute and difference spectra of cytochrome *c* oxidase  $aa_3$  from mammalian mitochondria (A) and difference spectra from *P. denitrificans* (B) in the reduced state with increasing quantities of nitric oxide (NO). (C) Relative amplitude of NO binding to reduced cytochrome *c* oxidase from mitochondria (filled squares) and from *P. denitrificans* (open circles).

However, their overall binding properties are quite different (Fig. 1C). In the sub-stoichiometric range, NO binding to  $CcO_m$  is linear at low NO concentrations and saturates at 1:1 stoichiometry, implying that the NO affinity is less than  $\sim 150$  nM, consistent with earlier reports [4,11]. By contrast, the titration curve for  $CcO_{Pd}$  is more sluggish and the fully bound spectrum is only observed at a  $\sim 7:1$  NO:enzyme ratio. We have previously suggested that this behavior is due to high-affinity ( $< 150$  nM) uptake of one NO by the binuclear site and its equilibration between binding to heme  $a_3$  and accommodation at another site (likely  $Cu_B$ ), as well as a lower-affinity ( $\sim 1$   $\mu$ M) uptake of a second NO molecule [14,15]. A very low NO-reductase activity of the bacterial enzyme might also contribute to the sluggish shape of the titration curve, although we are not aware of a report of such an activity in  $CcO_{Pd}$ , and our overall analysis is consistent with a lack of NO-reductase activity (see Discussion).

### 3.2. Picosecond NO Rebinding to $CcO$ $aa_3$ from beef heart mitochondria

The picosecond geminate rebinding kinetics of NO with heme  $a_3$  of  $CcO_{Pd}$  can be fitted with a 200-ps decay phase and a non-decaying phase. The relative amplitude of the decay phase

increases with NO concentration ([14], Fig. 2, inset). By contrast, our present kinetic measurements indicate that, in CcO<sub>m</sub>, the NO concentration does not affect the dynamics of NO rebinding (Fig. 2). For all NO concentrations tested (from substoichiometric to 2 mM), the kinetics can be fitted with a monoexponential decay with a  $250 \pm 30$  ps time constant (90%) and an asymptotic phase (10%). The shape of the transient spectra remains unchanged between 30 ps and 2 ns, as was observed for the bacterial enzyme [14]. Equally, the shape of the transient spectra and the quantum yield of NO photodissociation (based on comparison with the total heme bleaching immediately after excitation) is the same, independent of the source of the enzyme (data not shown).

### 3.3. EPR experiments

In the light of the results obtained on the *P. denitrificans* enzyme, the question arises whether the site from which the 250 ps rebinding phase originates in the bovine aa<sub>3</sub> oxidase harbors one or two NO molecules. We therefore registered EPR spectra in a range of sub- and superstoichiometric NO:enzyme ratios (Fig. 3) in a way similar to that previously reported for *P. denitrificans* oxidase [14]. At substoichiometric NO concentrations, a spectrum with  $g$ -values of  $g_z=2.09$ ,  $g_y=2.005$  and  $g_x=1.97$  was obtained, featuring a prominent 9-line hyperfine pattern on the  $g_y$  line. This spectrum is similar to those published in the past on the nitrosylated bovine aa<sub>3</sub> oxidase [26,27]. The 9-line structure is due to a hyperfine triplet [ $A_{\text{HF}}=20.5$  mT] brought about by interaction of the unpaired electron spin with the  $I=1$  nucleus of the NO moiety. This triplet is further split by a so-called superhyperfine pattern

( $A_{\text{SHF}}=6.6$  mT) due to unpaired electron density reaching through the heme plane towards the binding nitrogen atom of the axial histidine ligand. The observed spectrum is attributed to a 6-coordinated nitrosylated heme in which NO is positioned in a way that the nitrogen p-orbitals of the trans histidine ligand and the NO molecule are parallel to each other [14].

Similar  $g$ -values and a comparable hyperfine pattern were observed at highly substoichiometric NO/aa<sub>3</sub> ratios in the *P. denitrificans* oxidase [14]. Titrating a second NO molecule into the site, however, transformed the 9-hyperfine line spectrum into a 3-line spectrum (Fig. 3, lower part). It is of note that the 0.3:1 spectrum in Fig. 3 already contains contributions of a population where two NO molecules occupy the pocket, as discussed in [14]. The observed spectral change was interpreted as resulting from steric hindrance imposed by this second NO molecule (most likely bound to Cu<sub>B</sub>) on the heme-nitrosyl. We proposed that rotating the nitrosyl ligand away from its unperturbed position abolishes the superhyperfine trans-effect and thus wipes out the 6.6 mT interaction with the histidine nitrogen nucleus [14].

For the bovine enzyme, by contrast, such spectral changes were not observed (Fig. 3, upper part). The 9-line spectrum only grew in intensity without changing shape. After reaching saturation, the spectrum remained unchanged up to at least 20  $\mu\text{M}$  NO. At 2 mM NO, the signal completely disappeared and only a simple spectrum remained in the  $g=2$  region. As demonstrated in Fig. 3, this feature is essentially due to the ruthenium ion present as mediator in this sample. The disappearance of the heme-nitrosyl EPR spectrum at very high NO concentrations has already been reported in the past [13] and is considered to result from the antiferromagnetic

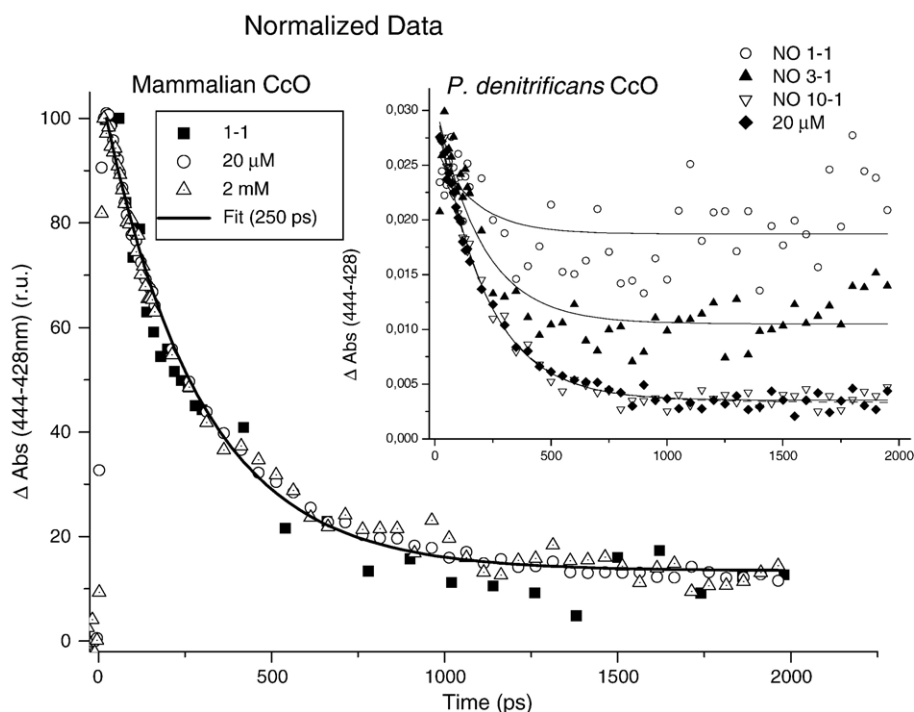


Fig. 2. Kinetics of geminate recombination of NO and heme a<sub>3</sub> after photodissociation at various NO:CcO ratios in CcO aa<sub>3</sub> from mammalian mitochondria and *P. denitrificans* (inset). In both panels, the amplitude of the kinetics are normalized at  $t=30$  ps. Lines are fits.



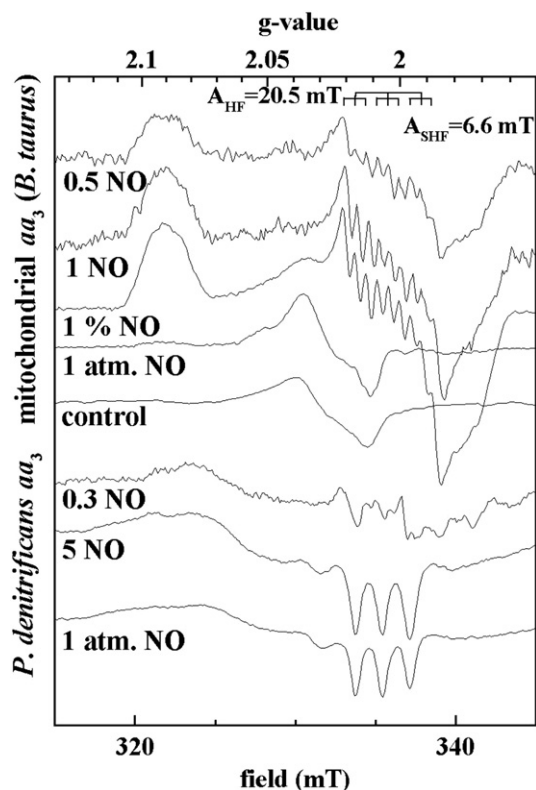


Fig. 3. EPR spectra recorded on reduced CcO  $aa_3$  from beef heart mitochondria (top spectra) and from *P. denitrificans* (bottom spectra) at various NO concentrations, as indicated for each spectrum either as NO:enzyme ratio or as NO concentration in the solution. The top two spectra are taken from a series of experiments on samples equilibrated overnight without redox mediators whereas the remaining three spectra on the bovine enzyme are part of a different series containing ruthenium hexamine as mediator to facilitate equilibration. Both series overlap in the 1 to 10 NO:CcOm region. The control sample for the mitochondrial oxidase experiments contained the buffer, 10 mM sodium ascorbate and 10  $\mu$ M ruthenium hexamine, the latter yielding the remnant EPR signal (cf. [37,38]). Instrument settings: temperature, 50 K; microwave frequency, 9.42 GHz; microwave power, 6.7 mW; modulation amplitude, 0.1 mT.

interaction of two NO moieties [26]. A signal in the  $g=4$  region as observed in the *P. denitrificans* enzyme at superstoichiometric NO concentrations and attributed to NO bound to reduced  $Cu_B$  [14] was absent at all NO concentrations analysed.

In conclusion, the results presented here demonstrate that only one NO molecule is present in mammalian cytochrome  $c$  oxidase  $aa_3$ , bound to heme  $a_3$  under moderate NO concentrations, up to the micromolar regime.

## 4. Discussion

### 4.1. NO geminate recombination in mitochondrial CcO

Our results provide a first assessment of sub-nanosecond NO geminate recombination in mammalian  $aa_3$  oxidase. They show that 90% of the dissociated NO rebinds in 250 ps, presumably from the active site pocket or its close environment. Previous nanosecond spectroscopic measurements have shown that additional partial recombination phases occur with time

constants of  $\sim 10$  ns and 100 ns [39]. These phases correspond to the 10% NO escaping from the active site and may reflect the presence of additional intra-protein docking sites. Recombination phases on similar time scales have also been observed in heme-copper oxidases from *P. denitrificans* and *Thermus thermophilus* [14].

### 4.2. Accommodation of NO in the active site of mitochondrial CcO

Previous experiments on CcO  $aa_3$  from *P. denitrificans* have shown an NO:enzyme stoichiometry-dependent EPR signal, indicating that two NO molecules can bind simultaneously to heme  $a_3$  and to  $Cu_B$ , even at stoichiometric concentrations [14]. The kinetics of NO rebinding to heme  $a_3$  varied similarly with this stoichiometry [14,15]. The increased yield of picosecond recombination at higher NO concentrations indicates that occupation of the  $Cu_B$  site by NO closes the exit gate of the active site for heme-dissociated NO. In the present study, analogous experiments performed on mammalian CcO  $aa_3$  give different results. Here, EPR steady-state NO titrations of the reduced enzyme show a single type signal corresponding to that observed for substoichiometric NO concentrations in the bacterial enzyme, and very similar to that previously reported for low NO concentrations in the mitochondrial enzyme [13] that saturates at a 1:1 stoichiometry. Only at very high NO concentrations ( $\sim 2$  mM) the effect of a second NO accommodated in the active site is observed. The disappearance of the EPR signal in this case implies antiferromagnetic coupling between the two NO molecules. This is different from the two-NO effect observed already at much lower concentrations in the bacterial oxidase, where the heme-bound NO is thought to rotate under the effect of the  $Cu_B$ -bound NO [14]. Altogether this indicates that the mitochondrial active site is less prone to accommodate a second NO molecule at the  $Cu_B$  site under our experimental conditions.

Interestingly, the rebinding kinetics for mitochondrial CcO with one molecule NO ( $\sim 90\%$  rebinding in 250 ps), are very similar to those for *P. denitrificans* oxidase under conditions where two NO molecules are present. However, under conditions where one NO molecule is present in *P. denitrificans*  $aa_3$  oxidase and in *T. thermophilus*  $ba_3$  oxidase, where a two-NO containing active site cannot be built up, no picosecond rebinding is observed [14]. This is presumably because the dissociated NO molecule binds to the  $Cu_B$  site, from where it is not released on a picosecond timescale. These findings strongly suggest that in mitochondrial CcO, despite its  $Cu_B$  site being unoccupied, dissociated NO does not transiently bind to  $Cu_B$  within 250 ps: at NO concentrations up to 2 mM, the rebinding behavior in the mitochondrial enzyme is similar to that when the  $Cu_B$  site is occupied, as in the *P. denitrificans* enzyme at superstoichiometric NO concentrations. Therefore, the intrinsic affinity of NO to  $Cu_B$  appears to be much lower (at least  $\sim 1000$  fold) in the mitochondrial enzyme. The absence of the EPR signal provisionally attributed to  $Cu_B$ -NO [14] further indicates that the second NO in the mammalian oxidase does not bind at

the copper site even at 2 mM NO, at least not in the same way as in CcO from *P. denitrificans*.

One might suggest that only a different, more constrained and therefore lower-affinity, configuration of Cu<sub>B</sub>-NO can be populated in the mammalian enzyme. Here, the Cu<sub>B</sub>-bound NO would be in closer interaction with the heme-bound NO, resulting in the antiferromagnetic coupling responsible for the disappearance of the heme-NO EPR signal [13,26]. Such a configuration might also correspond to the infrared absorption assigned to Cu<sub>B</sub>-NO in dithionite-reduced mitochondrial CcO [12].

#### 4.3. Relation with NO reductase activity

Cytochrome *c* oxidase is thought to have evolved from nitric oxide reductase (NOR). This scenario is supported by the O<sub>2</sub>-reductase activity of some NOR [28] and the NO-reductase activity of several CcO [29,30]. The active site of NOR contains heme, and an Fe atom instead of Cu<sub>B</sub>. Its function (catalysis of the  $2\text{NO} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$  reaction) requires that two NO molecules can be accommodated in the fully reduced active site. Low NO reductase activity has been reported for the bacterial oxidases *ba*<sub>3</sub> and *caa*<sub>3</sub> from *T. thermophilus* [17], and *cbb*<sub>3</sub> from *Pseudomonas stutzeri* [29] at micromolar NO concentrations; we are not aware of any assessments for the *P. denitrificans* *aa*<sub>3</sub> oxidase. Low NO reductase activity has also been reported for mammalian CcO at high NO concentrations [13]; a report of such activity at low NO concentrations using infrared spectroscopy [31] appears to be in contrast with later amperometric measurements [18]. Our conclusion that two NO molecules can be co-present in the active site of the bacterial enzyme at relatively low concentrations, but in the mammalian enzyme only at very high concentrations are in general agreement with the ensemble of these findings.

#### 4.4. Differences in interaction with NO

Altogether our findings indicate a difference in interaction with NO between reduced mammalian and bacterial cytochrome *c* oxidase *aa*<sub>3</sub>. Possible reasons for this difference will be discussed below.

Bacterial respiratory-chain complexes are considered good models for their mitochondrial counterparts as e.g. their terminal electron acceptor complex, CcO, reduces O<sub>2</sub> in the same way as the mitochondrial enzyme [32], although differences have been proposed on the level of proton channeling [20] or, as described in the present work, NO binding. Indeed, close inspection of the aligned structures (not shown) of the fully oxidized (structures of other forms are not available for the bacterial enzyme) active sites of CcO<sub>m</sub> (PDB entry 1V54 [33]) and the four subunit form of CcO<sub>pd</sub> (PDB entry 1QLE) revealed very similar relative positions of the catalytic residues and cofactors, considering that the now-established [34] cross-link of His<sub>276</sub> and Tyr<sub>280</sub>, was not yet taken into account during refinement of the four subunit structure. If the observed differences in interaction with NO are due to structural parameters, these steric differences must be

more subtle than can be retrieved from the present X-ray structures. More relevant comparisons must also await the availability of high-resolution structures of the respective nitrosylated forms. We note, however, that significant changes in the active site have been reported between the four- and two-subunit complexes of the *P. denitrificans* enzyme [34]. It therefore seems possible that differences in the environment of the catalytic subunit I between the four-subunit bacterial and the thirteen-subunit mammalian enzyme give rise to differences in the flexibility of the active site.

The differences observed also raise the question of a possible physiological importance of our observations. In *P. denitrificans* NO is expected to have only a marginal regulatory role at the level of CcO *aa*<sub>3</sub> [35]. Differently, in mitochondrial oxidase a strong pressure exists to avoid the stable binding of NO to Cu<sub>B</sub>, and thus the putative interaction with O<sub>2</sub> or an O<sub>2</sub>-reduction intermediate, susceptible to produce strong oxidant radical NO<sub>x</sub> species [36]. Such a selection pressure is clearly not present in *P. denitrificans*.

#### References

- [1] M. Brunori, A. Giuffrè, E. Forte, D. Mastronicola, M.C. Barone, P. Sarti, Control of cytochrome *c* oxidase activity by nitric oxide, *Biochim. Biophys. Acta* 1655 (2004) 365–371.
- [2] S. Moncada, J.D. Erusalimsky, Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat. Rev., Mol. Cell Biol.* 3 (2002) 214–220.
- [3] D.F. Wilson, A. Mokashi, D. Chugh, S. Vinogradov, S. Osanai, S. Lahiri, The primary oxygen sensor of the cat carotid body is cytochrome *a*<sub>3</sub> of the mitochondrial respiratory chain, *FEBS Lett.* 351 (1994) 370–374.
- [4] M.G. Mason, P. Nicholls, M.T. Wilson, C.E. Cooper, Nitric oxide inhibition of respiration involves both competitive (heme) and non-competitive (copper) binding to cytochrome *c* oxidase, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 708–713.
- [5] W.W. Wainio, Reactions of cytochrome oxidase, *J. Biol. Chem.* 212 (1955) 723–734.
- [6] C.E. Cooper, J. Torres, M.A. Sharpe, M.T. Wilson, Nitric oxide ejects electrons from the binuclear centre of cytochrome *c* oxidase by reacting with oxidised copper: a general mechanism for the interaction of copper proteins with nitric oxide? *FEBS Lett.* 414 (1997) 281–284.
- [7] A. Giuffrè, P. Sarti, E. D'Itri, G. Buse, T. Soulimane, M. Brunori, On the mechanism of inhibition of cytochrome *c* oxidase by nitric oxide, *J. Biol. Chem.* 271 (1996) 33404–33408.
- [8] J. Hendriks, A. Oubrie, J. Castresana, A. Urbani, S. Gemeinhardt, M. Saraste, Nitric oxide reductases in bacteria, *Biochim. Biophys. Acta* 1459 (2000) 266–273.
- [9] P. Moenne-Loccoz, S. de Vries, Structural characterization of the catalytic high-spin heme b of nitric oxide reductase: a Resonance Raman Study, *J. Am. Chem. Soc.* 120 (1998) 5147–5152.
- [10] E. Pinakoulaki, S. Gemeinhardt, M. Saraste, C. Varotsis, Nitric-oxide reductase. Structure and properties of the catalytic site from resonance Raman scattering, *J. Biol. Chem.* 277 (2002) 23407–23413.
- [11] C.E. Cooper, Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? *Trends Biochem. Sci.* 27 (2002) 33–39.
- [12] X.J. Zhao, V. Sampath, W.S. Caughey, Infrared characterization of nitric oxide bonding to bovine heart cytochrome *c* oxidase and myoglobin, *Biochem. Biophys. Res. Commun.* 204 (1994) 537–543.
- [13] G.W. Brudvig, T.H. Stevens, S.I. Chan, Reactions of nitric oxide with cytochrome *c* oxidase, *Biochemistry* 19 (1980) 5275–5285.
- [14] E. Pilet, W. Nitschke, F. Rappaport, T. Soulimane, J. Lambry, U. Liebl, M.H. Vos, NO binding and dynamics in reduced heme-copper oxidases *aa*<sub>3</sub> from *Paracoccus denitrificans* and *ba*<sub>3</sub> from *Thermus thermophilus*, *Biochemistry* 43 (2004) 14118–14127.

- [15] M.H. Vos, G. Lipowski, J.-C. Lambry, J.-L. Martin, U. Liebl, Dynamics of nitric oxide in the active site of reduced cytochrome *c* oxidase *aa*<sub>3</sub>, *Biochemistry* 40 (2001) 7806–7811.
- [16] A. Giuffrè, M. Barone, Mastronicola, E.D. D'Itri, P. Sarti, M. Brunori, Reaction of nitric oxide with the turnover intermediates of cytochrome *c* oxidase: reaction pathway and functional effects, *Biochemistry* 39 (2000) 15446–15453.
- [17] A. Giuffrè, G. Stubauer, P. Sarti, M. Brunori, W.G. Zumft, G. Buse, T. Soulimane, The heme-copper oxidases of *Thermus thermophilus* catalyze the reduction of nitric oxide: evolutionary implications, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999) 14718–14723.
- [18] G. Stubauer, A. Giuffrè, M. Brunori, P. Sarti, Cytochrome *c* oxidase does not catalyze the anaerobic reduction of NO, *Biochem. Biophys. Res. Commun.* 245 (1998) 459–465.
- [19] M.M. Pereira, M. Santana, M. Teixeira, A novel scenario for the evolution of haem-copper oxygen reductases, *Biochim. Biophys. Acta* 1505 (2001) 185–208.
- [20] S. Yoshikawa, A cytochrome *c* oxidase proton pumping mechanism that excludes the O<sub>2</sub> reduction site, *FEBS Lett.* 555 (2003) 8–12.
- [21] B. Ludwig, G. Schatz, A two-subunit cytochrome *c* oxidase (cytochrome *aa*<sub>3</sub>) from *Paracoccus denitrificans*, *Proc. Natl. Acad. Sci. U. S. A.* 77 (1980) 196–200.
- [22] K. Van Buuren, Binding of cyanide to cytochrome *aa*<sub>3</sub>, PhD thesis, University of Amsterdam, 1972.
- [23] E. Pilet, A. Jasaitis, U. Liebl, M.H. Vos, Electron transfer between hemes in mammalian cytochrome *c* oxidase, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 16198–16203.
- [24] J.-L. Martin, M.H. Vos, Femtosecond measurements of geminate recombination in heme proteins, *Methods Enzymol.* 232 (1994) 416–430.
- [25] M.H. Vos, J.-L. Martin, Femtosecond processes in proteins, *Biochim. Biophys. Acta* 1411 (1999) 1–20.
- [26] T.H. Stevens, D.F. Bocian, S.I. Chan, EPR studies of <sup>15</sup>NO-ferrocyclochrome *a*<sub>3</sub> in cytochrome *c* oxidase, *FEBS Lett.* 97 (1979) 314–316.
- [27] T.H. Stevens, S.I. Chan, Histidine is the axial ligand to cytochrome alpha 3 in cytochrome *c* oxidase, *J. Biol. Chem.* 256 (1981) 1069–1071.
- [28] F. Fujiwara, Cytochrome *cb*-type nitric oxide reductase with cytochrome *c* oxidase activity from *Paracoccus denitrificans* ATCC 35512, *J. Bacteriol.* 178 (1996) 1866–1871.
- [29] E. Forte, A. Urbani, M. Saraste, P. Sarti, M. Brunori, A. Giuffrè, The cytochrome *cbb*<sub>3</sub> from *Pseudomonas stutzeri* displays nitric oxide reductase activity, *Eur. J. Biochem.* 268 (2001) 6486–6491.
- [30] A. Giuffrè, E. Forte, G. Antonini, E. D'Itri, M. Brunori, T. Soulimane, G. Buse, Kinetic properties of *ba*<sub>3</sub> oxidase from *Thermus thermophilus*: effect of temperature, *Biochemistry* 38 (1999) 1057–1065.
- [31] X.J. Zhao, V. Sampath, W.S. Caughey, Cytochrome *c* oxidase catalysis of the reduction of nitric oxide to nitrous oxide, *Biochem. Biophys. Res. Commun.* 212 (1995) 1054–1060.
- [32] O. Richter, B. Ludwig, Cytochrome *c* oxidase—structure, function, and physiology of a redox-driven molecular machine, *Rev. Physiol., Biochem. Pharmacol.* 147 (2003) 47.
- [33] T. Tsukihara, K. Shimokata, Y. Katayama, H. Shimada, K. Muramoto, H. Aoyama, M. Mochizuki, K. Shinzawa-Itoh, E. Yamashita, M. Yao, Y. Ishimura, S. Yoshikawa, The low-spin heme of cytochrome *c* oxidase as the driving element of the proton-pumping process, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 15304–15309.
- [34] C. Ostermeier, A. Harrenga, U. Ermler, H. Michel, Structure at 2.7 Å resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody FV fragment, *Proc. Natl. Acad. Sci. U. S. A.* 94 (1997) 10547–10553.
- [35] W.G. Zumft, Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type, *J. Inorg. Biochem.* 99 (2005) 194–215.
- [36] M. Palacios-Callender, M. Quintero, V.S. Hollis, R.J. Springett, S. Moncada, Endogenous NO regulates superoxide production at low oxygen concentrations by modifying the redox state of cytochrome *c* oxidase, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 7630–7635.
- [37] M. Fabre, J. Bonvoisin, Electronic and magnetic communication in mixed-valent and homovalent ruthenium complexes containing phenylcyanamide type bridging ligands, *J. Am. Chem. Soc.* 129 (2007) 1434–1444.
- [38] M. Sieger, B. Sarkar, S. Záli, J. Fiedler, N. Escola, F. Doctorovich, J.A. Olabe, W. Kaim, Establishing the NO oxidation state in complexes [C15(NO)M]<sup>n+</sup>, M=Ru or Ir, through experiments and DFT calculations, *Dalton Trans.* (2004) 1797–1800.
- [39] R.S. Blackmore, C. Greenwood, Q.H. Gibson, Studies of the primary oxygen intermediate in the reaction of fully reduced cytochrome oxidase, *J. Biol. Chem.* 266 (1991) 19245–19249.