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Small Molecule Signaling Agents: The Integrated Chemistry and Biochemistry of Nitrogen Oxides, Oxides of Carbon, Dioxygen, Hydrogen Sulfide, and Their Derived Species

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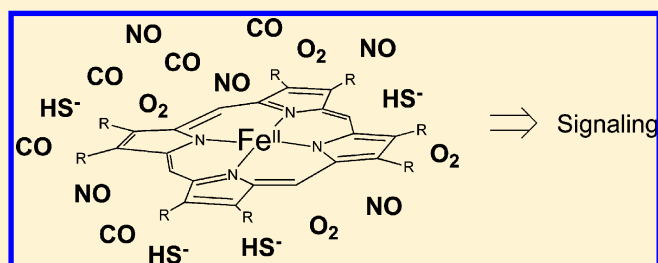
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ABSTRACT: Several small molecule species formally known primarily as toxic gases have, over the past 20 years, been shown to be endogenously generated signaling molecules. The biological signaling associated with the small molecules NO, CO, H₂S (and the nonendogenously generated O₂), and their derived species have become a topic of extreme interest. It has become increasingly clear that these small molecule signaling agents form an integrated signaling web that affects/regulates numerous physiological processes. The chemical interactions between these species and each other or biological targets is an important factor in their roles as signaling agents. Thus, a fundamental understanding of the chemistry of these molecules is essential to understanding their biological/physiological utility. This review focuses on this chemistry and attempts to establish the chemical basis for their signaling functions.



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■ INTRODUCTION

Since the discovery of nitric oxide (NO) as an endogenously generated signaling agent,¹ it has become increasingly evident that other endogenously generated small molecule species such as carbon monoxide (CO) and hydrogen sulfide (H₂S) as well as NO-derived species have important physiological signaling functions as well.^{2–5} Interestingly, all of these species were previously well-known toxins of significant industrial and/or environmental concern. Moreover, it is well established that dioxygen (O₂) and O₂-derived species such as superoxide (O₂^{•−}) and hydrogen peroxide (H₂O₂) can be vital cell signaling agents as well.^{6,7} Thus, there appears to be a wide array of small molecules that are utilized in biological systems as regulators and effectors of physiological function. Indeed, the term “gasotransmitter” has been coined recently^{8,9} to specifically denote this group of small molecule signaling agents. However, this term can be very misleading since all of these small molecule agents are completely soluble at the concentrations that are physiologically relevant and cannot be considered as gases in these situations. Thus, use of this term is somewhat unfortunate since it does not properly reflect the physical state of the species when they act in cell signaling. Regardless, these small molecule agents represent a relatively new and important cell signaling paradigm. It is the tenet of this review that the chemical properties and reactivity of the small molecule signaling species are of paramount importance to their biological function/utility and that Nature has evolved around these agents to form an integrated signaling web based on this chemistry.

One of the most intriguing and crucial aspects of the cell signaling associated with these small molecule species (i.e., NO, CO, H₂S, O₂, H₂O₂, O₂^{•−}, etc.) is the fact that, in many cases, there is significant commonality in their biochemical targets. That is, the macromolecular and biologically relevant reactive centers for almost all of these species includes redox metals (i.e., iron and/or copper proteins) and redox active amino acids (such as cysteine thiols or tyrosine phenols) as predominant sites of action. However, the intimate chemical interactions between these small signaling species and their biological targets can be kinetically distinct, and the nature of the products can exhibit significant differences with regards to further chemistry, stability, and/or structure. Indeed, these differences at the fundamental chemical level represent the genesis and nature of the biological response. Many of these agents also participate in or affect oxidative, reductive, and/or free radical chemistry. Clearly, the fact that these agents share common reactive targets and, in some cases, react with each other is not a coincidence but, rather, represents an integrated signaling system that has evolved around this chemistry. The integrated physiological signaling associated with these species has been discussed in numerous recent reviews,^{2,3,5,8,9} and it is not the intent of this review to further elaborate on this aspect of their biology. Thus, this review focuses first on the fundamental and biologically relevant chemistry of these individual small molecules, and their derivatives, followed by a brief discussion of examples of how this chemistry can integrate within the context of a biological system to form an intricate and integrated signaling system.

■ BIOLOGICAL CHEMISTRY OF DIOXYGEN (O₂) AND RELATED SPECIES

Dioxygen. Among all signaling agents discussed herein, O₂ has been the most extensively studied. Indeed, much of free radical biology was discovered or is derived from the study of O₂

and its reduction to reactive oxygen species (ROS) in biological systems. (At the risk of being heretical, the term “reactive oxygen species” or ROS is clearly one of the most misused and misunderstood monikers in the lexicon of biology/biochemistry. From a chemical perspective, all chemicals are potentially “reactive” depending on their environment or proximity to other reactive partners. More importantly, the inherent chemical properties/reactivities of the ROS are very distinct, and we feel it unwise and even misleading to lump all these species together under this somewhat meaningless descriptor.) There are numerous books and reviews that discuss the chemistry, biology, physiology, and pathophysiology associated with O₂.^{10–13} Thus, the focus of this review will be to discuss only the most salient and fundamental features of the chemistry associated with these species.

Dioxygen is a chemically unique and fascinating molecule. Unlike most other diatomics, its electron configuration cannot be accurately described using valence bond or Lewis structure formalisms (Figure 1a). Examination of the molecular orbital

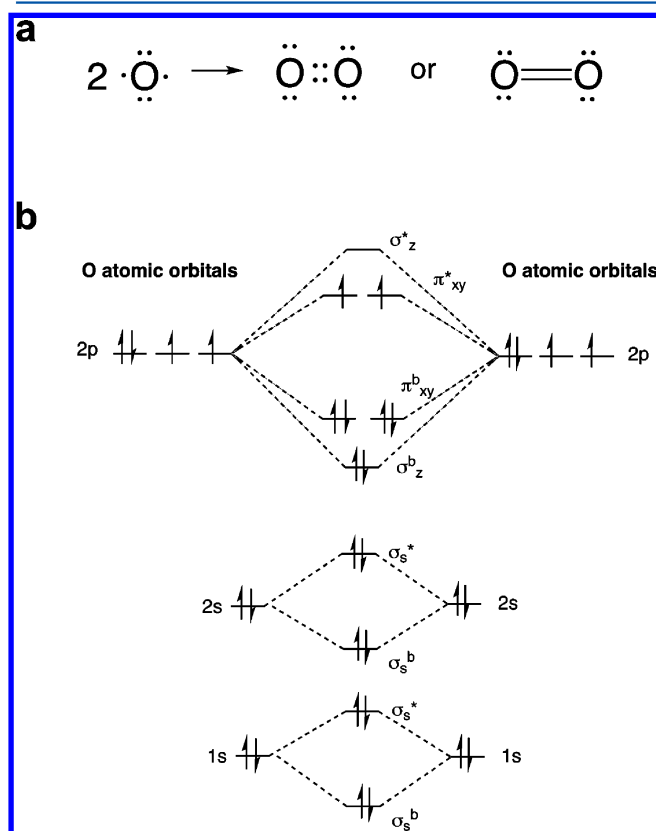


Figure 1. (a) Lewis and valence bond depiction of O₂. (b) Molecular orbital diagram for O₂.

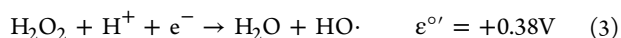
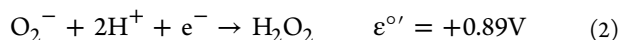
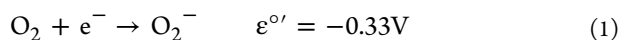
diagram for O₂ (Figure 1b) reveals that it actually has two unpaired electrons with the same spin occupying degenerate π* antibonding orbitals. Thus, O₂ has a triplet electronic ground state.

The fact that ground state O₂ has unpaired electrons allows it to react with other species with an unpaired electron. Indeed, O₂ is often viewed as a diradical and can react accordingly, mainly reacting with other radicals. Before continuing the discussion of the chemistry of O₂ and derived species, it is worthwhile to first define the term “radical” and/or “free radical” as used herein. For future discussions, we have adopted the general definition

prescribed by Halliwell and Gutteridge¹³ for a free radical as being “any species capable of independent existence that contains one or more unpaired electrons”. Although the term “free” is meant to represent the property of independent existence, it is oftentimes omitted and considered inherent to some of the agents discussed herein. It needs to be stressed, however, that this definition is not rigorous. For example, many stable transition metal complexes have unpaired electrons (and are thus paramagnetic) and are not typically considered as free radicals, although they are capable of independent existence. So what distinguishes a free radical such as the hydroxyl radical ($\text{HO}\cdot$, vide infra) from, for example, the paramagnetic ferric iron in hemoglobin? One distinction is that $\text{HO}\cdot$ is a main group (i.e., exclusion of transition elements) molecular species with an open shell configuration (i.e., there is a single unpaired electron in a valence shell orbital and therefore does not satisfy the octet rule for main group elements). Also, particularly important to the free radical designation is the implication of a certain minimum of reactivity, such as the reaction with other radical species (although this is also not a rigorously observed distinction, vide infra). Thus, “free radical” is typically used to describe atoms/molecules made up of main group elements with an unpaired electron in a valence shell orbital and that may react readily with other radicals. Therefore, designating O_2 as a free radical using these criteria is at least partially valid (although the Lewis structure for O_2 does satisfy the octet rule, it possesses unpaired electrons, and it can react with other radicals).

Historically, the term radical, when related to biological systems, was associated with extreme reactivity leading to indiscriminant and potentially deleterious chemistry. However, biologically relevant radicals exhibit many degrees of reactivity, and as we shall discuss herein, these radicals span the entire range of reactivity from virtually nonoxidizing (and even reducing) to extremely oxidizing. Moreover, it is now clear that biology utilizes many radical processes to accomplish otherwise difficult biochemical transformations.¹⁴ Many radicals are electron poor and therefore are good oxidants. For example, the hydroxyl radical ($\text{HO}\cdot$, a species to be discussed in more detail later) is a strong one-electron oxidant, as evidenced by a reduction potential of 2.31 V (vs NHE, pH 7.0). By comparison, O_2 is a poor one-electron oxidant. The reduction potential for the O_2/O_2^- couple is -0.33 V (vs NHE, 1 atm O_2 , pH 7.0). (It should be noted that the reduction potential for O_2 is also reported to be -0.16 V (vs NHE, pH 7.0), which represents the value if O_2 were at 1 M, rather than 1 atm.) Thus, although both $\text{HO}\cdot$ and O_2 possess unpaired electrons, $\text{HO}\cdot$ is an extremely strong one-electron oxidant, while O_2 is not.

At first glance, the fact that O_2 is a poor one-electron oxidant may appear to be contradictory to its role as the ultimate electron acceptor (oxidant) in aerobic organisms. That is, it is the energetically favorable reduction of O_2 to H_2O that serves as the basis for aerobic life. The reduction potentials for the individual steps in the overall reduction of O_2 to H_2O are shown below (reactions 1–4, all vs NHE and pH 7).¹⁰



The first one-electron reduction of O_2 to O_2^- is relatively unfavorable as evidenced by a reduction potential of -0.33 V. However, the reduction potential for the conversion of O_2 to H_2O is overall very favorable with an $\varepsilon^{\circ'}$ for the 4-electron reduction of $+0.81$ V (remember that this reduction potential represents an average “per electron” value). Thus, O_2 is a poor one-electron oxidant but a very good 4-electron oxidant. This thermodynamic barrier to the first one-electron reduction of O_2 to give O_2^- serves to restrict, to a certain degree, indiscriminant and unwanted oxidations carried out by O_2 .

Another way of evaluating the one-electron oxidizing potential of O_2 and related radical species is to examine their ability to perform hydrogen atom abstraction chemistry since this reaction represents the transfer of an electron (along with a proton) to the hydrogen abstracting oxidant. If O_2 and $\text{HO}\cdot$ were to abstract hydrogen atoms from a substrate R-H (reactions 5 and 6), the O-H bonds formed have bond dissociation energies (BDE) of 47 and 119 kcal/mol, respectively, indicating that this reaction is much more favorable for $\text{HO}\cdot$. Indeed, since the strength of most C-H or N-H bonds in biological molecules is >80 kcal/mol, this reaction is thermodynamically very unfavorable for O_2 but generally very favorable for $\text{HO}\cdot$.



Thus, it is clear that O_2 is a poor one-electron oxidant in spite of the fact that it has radical character.

The fact that O_2 has a triplet electronic ground state (i.e., $^3\text{O}_2$) is significant since it kinetically restricts the types of reactions that it can participate in. For example, the reaction of O_2 with singlet organic molecules (all electrons are spin paired in bonds or as lone pairs) to generate singlet products is “spin forbidden”. That is, the reaction of triplet O_2 with singlet molecules to give singlet products would require a spin “flip” (requiring two steps and high energy intermediates) and is very slow.¹⁵ Thus, spontaneous reactions of O_2 with organic molecules are very restricted both thermodynamically (due to the unfavorable one-electron reduction chemistry) and kinetically (due to the spin restriction). These fundamental properties of O_2 have allowed Nature to reductively harvest its tremendous thermodynamic potential without having to cope (for the most part, vide infra) with an abundance of indiscriminant and/or potentially uncontrollable processes.

Although O_2 is poor at initiating radical chemistry (vide supra), it can rapidly react with other, existing radicals. That is, if a radical center is present (for example an alkyl radical, $\text{R}\cdot$), it can rapidly react with O_2 (reaction 7).



This reaction has no spin restriction and therefore can be very fast. The product of the reaction of O_2 with $\text{R}\cdot$ still has an unpaired electron and, therefore, maintains radical character. If the radical–radical coupling reaction occurs with a carbon-centered radical, the product is an alkylperoxyl radical ($\text{ROO}\cdot$), which is a relatively strong one-electron oxidant (ε° for the $\text{ROO}\cdot/\text{H}^+/\text{ROOH}$ couple is around 1 V, pH 7.0 vs NHE).¹⁶ In the presence of easily oxidized C-H bonds, such as those found in polyunsaturated fatty acids, the alkylperoxyl radical

can react further leading to a chain reaction, where O_2 is consumed, and numerous oxidized products containing oxygen are generated. When this process occurs in lipids, it is referred to as a lipid peroxidation (Figure 2).

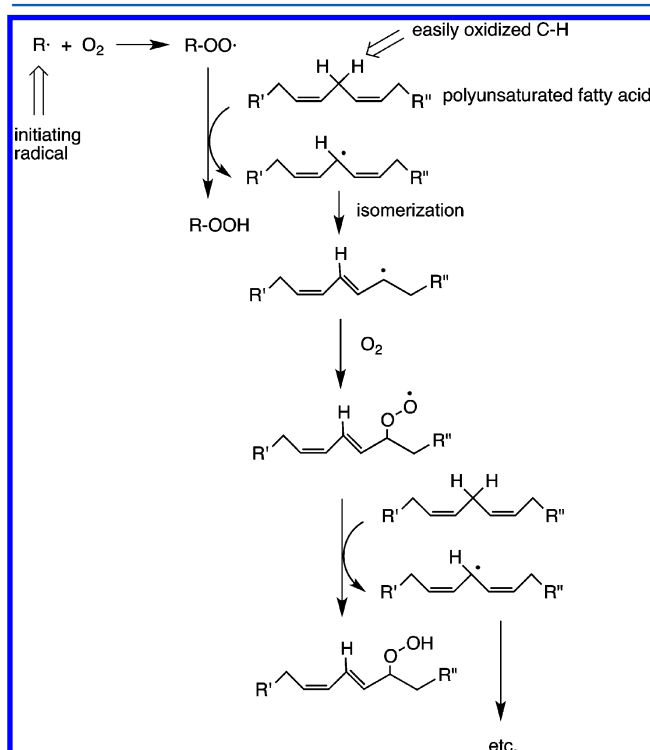


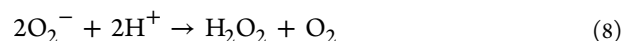
Figure 2. Lipid peroxidation. Numerous oxidized products can be generated. Only the simplest alkylperoxide product is shown.

The bis-allylic C–H bond (the “easily oxidized” C–H shown in Figure 2) is relatively weak (approximately 76 kcal/mol).¹⁷ Thus, initial abstraction of this hydrogen is facile as is the subsequent abstraction in the chain-carrying steps of the process (the BDE for ROO–H is approximately 87 kcal/mol).¹⁸ The reduction potential for the bis-allylic radical has been calculated to be a relatively low 0.6 V (pH 7), also consistent with the propensity for this position to be oxidized.¹⁶ It is important to note that this process represents a chain reaction, meaning that a single initiating event (formation of the initial radical) can lead to the destruction of numerous lipid molecules. Also, the presence of polyunsaturated fatty acids greatly increases the susceptibility of membranes to this process since the bis-allylic motif is so easily oxidized. Finally, since O_2 is very lipid soluble and therefore will favorably partition into lipid components, membranes appear to be major sites of free radical damage. To be sure, the chemistry depicted in Figure 2 is greatly simplified as numerous products resulting from the autooxidation of lipids can be generated. In fact, some of the more prevalent products, such as 5-hydroxynonenal, appear to be important signaling molecules that are indicators of cellular oxidative stress.¹⁹

Singlet Oxygen (1O_2). As discussed above, dioxygen is a ground state triplet. There are two relatively low-lying excited singlet electronic states associated with 3O_2 designated as $^1\Sigma_g^+$ (representing electrons of opposite spins occupying each of the π^* orbitals, orbitals depicted in Figure 1) and $^1\Delta_g$ (paired electrons in a π^* orbital).^{20,21} The $^1\Sigma_g^+$ is very short-lived, and it is generally considered that all reactions in biological

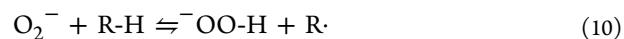
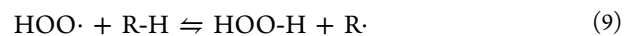
solutions occur via the $^1\Delta_g$ species. 1O_2 ($^1\Delta_g$) is 22 kcal/mol higher in energy than 3O_2 , and its lifetime in solution can range from 4–16,000 μ s, depending on the solvent. Unlike 3O_2 , reactions of 1O_2 do not have the spin restriction discussed above. Thus, 1O_2 can react spontaneously with a variety of biological nucleophiles, including molecules with unsaturations and thiols. Although 1O_2 has apparent signaling functions in plants²² and has tremendous utility in medicine (e.g., as an effector species in photodynamic therapy),²³ it is currently not known to have signaling functions in mammalian systems (at least not to the extent of the other small molecule species discussed herein). Therefore, only this brief introduction to 1O_2 will be given.

Superoxide (O_2^-). One-electron reduction of O_2 generates O_2^- . By examining the thermodynamics of combining reaction 1 (in reverse) and reaction 2, it is clear that O_2^- is unstable with respect to disproportionation (reaction 8). That is, two O_2^- molecules can react, in the presence of protons, to give one molecule each of O_2 and H_2O_2 .



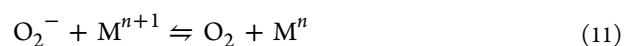
This second-order process, also referred to as a dismutation, occurs spontaneously with the fastest rate at a pH of 4.7. Since the pK_a of the conjugate acid of O_2^- , $HOO\cdot$, is also 4.7, the reaction is fastest when one molecule of $HOO\cdot$ reacts with one molecule of O_2^- (since at pH 4.7, 50% of each is present). Importantly, this reaction is catalyzed by a class of enzymes called superoxide dismutases (SOD), which keep biological concentrations of O_2^- very low.²⁴

O_2^- possesses a single unpaired electron and, therefore, is formally a radical species and capable of reacting as such. For example, O_2^- can react with other radicals such as NO leading to potentially reactive species (vide infra). The reduction potential for the $O_2^-, 2H^+/H_2O_2$ couple (reaction 2) is 0.89 V (vs NHE, pH 7) indicating that O_2^- can be a decent one-electron oxidant if protons are present. Indeed, the oxidizing capabilities of O_2^- are highly proton-dependent as indicated by the changes in its reduction potential as a function of pH ($\epsilon^\circ = +1.44$ V at pH 0, +0.89 V at pH 7, and +0.2 V at pH 14).¹⁰ Consistent with the idea of protons being crucial to the oxidizing capability of O_2^- , the BDEs of the O–H bond of $HOO\cdot$ and $^-\text{OO}\cdot$ are 89 and 63 kcal/mol, respectively. Thus, H-atom abstraction by protonated O_2^- (reaction 9) is much more favorable than H-atom abstraction by O_2^- itself (reaction 10). Significantly, the BDE for a thiol (RS–H bond) is approximately 85–90 kcal/mol indicating $HOO\cdot$ will be capable of oxidizing thiols (although this is potentially a very complex process).²⁵



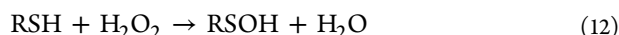
It needs to be stressed, however, that the concentrations of $HOO\cdot$ at the physiological pH of 7 are apt to be very low since its pK_a is only 4.7, making the conjugate base, O_2^- , the predominant species present (although in acidic intracellular compartments, this chemistry can become relevant).

As indicated by the relatively unfavorable reduction potential for O_2 (reaction 1), O_2^- is also a good reductant capable of, for example, reducing redox metals (reaction 11).



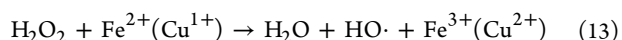
The metal (M in reaction 11) is typically Fe^{3+} or Cu^{2+} in biological systems. O_2^- is also capable of reducing organic-type redox species such as quinones. It should be noted, however, that the reduction potentials for the same metal can vary considerably depending on the coordination or chemical environment and that quinones have widely varying reduction potentials depending on the structure and environment. Thus, O_2^- can be a one-electron reductant and in the presence of protons can be a reasonable one-electron oxidant.

Hydrogen Peroxide (H_2O_2). Unlike O_2 and O_2^- , H_2O_2 has no unpaired electrons. Thus, it does not directly participate in radical processes of the type described above for O_2 and O_2^- . H_2O_2 is best characterized as a two-electron oxidant that reacts with nucleophilic reductants. For example, the reaction of H_2O_2 with nucleophilic thiols generates the corresponding sulfenic acid (which represents a two-electron oxidation of sulfur) (reaction 12).



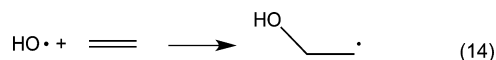
In this reaction, H_2O_2 is an electrophile, reacting with a nucleophilic thiol resulting in a net 2-electron oxidation of the thiol. Since all the reactants are electronic singlets, these reactions are spin "allowed", unlike the direct reactions of O_2 described above, and there are no radical intermediates. Although this reaction has been proposed extensively in the biological literature for explaining the effect of H_2O_2 on the activity of a variety of thiol proteins and peptides, it is worth noting that this reaction is generally very slow at physiological pH in the absence of any catalytic assistance. For example, the rate constants for the noncatalyzed reaction of H_2O_2 with cysteine, glutathione, and even dithiothreitol under physiological conditions (pH 7.4, 37 °C) are all $<5 \text{ M}^{-1} \text{ s}^{-1}$.²⁵ The reaction of H_2O_2 with a thiol occurs primarily via the more nucleophilic ionized thiolate species, and the rate constant for this reaction is slightly greater ($18\text{--}26 \text{ M}^{-1} \text{ s}^{-1}$).²⁵ In a test tube, where there are no consumptive processes for H_2O_2 occurring, oxidation chemistry can be observed. However, in a biological system where numerous processes exist that degrade H_2O_2 , the slow kinetics preclude many of these reactions from being relevant. Thus, oxidation by H_2O_2 should exhibit selectivity for thiols that are primarily ionized, although the low magnitude of these rate constants limits the physiological relevance of this reaction as a general process. There are, however, thiol proteins with significant rate constants for the reaction with H_2O_2 ($>10^5\text{--}10^6 \text{ M}^{-1} \text{ s}^{-1}$)²⁶ indicating that H_2O_2 can be a signaling molecule capable of selectively reacting with certain thiol proteins that possess catalytic entities that accelerate this reaction. It appears likely that significant increases in the rate constants for the reactions of biological nucleophiles with H_2O_2 will require acid assistance (Lewis or Lowry–Bronsted), which will enhance the electrophilicity of H_2O_2 and preclude the unfavorability of a hydroxide leaving group.

One-electron reduction of hydrogen peroxide occurs with a reduction potential of +0.38 V ($\text{H}_2\text{O}_2, \text{H}^+/\text{H}_2\text{O}, \text{HO}\cdot$, pH 7, vs NHE) (reaction 13). The products of this reduction are H_2O and $\text{HO}\cdot$ (the generation of extremely stable species H_2O helps drive the formation the highly reactive $\text{HO}\cdot$). Thus, H_2O_2 is not a radical species but upon reduction generates a very oxidizing radical, $\text{HO}\cdot$. The reduction of H_2O_2 in biological systems can occur via reaction with the reduced forms of several redox-active metals such as the ferrous ion (Fe^{2+}) or cuprous ion (Cu^{1+}) (reaction 13).



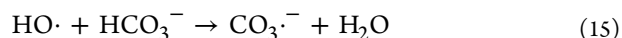
This reaction, referred to as the Fenton reaction, has been reported to be responsible for some of the toxicity associated with H_2O_2 since it generates a potent and indiscriminant oxidant, $\text{HO}\cdot$. However, it should be realized that this form of H_2O_2 toxicity is highly dependent on the presence/location of reactive forms of Fe^{2+} or Cu^{1+} ions, which can have widely variant reactivities in this regard.²⁷

Hydroxyl Radical ($\text{HO}\cdot$). As discussed briefly above, $\text{HO}\cdot$ is a potent one-electron oxidant. This is easily seen from its reduction potential (2.31 V for the $\text{HO}\cdot, \text{H}^+/\text{H}_2\text{O}$, pH 7, vs NHE) and the fact that the reduced species, H_2O , has an O–H bond dissociation energy of 119 kcal/mol. That is, one-electron reduction of $\text{HO}\cdot$ is highly favorable, and abstraction of a hydrogen atom by $\text{HO}\cdot$ generates a very strong bond. Indeed, there are very few biological molecules or functional groups that cannot be oxidized by $\text{HO}\cdot$, making it a focal point in discussions of the deleterious aspects of O_2 -derived species. As with all oxidizing radicals, $\text{HO}\cdot$ not only abstracts hydrogen atoms as a mechanism to gain an electron, but it can also add to unsaturations, a reaction that also results in the generation of another radical (reaction 14). Because of its extreme reactivity,



$\text{HO}\cdot$ has a fleeting lifetime and therefore is unable to travel significant distances in a biological milieu since it quickly reacts with nearly the first molecule it encounters. The second order rate constants for the reaction of $\text{HO}\cdot$ with a variety of biologically relevant molecules are typically near the diffusion-controlled limit ($>10^9 \text{ M}^{-1} \text{ s}^{-1}$), indicating that very few collisions are required for a reaction to occur.²⁸ Thus, it will be expected that biological damage by $\text{HO}\cdot$ will be localized to its site of generation, which in many cases means the site where a catalytic metal is present (reaction 13). In fact, evidence suggests that under certain conditions "free" $\text{HO}\cdot$ is not generated in the Fenton reaction but that rather a metal-bound oxidant is made, and this is the ultimate oxidant.²⁹

In biological systems, $\text{HO}\cdot$, or the Fenton reaction product, can react with bicarbonate (HCO_3^- , a very abundant species in biological systems) to give the carbonate radical anion ($\text{CO}_3^{\cdot-}$) (reaction 15).



Unlike most reactions of $\text{HO}\cdot$ with biological molecules, which have near diffusion controlled rate constants, the rate constant for the reaction of $\text{HO}\cdot$ with HCO_3^- is only about $8.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The pK_a of HCO_3^- is reported to be <0 , indicating that the radical anion is the near exclusive species present at physiological pH.³⁰ Although less oxidizing than $\text{HO}\cdot$, $\text{CO}_3^{\cdot-}$ is a strong one-electron oxidant as indicated by a reduction potential for the $\text{CO}_3^{\cdot-}, \text{H}^+/\text{HCO}_3^-$ couple of 1.78 V (vs NHE, pH 7).³¹ The significance of biological $\text{CO}_3^{\cdot-}$ formation resulting from Fenton chemistry is that $\text{CO}_3^{\cdot-}$ can better diffuse from the site of $\text{HO}\cdot$ formation (or formation of the highly oxidizing metal oxidant) due to its lessened oxidative reactivity, possibly allowing oxidation chemistry to occur that is remote from the site of the initial $\text{HO}\cdot$ generation.

Coordination Chemistry of O_2 . Dioxxygen coordinates to a variety of metal centers in biological systems.^{32,33} Examples of O_2 -metal binding in biological systems include the binding and transport of O_2 using the ferrous heme moiety in hemoglobin, the biochemical reduction of O_2 that occurs during

mitochondrial respiration via an O₂–metal complex in cytochrome *c* oxidase, the binding and reductive activation of O₂ via a ferrous heme prosthetic group of the metabolic enzyme cytochrome P450, and the reductive activation of O₂ using a nonheme ferrous ion of the O₂-sensing enzyme prolyl hydroxylase (vide infra). A variety of copper proteins also bind and/or activate O₂ similarly.³⁴ Thus, the binding to protein metal centers, especially iron and copper, represents a primary biological target/fate for O₂. For the reasons described below, O₂ binds to metals in lower oxidation states. For example, ferric (Fe³⁺) and cupric (Cu²⁺) proteins do not bind to O₂, whereas binding can occur with ferrous (Fe²⁺) and cuprous (Cu¹⁺) proteins (providing there is an open coordination site, among other things). The chemistry of O₂ binding to metals is complex and typically involves at least two types of bonding interactions. Donation of a lone pair of electrons residing in an sp²-hybridized orbital on O₂ into a metal d-orbital of appropriate symmetry generates a σ -bond. The spatial orientation of the lone pair sp² orbital predicts the bonding of O₂ to the metal to be end-on and bent (Figure 3). An antibonding π^* orbital on O₂

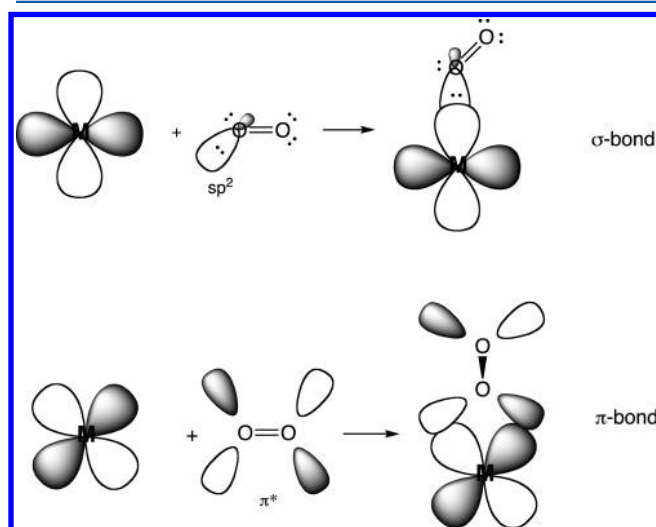


Figure 3. Bonding schemes for end-on O₂ binding to metals.

also has the proper symmetry to overlap with another d-orbital on the metal, leading to a bonding interaction made up of the donation of electrons from the metal to the partially filled π^* orbital on the O₂ ligand (Figure 3).

The donation of electrons from the metal into the π^* orbital of O₂ (often referred to as back-bonding) leads to a weakening of the O–O bond, and the complex is often viewed as an oxidized metal–superoxide complex (M⁺–O₂[–]). Depending on the metal and its coordination environment, the degree of back-donation from the metal to the ligand can vary considerably.³⁵ When O₂ is bound in an end-on/bent geometry (as is often the case and shown in Figure 3), there can also be a σ -type interaction between a π^* antibonding orbital (the one orthogonal to the π^* orbital depicted in Figure 3) and the d_{z2} orbital on the metal. In cases where there is an unpaired electron in the d_{z2} orbital, this electron can spin pair with an electron in the O₂ π^* orbital leading to a strong bonding interaction.³⁵

It should be noted that O₂ can bind to metals in other ways besides that described above. For example, O₂ can bind “side-on” where both oxygen atoms ligate the metal and in cases where two coordinating metals exist in close proximity, the

oxygen atoms of O₂ can bind both metal centers giving an –O–O– bridge between the metals. To be sure, these other binding modes are biologically relevant and important. However, for the purposes of illustrating how the coordination chemistry of O₂ can be compared and related to that of the other signaling species of interest, we limit our discussion to the type of coordination shown in Figure 3.

■ BIOLOGICAL CHEMISTRY OF NITRIC OXIDE AND RELATED SPECIES

Nitric Oxide. Unlike O₂, the Lewis structure/valence bond depictions of NO predict that it possesses an unpaired electron (Figure 4a). The existence of the unpaired electron is also

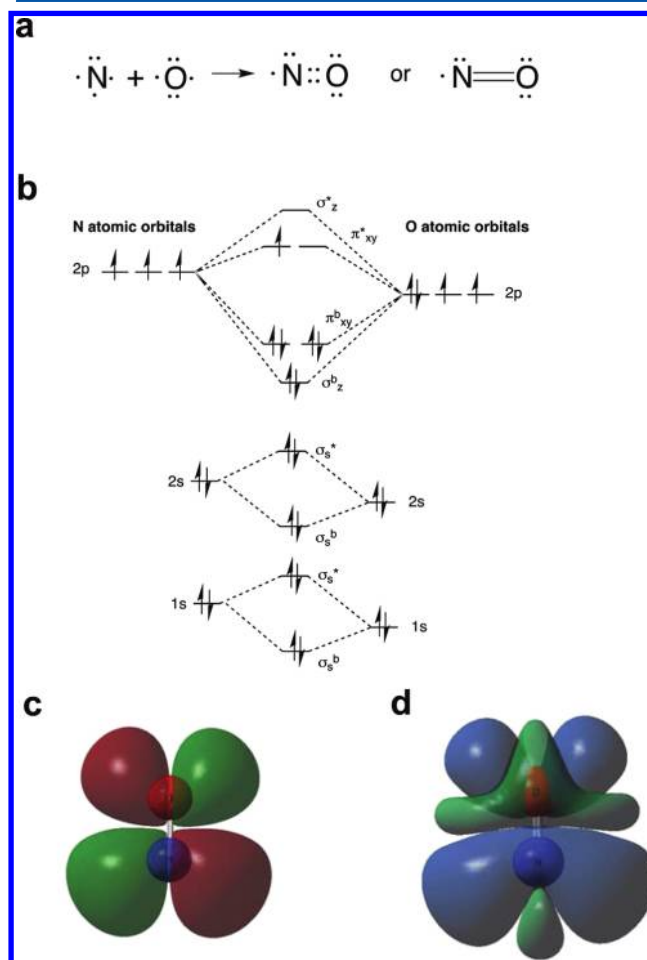


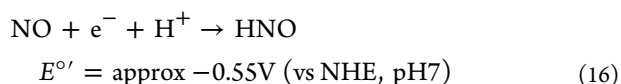
Figure 4. (a) Lewis structure/valence bond depiction of NO, (b) molecular orbital diagram for NO, (c) singly occupied molecular orbital (SOMO) of NO, and (d) NO spin density. Note: panels c and d were calculated at the CCSD(T)/6-311++G(3df,3pd)//MP2/6-311++G(3df,3pd) level (isovalue of 0.0004 and 0.02, respectively).

confirmed by the molecular orbital diagram (Figure 4b). However, like O₂, the unpaired electron exists in a π^* orbital and is not strictly localized on the nitrogen atom (i.e., delocalized on both the N and O atoms) (Figure 4c,d). The geometries of the molecular orbitals of NO are qualitatively similar to those of O₂.

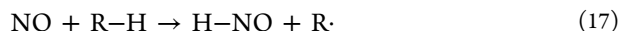
As indicated by its Lewis structure, NO does not obey the octet rule for main group elements. Thus, it may be asked why NO exists primarily as a monomer at room temperature and pressure when it can dimerize to give a species (NO)₂ that

would appear to be more stable (or will at least satisfy the octet rule). Indeed, at low temperatures NO does dimerize, with the most stable structure being a cis-N-N bonded species. The N-N bond strength is extremely low (approximately 2 kcal/mol), only slightly greater than van der Waals forces.³⁶ The extremely weak (and long) N-N bond of the NO dimer is generally attributed to 1) only partial σ N-N bond formation due to delocalization of the bonding electron over both the N and O atoms of the monomer (the π^* electron)³⁷ and 2) repulsion between the adjacent nitrogen lone pairs in the dimer.³⁸

Thus, NO is a radical without any significant tendency to dimerize under biological conditions, and this chemical property is one of the important factors in its biology. However, unlike HO \cdot and similar to O $_2$, NO is not a good one-electron oxidant. In fact, the reduction potential for NO predicts that biological one-electron reduction of NO will be significantly more difficult than O $_2$ (reaction 16).^{39,40}



Consistent with the lack of one-electron oxidizing potential for NO, the H-NO bond dissociation energy is only 47 kcal/mol⁴¹ indicating that NO will be very poor at abstracting H-atoms from biological substrates (reaction 17).



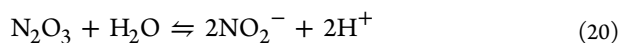
Although NO is a poor one-electron oxidant (and therefore, will not spontaneously initiate radical chemistry), it will react with existing radicals such as O $_2$. NO readily reacts with O $_2$ in solution to give, initially, nitrogen dioxide (NO $_2$) (reaction 18).



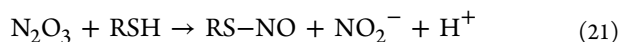
Nitrogen dioxide also has an unpaired electron (and therefore a radical species) and is much more oxidizing than either precursor, NO or O $_2$ (vide supra). The fate of NO $_2$, when it is generated from the autoxidation of NO in pure water, is to react with another equivalent of NO (since both are radical species) to give dinitrogen trioxide, considered the anhydride of nitrous acid, N $_2$ O $_3$ (reaction 19).



N $_2$ O $_3$ then reacts with water to give two equivalents of nitrite (NO $_2^-$) (reaction 20).



Thus, the ultimate fate of NO in a pure aerobic aqueous solution is the generation NO $_2^-$. It is worth noting that reactions 19 and 20 are readily reversible, indicating that an acidified and concentrated solution of NO $_2^-$ can generate N $_2$ O $_3$, NO, and NO $_2$. N $_2$ O $_3$ can also react with other nucleophiles besides H $_2$ O. For example, the reaction with thiols results in the formation of S-nitrosothiols (RSNO) (reaction 21).



S-Nitrosothiols have been reported to be an important redox form of biological thiols and thiol proteins (vide infra). Considering their proposed regulatory function in thiol proteins, a thorough understanding of the chemistry of their formation and degradation is important to evaluate their relevance or likelihood as intermediates in signaling pathways. A discussion

of this will be given later, once other reactions potentially responsible for their generation have been discussed.

A particularly important aspect of the autoxidation of NO is the fact that the rate is second order in NO ($-\text{d}[\text{NO}]/\text{dt} = 4k[\text{NO}]^2[\text{O}_2]$),⁴² meaning that it will only be significant at high NO concentrations. Thus, the formation of NO $_2$, N $_2$ O $_3$, and NO $_2^-$ from the NO-O $_2$ reaction will only occur when NO concentrations are very high and under aerobic conditions. In biological systems, however, NO and O $_2$ partition favorably into hydrophobic environments (i.e., lipid bilayers) resulting in much greater concentrations compared to the mostly aqueous compartments. This favorable partitioning into hydrophobic compartments greatly accelerates the NO/O $_2$ chemistry.⁴³

NO will also react with other oxidizing radicals, such as those generated during lipid peroxidation (Figure 2). However, unlike O $_2$, the reaction of NO with a radical leads to a quenching of all radical character and a cessation of the radical chain chemistry characteristic of lipid peroxidation (reaction 22).



The ability of NO to rapidly react with and quench oxidizing radicals indicates that it can be a good antioxidant.⁴⁴ Significantly, the rate constants for the reaction of NO with a variety of biologically relevant radical species are all near diffusion controlled: peroxy (ROO \cdot), $(1-3) \times 10^9 \text{ M}^{-1}\text{s}^{-1}$;⁴⁵ thiyl (RS \cdot), $(2-3) \times 10^9 \text{ M}^{-1}\text{s}^{-1}$;⁴⁶ and tyrosyl (tyr \cdot), $(1-2) \times 10^9 \text{ M}^{-1}\text{s}^{-1}$.⁴⁷ Thus, one-electron oxidation chemistry occurring in the presence of NO dramatically alters the nature of the products formed, and NO generally antagonizes the radical chain chemistry.

One of the most studied of all radical reactions of NO is its reaction with O $_2^-$. As described above, O $_2^-$ is a radical species, and reaction with NO also occurs with a near diffusion-controlled rate constant ($6.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$)⁴⁸ (reaction 23).



The product of reaction 23 is peroxynitrite (ONOO $^-$), and the chemistry of this species will be discussed in detail below.

As discussed throughout this review, numerous reports indicate that thiols/thiol proteins can be major targets for the biological actions of nitrogen oxides. Although several studies allude to a direct reaction between NO and thiols,^{49,50} this appears to occur only under high concentrations of NO (nonphysiological) and at a very low rate (if at all). If the reaction is viewed as a nucleophilic attack of a thiol on an electrophilic NO, akin to the attack of a nucleophile on a carbonyl function, then the reaction would involve overlap between the molecular orbital containing the nucleophilic lone pair of electrons and the π^* orbital of NO. Since the π^* orbital of NO has an electron (Figure 4b) (unlike the π^* orbital of a carbonyl function or, as discussed below, HNO, which is empty), the reaction should not occur in this way (Figure 5). Moreover, the radical product of nucleophilic addition to NO would be thermodynamically unstable. Finally, HNO has significantly greater negative charge on oxygen compared to NO, making the nitrogen atom of HNO a better electrophile. Thus, it is generally accepted that direct reactions of NO with thiols (of the type shown in Figure 5) are not physiologically relevant.

It is worth mentioning, however, that the NO dimer (NO) $_2$ is very electrophilic and has been proposed to be capable of reacting readily with nucleophiles (e.g., thiols or phosphines), leading directly to oxidized products.⁵¹ However, as discussed above, the presence of even scant levels of NO dimers are unlikely under most biological conditions. Therefore, in the

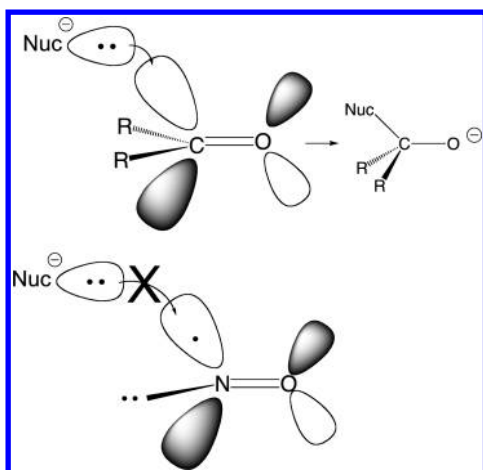


Figure 5. Attack of a nucleophile on a carbonyl versus attack on NO (note: electron in the NO π^* orbital is not localized in the lobe shown but is distributed throughout the π^* orbitals).

absence of any special conditions (e.g., proximal metal binding), NO dimer chemistry is likely to be inaccessible in biological systems.

Nitrogen Dioxide (NO_2). As discussed above, NO_2 can be generated during the autoxidation of NO (reaction 18), especially in hydrophobic compartments. Other possible mechanisms for NO_2 generation in biological systems exist and will be discussed later. NO_2 is a radical as shown by the valence bond depiction (Figure 6).

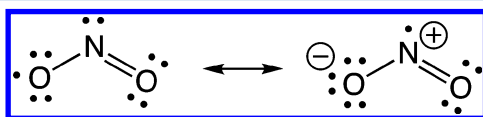


Figure 6. Valence bond depiction of NO_2 (note: only two of several resonance forms are shown).

The reduction potential for the $\text{NO}_2/\text{NO}_2^-$ couple is 1.04 V (vs NHE)⁵² indicating that NO_2 is a reasonable one-electron oxidant. The H-ONO bond dissociation energy is calculated to be 76 kcal/mol,⁵³ which is consistent with the idea that NO_2 could abstract activated hydrogens (centers with a relatively weak bond to hydrogen). It should be noted that the H- NO_2 bond of hydrogen nitril is calculated to be approximately 10 kcal/mol weaker than the H-ONO bond,⁵³ indicating that the O-atom of NO_2 is the hydrogen atom abstractor or oxidizing center. NO_2 has been reported to be capable of oxidizing a variety of biologically relevant functional groups. For example, phenols can be oxidized by NO_2 forming an intermediate phenoxyl radical which can further react with another NO_2 via a radical-radical combination and tautomerization reaction to generate a nitrated phenol⁵⁴ (Figure 7).

This reaction is pH dependent with increasing rates at higher pH ($k = 3.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.5 and $k = 2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 11.3 for the reaction of NO_2 with tyrosine containing

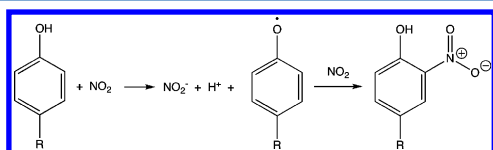
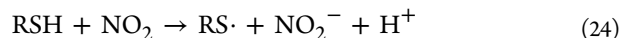


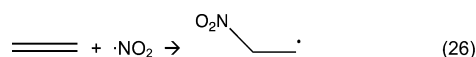
Figure 7. Oxidation of substituted phenol by NO_2 .

dipeptides)⁵⁴ indicating a faster reaction via the deprotonated phenoxide species. Thiols are also oxidized by NO_2 ($k = (2-5) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4), giving as an immediate product the thiyl radical (reaction 24).⁵⁵



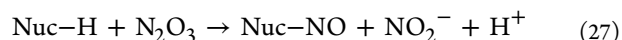
Like phenol oxidation by NO_2 , oxidation of thiols is also pH dependent indicating a faster reaction with the deprotonated thiolate species.

NO_2 is capable of oxidizing substrates via either H-atom abstraction⁵⁶ (reaction 25) or addition across unsaturations⁵⁷ (reaction 26). Either mechanism results in the generation of radical intermediates that can react further with other radical species.



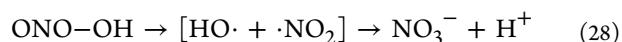
Most studies examining the addition of NO_2 to unsaturated bonds (reaction 26) report the addition reaction to occur via the nitrogen-centered radical species as opposed to the oxygen-centered radical (Figure 6). However, the addition of NO_2 to a nitron spin trap has been reported to occur via the oxygen atom,⁵⁸ indicating the possibility of O-atom attack on unsaturated systems. Moreover, trapping of radicals generated via an initial NO_2 addition to an unsaturation (reaction 26) can occur via the O- or N-atom of NO_2 ,⁵⁹ indicating that the chemistry of either the nitrogen-centered radical species ($\text{O}_2\text{N}\cdot$) or the oxygen-centered radical ($\text{ONO}\cdot$) can occur.

Dinitrogen Trioxide (N_2O_3). As described above (reaction 19), N_2O_3 can be generated from the reaction of NO_2 with NO in a facile radical-radical coupling reaction. Besides reaction with water to give NO_2^- (reaction 20), N_2O_3 can react with other nucleophiles (such as thiols or amines) leading to nitrosated (addition of “+NO”) products (reaction 27).



N_2O_3 can be viewed as the anhydride of nitrous acid (HONO),⁶⁰ and therefore, acidic solutions of NO_2^- can generate N_2O_3 via a simple equilibrium reaction (reverse of reaction 20). Therefore, at high concentrations of NO_2^- and under acidic conditions (note that the reverse of reaction 20 is second order in NO_2^- and is proton dependent) nitrosation reactions can occur via N_2O_3 formation.

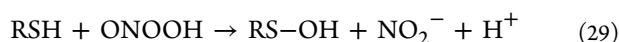
Peroxynitrite (ONOO^-). Because of its possible biological relevance, ONOO^- has been studied extensively.^{61,62} The conjugate acid of ONOO^- , peroxynitrous acid (ONOOH), has a pK_a of 6.8 indicating a predominance of the anionic species at physiological pH. *cis*-Peroxynitrous acid will undergo rearrangement to give nitrate, possibly via homolytic cleavage of the O-O bond and reattachment of the radical pair (reaction 28).⁶³



Although radical intermediates in the rearrangement of ONOOH to NO_3^- are generated, they are bound initially in a solvent cage that limits their reactivity with other species resulting in primarily reaction with each other giving the isomeric NO_3^- product. To be sure, the homolytic cleavage of the O-O bond for this rearrangement has been questioned,^{64,65}

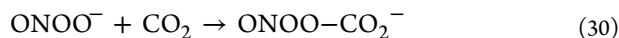
and the oxidation chemistry associated with peroxynitrite (see below) proposed to occur through other species in the peroxynitrous acid-to-nitrate rearrangement.⁶¹

Peroxynitrite is capable of oxidizing a variety of substrates, a property that has led to hypotheses regarding its possible pathophysiological role in a variety of diseases and disorders. An important target for ONOOH/ONOO[−] has been proposed to be tyrosine residues. The reaction of peroxynitrite with tyrosine results in nitration, forming 3-nitrotyrosine. The mechanism for this process may proceed through an initial one-electron tyrosine oxidation (by the HO· formed in reaction 28), followed by trapping of the tyrosyl radical by NO₂ (similar to what is depicted in Figure 7).⁶⁶ Again, oxidation processes not involving HO· have been proposed as well (vide supra). Peroxynitrite also oxidizes thiols to the corresponding sulfenic acids (reaction 29) in a manner similar to that previously discussed for peroxide.

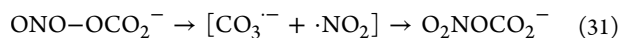


As with tyrosine nitration, it is proposed that thiol oxidation can also occur via a radical pathway involving the thiyl radical.⁶⁷ Peroxynitrous acid can directly perform 2-electron oxidations (i.e., sulfide to sulfoxide) as well,⁶² indicating that it can be a versatile oxidant capable of both one- and two-electron oxidations.

One of the most relevant reactions of peroxynitrite under physiological conditions is with carbon dioxide (CO₂), giving as an intermediate nitrosoperoxy carbonate (ONOOOCO₂[−]) (reaction 30).



Considering the moderately high rate constant for reaction 30 ($5.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) and the typically high levels of CO₂ in most tissues (>1 mM), this reaction is likely to be a primary fate of physiologically generated peroxynitrite.⁶⁸ Similar to the spontaneous decomposition of ONOOH, nitrosoperoxy carbonate also decomposes via homolytic O–O bond cleavage, generating the carbonate radical anion (CO₃^{·−}, vide infra) and NO₂, which can recombine to give nitrocarbonate (O₂NOCO₂[−]) (reaction 31). Hydrolysis of nitrocarbonate yields NO₃[−] and carbonate (CO₃^{2−}).



Since both CO₃^{·−} (CO₃^{·−}/HCO₃[−], $\epsilon^0 = 1.78 \text{ V}$, vs NHE at pH 7)³¹ and NO₂ (NO₂/NO₂[−], $\epsilon^0 = 1.04 \text{ V}$ vs NHE) are both reasonable one-electron oxidants, the products of the reaction of CO₂ with ONOO[−] can still elicit oxidation chemistry.⁶⁹

Nitrite (NO₂[−]). As discussed above, NO₂[−] is generated from the autoxidation of NO (reactions 18, 19, and 20). Because of the high order kinetics associated with the generation of NO₂[−] from NO, this chemistry will only be physiologically relevant at high concentrations of NO.⁷⁰ Also mentioned above, NO₂[−] can be a source of NO under acidic conditions and at high concentrations (due to the reversibility of reactions 20 and 19). However, more recent studies allude to other pathways for the generation of NO from NO₂[−] via one-electron reduction pathways. The one electron reduction potential for NO₂[−] is highly proton dependent and, under appropriately acidic conditions, very favorable (HNO₂/H⁺/NO, $\epsilon^0 = 0.98 \text{ V}$ vs NHE)⁷¹ yielding NO. It is reported that xanthine oxidase,⁷²

cytochrome c,⁷³ and hemoglobin^{74,75} are capable of reducing NO₂[−] to NO.

Coordination of NO₂[−] to metal centers (e.g., iron hemes) can result in either an N-bound “nitro” complex or an O-bound “nitrito” complex (Figure 8).⁷⁶

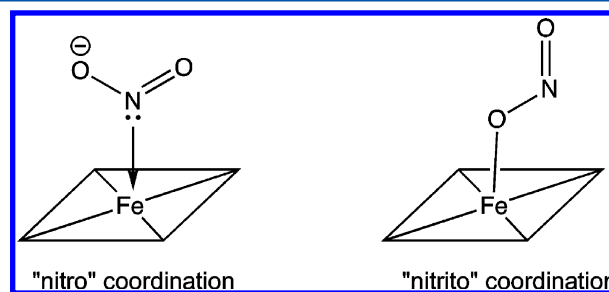


Figure 8. Nitro and nitrito coordination of NO₂[−] to the iron center.

In most cases, the nitro complex is observed in both Fe(II) and Fe(III) heme proteins,⁷⁷ although the nitro complex appears to be only slightly favored over the nitrito complex.⁷⁸ However, NO₂[−] binding to Fe(III)Mb gives the nitrito complex,⁷⁷ which is stabilized via a hydrogen bonding residue proximal to the bound NO₂[−].⁷⁶ Because of a great and recent interest in mammalian mechanisms of NO generation via NO₂[−] reduction,⁷⁹ numerous recent studies have examined the reduction of heme-bound nitrite. Indeed, it is known that reduction of ferrous heme-bound NO₂[−] can lead to the generation of NO. Significantly, computational examination of the O-bound nitrito complex of cytochrome *cd1* nitrite reductase indicates that a hydrogen bond from a protein residue (e.g., histidine) to the metal-bound oxygen allows facile NO generation with subsequent formation of a ferric hydroxo species.⁷⁸ Similarly, proton donation to an oxygen atom of the nitro complex can also result in decomposition with generation of a ferric-nitrosyl and water.^{79–81} These processes are schematically depicted in Figure 9.

Consistent with the idea that proton donation to the bound nitrite is an important factor in the reduction to NO, mutation studies that eliminate the H-bonding histidine in myoglobin report a significantly decreased rate of NO₂[−] reduction.⁷⁶ The physiological relevance of the NO₂[−] reduction chemistry discussed above remains to be established. Moreover, other chemistries associated with the interaction of NO₂[−] with hemoglobin (not discussed herein) may also be relevant to the generation of NO (or equivalent) from NO₂[−].⁸²

Nitroxyl (HNO). All of the above-mentioned nitrogen oxides are oxidized with respect to NO. Recent reports allude to the possibility that reduced forms such as HNO can also be generated in mammalian systems,⁸³ although this has not been established. As with many nitrogen oxides, the biological chemistry of HNO likely involves reaction with thiols and metals. Indeed, HNO appears to be particularly thiophilic.⁸⁴ It should be mentioned here that the reaction of a nucleophilic thiol with HNO is distinct from the reaction of a thiol with NO since HNO does not have a partially filled π^* orbital (Figure 4). The reaction of a thiol with HNO generates as an intermediate an *N*-hydroxysulfenamide (reaction 32, RSNHOH). This intermediate can then further react via two pathways, depending on the availability of other reactive thiols. In the presence of excess thiols (or vicinal thiols), further reaction occurs leading to the formation of the disulfide and

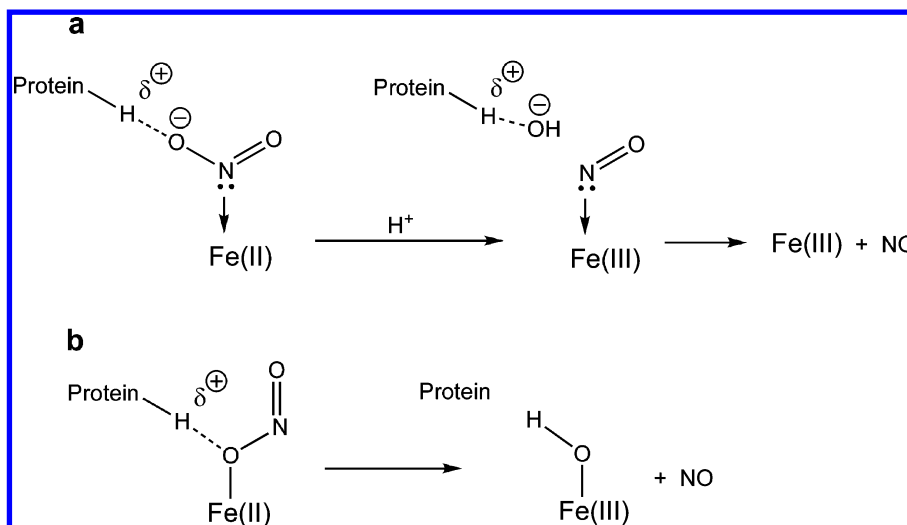
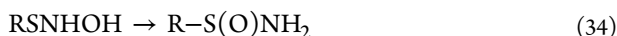
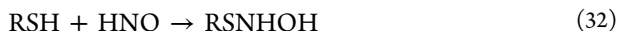


Figure 9. Possible mechanisms for the generation of NO via the reduction of ferrous-heme-bound nitrite. (a) Ferrous ion reduction of the nitro complex and (b) reduction of the nitrito complex.

hydroxylamine (NH₂OH) (reaction 33). Presumably, other strong nucleophiles can react similarly. In the absence of another thiol, the intermediate *N*-hydroxysulfenamide rearranges to generate a sulfinamide (reaction 34, RS(O)-NH₂).⁸⁵ Of particular significance with regard to HNO-mediated thiol modification is that HNO can oxidize the sulfur atom by 4 electrons in a single sequence when the sulfinamide is formed.



HNO also reacts readily with metalloproteins. For example, a stable ferrous-HNO complex (N-bound) with myoglobin has been generated and characterized⁸⁶ (reaction 35), and although not unequivocally demonstrated, others have alluded to a similar reaction with the ferrous heme of the enzyme guanylate cyclase, leading to activation of the enzyme.⁸⁷ Also, the reaction of HNO with ferric heme proteins can result in the generation of the corresponding ferrous-NO complex via reaction 36.



Unlike most other commonly studied nitrogen oxides, HNO cannot be stored due to a self-dimerization reaction, ultimately leading to the formation of nitrous oxide (N₂O) and H₂O (reaction 37).

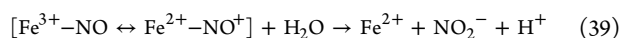


Thus, HNO is typically examined using donor compounds,⁸⁸ especially in biological systems. The HNO dimerization reaction precludes the generation of highly concentrated solutions of HNO. However, due to many other possible reactions with biological species (thiols), this reaction is likely to be scarce in biological systems due to the second order kinetics.

Although HNO reacts with O₂, it is generally thought that the relatively slow kinetics precludes this reaction from being biologically relevant.⁸⁹ Moreover, HNO does not hydrate appreciably (unlike, for example, formaldehyde) indicating that the biological activity of HNO is not likely due to the hydrated species.⁸⁴ Finally, HNO can be converted to NO via simple one electron oxidation, a process that can be performed by, for example, the cupric form of CuZn SOD⁹⁰ (reaction 38).



Coordination Chemistry of NO. Although NO can form complexes with many metals,⁹¹ herein is discussed only its interactions with ironheme proteins since these appear to be a major target for NO in biological systems. Nitric oxide is an "amphoteric" ligand (properties of an acid and a base) as it can bind to a metal as a nitrosonium cation (NO⁺) or a nitroxyl anion (NO⁻). Since NO⁺ is isoelectronic with CO, the binding geometry of NO⁺ is linear (i.e., a linear geometry of the metal-nitrogen-oxygen atoms; an explanation for this is given below in the discussion of CO coordination chemistry). NO⁻, however, is isoelectronic with O₂ and therefore binds in a bent geometry (akin to that shown above for O₂; Figure 2). When NO binds to ferrous (Fe²⁺) heme proteins, it binds in a bent fashion, like O₂, indicating an Fe³⁺-NO⁻-like complex. However, when NO binds ferric (Fe³⁺) proteins, it binds in a linear fashion, indicating that the Fe³⁺-NO interaction is more appropriately represented by Fe²⁺-(NO⁺).⁹² Importantly, ferric-NO complexes can undergo reductive nitrosylation leading to the formation of an oxidized nitrogen oxide (when water reacts) and a ferrous-NO complex (reactions 39, 40).



One of the most physiologically important aspects of NO coordination chemistry is its preference to form 5-coordinate complexes with ferrous heme proteins. Unlike other small molecule ligands (i.e., O₂ and CO), the coordination of NO to a ferrous heme labilizes the proximal ligand, leading to the

release of the ligand and generation of a 5-coordinate, square pyramidal nitrosyl complex (Figure 10).⁹³

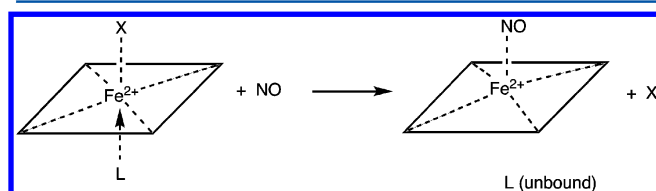


Figure 10. Proximal ligand release via the coordination of NO to a ferrous heme.

This aspect of NO coordination chemistry is likely the reason it is such a specific activator of the ferrous heme enzyme guanylate cyclase. That is, with other ligands (CO for example) the 6-coordinate, octahedral complex is stable, and the proximal ligand is not labile. Therefore, if the release of the proximal ligand is important to protein function, as in guanylate cyclase, then activation will be fairly specific to NO.

The ability for NO to weaken the proximal ligand bond in ferrous-heme complexes (often referred to as the “trans-effect”) has been attributed to electron donation from an energetically matched and the partially filled π^* orbital of ligated NO to the d_z^2 orbital on the metal. That is, when NO binds with a “bent” geometry, the two π^* orbitals are no longer degenerate. One of the π^* orbitals remains available for back-bonding with the d_{yz} or d_{xy} orbitals on the metal, while the other is now able to form a σ -bond with the d_{z^2} orbital on the metal.⁹⁴ If the metal complex possesses a ligand that is proximal or trans to the NO ligand, the d_{z^2} is already filled with electrons from this ligand. Thus, the electron from the NO π^* orbital can only occupy the σ^* orbital associated with the σ -bond of the trans ligand. Populating this antibonding orbital of the metal–proximal ligand interaction leads to a weakening of this bond to the metal resulting in the loss of the ligand and a preference for a 5-coordinate complex^{95–97} (Figure 11).

Significantly, a trans effect is weak or nonexistent with CO and O₂ due to mismatched energies of the relevant orbitals on the metal and ligand as well as other overriding effects.

S-Nitrosothiols (RS-NO). As mentioned above, S-nitrosothiol formation has been proposed to be an important event in the regulation/function of a variety of thiol proteins.⁹⁸ Therefore, it is reasonable to devote a modicum of space discussing the possible mechanisms of their formation and degradation (or at least review and collate the chemistry already discussed). However, prior to embarking on this chemical discussion, it is worthwhile to first address the often-confusing nomenclature and terminology associated with S-nitrosothiols, their formation, and related species. Currently, several terms are used almost interchangeably, some of which allude to specific chemical processes, and some of which are general descriptors of structure or bonding. For example, the term “nitrosation” (i.e., S-nitrosation) in the strictest chemical sense refers to a process by which nitrosonium ion (NO⁺) (or reactive equivalent) reacts with a nucleophile (e.g., a thiol) (reactions 21 and 27). Thus, when the term S-nitrosation is used, it is implied that an electrophilic nitrogen species with nitrosonium ion character (discussed below) has reacted with a thiol to form an S-nitrosothiol. Another term often used is “S-nitrosylation”. This merely implies that a bond between a sulfur atom and the nitrogen atom associated with NO has been formed and does not imply a mechanism of bond formation. Inorganic and

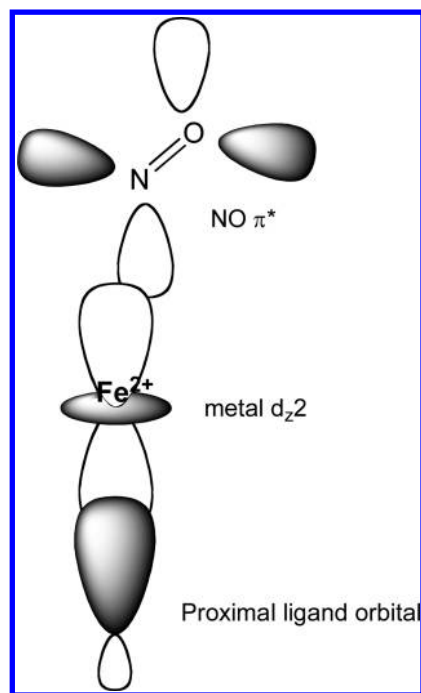


Figure 11. Donation of electrons to the metal from the NO π^* orbital in the bent geometry leading to a weakening of the trans-ligand bond (note: other bonding interactions are not shown).

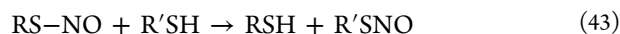
organometallic chemists originally used “nitrosyl” to describe metal nitrosyls which are coordination complexes where the nitrogen atom of NO is bound to a metal center (M-NO, M = metal). In many cases, metal “nitrosylation” refers to processes where a direct reaction of NO with a metal center generates a metal nitrosyl, although, for the most part, the term does not imply a chemical mechanism by which this complex forms or any other chemistry associated with the complex. That is, metal nitrosyls can be made via a variety of distinct processes and can have metal-NO⁺ character or metal-NO[−] character;⁹⁹ the term “nitrosyl” does not distinguish between any of these. The term “nitroso” is typically used to describe a functional group whereby a bond between the nitrogen of NO and, most often, main group elements is present (e.g., C-nitroso for C-NO, S-nitroso for S-NO, and N-nitroso for N-NO). However, there is no strict adherence of the use of the term “nitroso” to describe the bonding in main group elements. For example, nitrosyl chloride (NOCl) and nitrosyl tetrafluoroborate (NOBF₄ and also referred to as nitrosonium tetrafluoroborate) are often used.

It is important to remember that NO will not react directly with thiols under biological conditions (*vide supra*). If a direct reaction between a thiol and NO did occur (hypothetically), the generation of an S-nitrosothiol would require the loss of an electron (i.e., RSH + NO → RSNO + e[−] + H⁺), a process that will not happen without an electron acceptor present. Thus, S-nitrosothiol formation can readily occur if either the thiol or NO is first oxidized. Described above are several processes where this is the case. For example, species such as N₂O₃ (see reactions 21 and 27) or Fe²⁺-NO⁺ (see reaction 39) all represent nitrogen oxide species whereby the NO moiety is electron poor (and can be viewed as partially oxidized) and, therefore, capable of nitrosating a thiol. However, a one-electron oxidized thiol species, a thiyl radical (RS·), will directly react with NO to give an S-nitrosothiol (akin to reaction 22). Thus, NO can be oxidized by O₂ to generate a thiol nitrosating

species, N_2O_3 (reactions 18 and 19–21), NO can coordinate an oxidizing metal, such as Fe^{3+} , to give it nitrosonium-like character ($\text{Fe}^{2+}\text{-NO}^+$, which can nitrosate a thiol (reaction 41) or a thiol can be oxidized to a thiyl radical (for example, by NO_2 , reaction 24, or other oxidant) which can directly react with NO to give an S-nitrosothiol). Although one can envision other possible mechanisms for S-nitrosothiol formation involving other nitrogen oxides and/or thiol redox states, those mentioned immediately above would appear to be the ones to consider first.

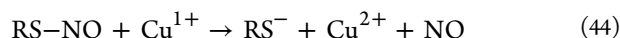


If indeed S-nitrosothiol formation is an important biological regulatory pathway, the possible mechanisms of its degradation are equally important as the mechanisms of formation. There are several established chemical mechanisms by which an S-nitrosothiol can be degraded. One way is via a reaction with another thiol. This reaction can follow two pathways that result in two distinct products: one pathway involves an attack of the nucleophilic thiol at the sulfur atom of the RS-NO reactant and generates a disulfide and HNO (reaction 42), and the other pathway involves an attack of the nucleophilic thiol at the nitrogen atom of the RS-NO species and results in the simple transfer the NO function (or NO^+) from one thiol to the other (referred to as transnitrosation) (reaction 43).⁸⁵



Although both reactions are well documented, the factors that govern the site of nucleophilic attack are not, as yet, known. Although reaction 43 does not lead to the overall destruction of an S-nitrosothiol per se, it does lead to the loss of this functionality in the original RS-NO species. It would not be surprising to find that other potent nucleophiles react similarly with S-nitrosothiols.

S-Nitrosothiols can also be degraded reductively. For example, cuprous ion (Cu^{1+}) can reduce an S-nitrosothiol to give a thiolate and NO (reaction 44).¹⁰⁰ This system is potentially catalytic since the thiolate product is capable of reducing the cupric ion (Cu^{2+}) back to cuprous ion.



Using cyclic voltammetry, the single reduction peak potential for a series of S-nitrosothiols has been determined to be -0.8 to -1.1 V (vs Ag/AgCl).¹⁰¹ These fairly negative values would indicate that RS-NO species are not easy to reduce under biological conditions.

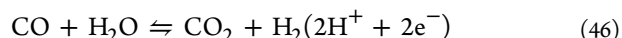
S-Nitrosothiols can also be degraded photochemically (reaction 45).¹⁰² Although the biological relevance of this process is questionable, this is clearly an important issue when using S-nitrosothiols in research. Significantly, photochemical NO release from RSNO occurs in both the UV and visible range, indicating that special care must be taken when using these compounds in the presence of light.¹⁰³



BIOLOGICAL CHEMISTRY OF CARBON MONOXIDE

Compared to NO and O_2 , the biological chemistry of CO is relatively simple. Most studies of CO biology (at least in mammalian systems) do not consider the possibility of redox

processes. However, it is worth mentioning that in some bacteria CO can be used as a source of carbon and electrons. For example, the bacterial enzyme CO-dehydrogenase is capable of oxidizing CO in a reaction equivalent to the water-gas-shift reaction (reaction 46).¹⁰⁴



Electrons derived from this process can be fed into a respiratory pathway, making this a potentially very important bacterial process. The reduction potential for the CO_2/CO couple ($\mathcal{E}^{\circ'} = -0.558$ V)¹⁰⁵ indicates that the oxidation of CO is very favorable, and therefore, it is not surprising that CO can be used as an electron source. Indeed, several studies have reported that $\text{CO}/\text{H}_2\text{O}$ can serve as a source of electrons for the reduction of mammalian heme proteins via this chemistry.^{106,107} However, the rate of reduction of, for example, cytochrome *c* oxidase, hemoglobin, or myoglobin is extremely slow (even under an atmosphere of CO) indicating that this chemistry may not be physiologically relevant in mammals.

Clearly, the most important biological aspect of CO chemistry is its ability to bind to metals. Although CO can form many types of coordination compounds, herein the focus will be on hemeprotein-CO interactions due to the known biological relevance. In general, the propensity of CO to form coordination complexes can be explained by its ability to form two types of bonds with metals (akin to O_2). Donation of a lone pair of electrons in an sp orbital on the carbon atom of CO to an empty d-orbital on the metal (typically the d_{z^2} orbital for heme proteins) generates a σ -bond between CO and the metal. Further bonding occurs when electrons in filled metal d-orbitals are donated "back" to the π -antibonding orbitals of CO. This bonding is analogous to that described previously for O_2 and is depicted in Figure 12.

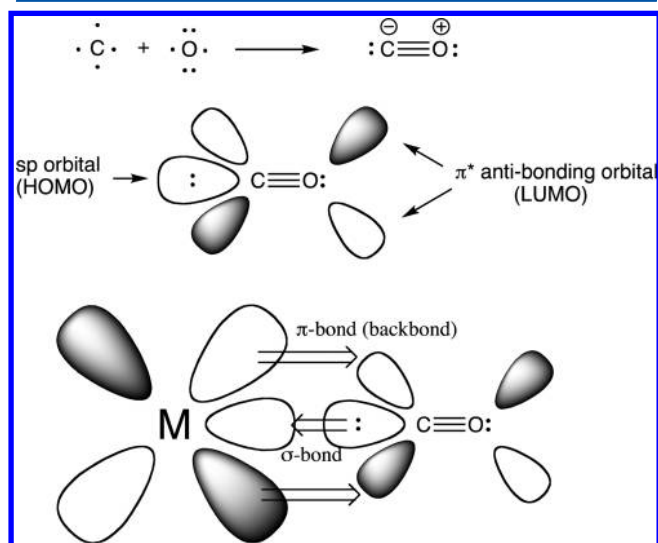


Figure 12. Bonding in CO and CO-metal complexes.

Since the σ -bonding interaction involves an electron pair in an sp hybridized orbital on CO, the preferred geometry for CO binding to a metal is linear. As with O_2 , the π -bonding (back-bonding) component of the metal-CO interaction is extremely important to the overall binding energetics. Indeed, CO and O_2 will only bind ferrous and not ferric heme proteins since the Fe^{2+} oxidation state is more electron rich and, therefore, better

able to donate electrons “back” to CO. Generally speaking, the metal bonding schemes for NO, CO, and O₂ are similar in that there are both σ and π components. The degree of back-bonding differs, however. In the case of O₂, the back-bonding is so significant that an Fe²⁺-O₂ complex is more accurately depicted as an Fe³⁺-O₂⁻ complex. This is not the case with CO, which has much less charge transfer from the metal. The degree of back-bonding for CO, NO, and O₂ is correlated to the relative energies of the p-orbitals, indicating that for X=O, the degree of charge transfer to the ligand is X = O > N > C.¹⁰⁸ The factors that allow heme protein discrimination between these ligands will be discussed later.

From a purely physiological perspective, the biological activity and function of endogenously generated CO is likely due to its ability to complex metal centers in mammalian systems. It is known that CO will react with, for example, HO·,¹⁰⁹ but this seems to be an unlikely fate/function for CO in light of the fact that HO· is such an indiscriminant oxidant and will react with almost all molecules in a cell. Thus, the biological targets for CO are likely to be metal centers (e.g., heme proteins), which may also bind O₂ and NO. Importantly, the biological activity of CO is not necessarily the result of direct actions associated with CO binding to a metal center but, rather, the ability of CO to block the actions of O₂ or NO binding. Indeed, the toxicity of CO is primarily the result of interference with O₂ transport via CO binding to hemoglobin. A comparison of the binding of these species to metal centers and the ability for proteins to discriminate between these species will be addressed below.

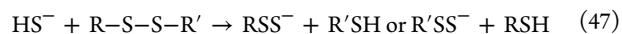
An important aspect of CO chemistry that sets it apart from the other signaling species (NO, O₂, and H₂S), is that it is resistant to conversion to other chemical species. As discussed above, O₂ and NO can be easily converted to numerous other species via myriad chemical/biochemical processes, and as will be discussed below, H₂S can be converted to a variety of oxidized species as well. In mammalian systems, CO is relatively inert and appears to only serve as a metal ligand for some metalloproteins. This aspect of CO may be an important factor in its biological utility. That is, CO is resistant to degradation and can “survive” conditions that would otherwise lead to the destruction of NO, O₂, or H₂S. Thus, the signaling of CO should be more robust than, for example, NO since it is less dependent on cellular conditions and the presence of oxidizing/reducing species. Whether this aspect of CO signaling is important remains to be determined.

■ BIOLOGICAL CHEMISTRY OF HYDROGEN SULFIDE AND RELATED SPECIES

The most recently proposed endogenously generated small molecule signaling agent is H₂S. Because of the paucity of current literature addressing the biologically/physiologically relevant chemistry of H₂S, a slightly more detailed discussion of this species will be given, along with brief introductions to some of its reported biochemistry. Hydrogen sulfide differs somewhat from O₂, NO, and CO in that it can be ionized in the physiological pH range. The pK_a of H₂S is 6.8, and the pK_a of HS⁻ is 14.1. Thus, at physiological pH, HS⁻ is the predominant species with vanishingly small amounts of S²⁻ present. It is important to note that the pK_a for H₂S is significantly lower than most alkylthiol species (RSH), which typically have values 1–2 pK_a units higher. Thus, there will be a greater proportion of HS⁻ in solution compared to RS⁻ under most conditions. It

should be realized, however, that cysteine thiols at some protein active sites can have extremely low pK_a values (much lower than H₂S) due to protein interactions that stabilize the thiolate anion.¹¹⁰ The reduction potential for the HS⁻/HS[•] and ·S⁻/HS⁻ couples are estimated to be approximately 0.9–1.1 V (vs NHE) similar to alkyl thiyl radicals.^{52,111} This indicates that the one-electron oxidized species are biologically accessible (like thiyl radicals of cysteine peptides and proteins)¹⁴ and are reasonable oxidants. The bond dissociation energy of the S–H bond in H₂S is 90 kcal/mol, a value also consistent with the oxidizing potential of HS⁻. Anionic HS⁻ is also a very nucleophilic species, on par with trialkyl phosphines and alkyl thiolates.¹¹²

The nucleophilicity of H₂S predicts that it will readily participate in polysulfide–thiol exchange chemistry of the type shown in reaction 47.^{113,114}

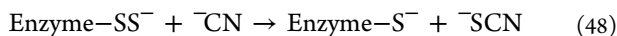


Reaction 47 depicts the reaction of HS⁻ with a disulfide electrophile. It is easy to envision an analogous reaction with higher polysulfides (i.e., RSSSR') as well. A product of reaction 47 is a hydropersulfide/persulfide anion (RSSH/RSS⁻). Hydropersulfides are important biological species known primarily for their ability to donate sulfane sulfur (the terminal sulfur atom) to a variety of biochemical cofactors and prosthetic groups.¹¹⁵ However, it appears likely that the biological chemistry of hydropersulfides has the potential to go beyond its ability to serve as a donor of sulfane sulfur. Indeed, literature precedence seems to indicate that the hydropersulfide oxidation state in proteins can have numerous activities (vide infra) including enhanced activity.^{116,117}

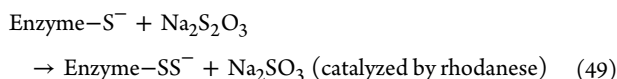
As with the other small molecule signaling agents (O₂, NO, and CO), another hallmark of H₂S (or HS⁻/S²⁻) biological chemistry is metal binding. Indeed, the toxicity associated with high level H₂S exposure is thought to be due primarily to its ability to bind to cytochrome *c* oxidase, leading to the inhibition of respiration. However, unlike O₂ and CO (and for the most part NO), which bind to metals in lower oxidation states (e.g., Fe²⁺ rather than Fe³⁺), H₂S binds metals as an anion, HS⁻, and tends to bind to oxidized forms of metals (e.g., Fe³⁺ rather than Fe²⁺). Particularly noteworthy with regards to S²⁻ metal ligation is the existence and importance of iron–sulfur (FeS) clusters in biology as electron transfer prosthetic groups and Lewis acids in enzymes.¹¹⁸ Although it is likely that a portion of the biological activity of H₂S can be a result of interactions with metal-containing systems, there has yet to be identified any metalloprotein that serves as a target for its reported physiological actions (although cytochrome *c* oxidase is a potential target and has been exploited pharmacologically to induce an H₂S-mediated hibernative state).¹¹⁹

As indicated above, an eventual fate of the reaction of H₂S with oxidized thiol species can be the generation of a hydropersulfide (for example, reaction 47).¹²⁰ Thus, it is not hard to imagine that hydropersulfide generation and chemistry can be involved in the mechanism(s) of H₂S-mediated biological activity. Indeed, several reports in the literature suggest that hydropersulfides can be important mediators of enzyme function. For example, several studies from Massey's group revealed an important hydropersulfide at the active site of xanthine oxidase.^{116,117} Cyanolysis of the hydropersulfide, giving the thiolate and thiocyanate (reaction 48), results in loss of enzyme activity, indicating a crucial role for the

hydropersulfide in catalysis.



Similarly, the enzyme aldehyde oxidase was found to contain an active site hydropersulfide that was also crucial for enzyme activity.¹²¹ When the mitochondrial enzyme malate dehydrogenase is reacted with thiosulfate and rhodanese (a system that will convert thiols to persulfides, reaction 49), a significant increase in activity was found.¹²²



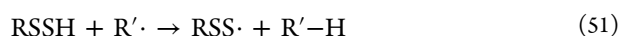
An enzyme involved in heme biosynthesis, aminolaevulinatase synthetase was also reported to be greatly activated in the presence of polysulfides (i.e., RSSSR and larger), possibly via eventual hydropersulfide formation at a protein thiol residue.¹²³ More recently, Kim and co-workers have reported the presence of an important hydropersulfide forming cysteine residue in a Ni carbon monoxide dehydrogenase.¹²⁴ The authors propose that this hydropersulfide may be important to the stability and generation of the active site Fe–Ni cluster and, possibly, as a redox species important for catalysis. Hydropersulfide modification of a solvent-exposed cysteine residue in the antioxidant enzyme CuZn SOD has been found and reported to have altered biophysical properties.¹²⁵ A recent and provocative study indicates that persulfide generation in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) leads to an increase in activity.¹²⁶ In this study, persulfide formation was proposed to occur on the active site cysteine, indicating an increased biochemical reactivity associated with the persulfide oxidation state. However, this study did not elaborate on the mechanism or origin of the activation. Discussed later, an increase in chemical reactivity of a persulfide compared to the corresponding thiol may be predicted. To be sure, protein hydropersulfide formation has been reported to lead to enzyme inhibition as well.¹²⁷

At this point, it seems likely that hydropersulfides are a part of the thiol redox reaction manifold that includes the more well studied and known thiols (RSH), thiyls (RS·), disulfides (RSSR), sulfenates (RSO⁻), sulfinates (RS(O)O⁻), sulfonates (RS(O)₂O⁻), and nitrosothiols (RSNO). It should be noted that there is a strong possibility that, along with the examples listed above, many other protein hydropersulfides exist. The reason for this statement is that a likely mechanism of endogenous protein persulfide formation involves an H₂S reaction with oxidized cysteine species and that this post-translational modification has not been examined thoroughly in in-vitro preparations. This is especially true since purification of thiol proteins is typically performed under reducing conditions that will reduce the oxidized species including hydropersulfides, precluding their isolation from natural sources. Hydropersulfides are also inherently unstable and will, among other decomposition pathways, disproportionate to sulfides (thiols) and elemental sulfur (S⁰)¹²⁸ (reaction 50) making their long-term storage difficult. Thus, it is possible that hydropersulfides are common, an inherent member of the thiol redox family, and serve specific functions as regulators of thiol protein activity.



The chemistry of hydropersulfides (especially biologically relevant hydropersulfides) is relatively unknown. Although they

have been observed in numerous enzymes (*vide supra*) and found to elicit both increases and decreases in activity, the chemistry associated with these effects is not established. In comparing the fundamental chemistry of a thiol to the corresponding hydropersulfide, it is known that hydropersulfides are significantly more acidic.¹²⁹ For example, the pK_a1 for HSSH is only 5 compared to a pK_a1 of 6.8 for H₂S. This trend holds true for alkyl hydropersulfides versus alkyl thiols as well.¹³⁰ That is, the pK_as of alkyl hydropersulfides (RSSH) are approximately 1–2 pK_a units lower than the corresponding alkyl thiols (RSH). Thus, the anionic form of a persulfide will be much more prevalent than the anionic form of the corresponding thiol. Persulfides are also better reductants than thiols, as evidenced by a significantly lower S–H bond dissociation energy (BDE) (RSS-H BDE = 70 kcal/mol, RS-H BDE = 92 kcal/mol).¹³¹ Accordingly, persulfides should more readily donate a hydrogen atom to one-electron oxidants (reaction 51) compared to the analogous reaction with thiols.



Moreover, it is likely that the persulfide anion is a better one-electron reductant compared to the corresponding thiolate as well (reaction 52) (although a reduction potential for RSS⁻ is not reported).



Analogous to the relationship between ammonia (NH₃) and substituted derivatives hydroxylamine and hydrazine (NH₂OH and NH₂NH₂), it is expected that hydropersulfides are also better nucleophiles than the corresponding thiols. That is, putting a lone pair of electrons adjacent to the nucleophilic pair of electrons greatly increases their nucleophilic reactivity. The effect (often referred to as the “alpha-effect”) has been proposed to be due to a number of factors including ground state destabilization via lone-pair repulsion, transition state stabilization by the adjacent lone pair, and reduced solvation (and therefore destabilization) of species with adjacent lone pairs.¹³²

Thus, compared to thiols, hydropersulfides possess increased acidity (indicating an increased concentration of the anionic species), greater inherent nucleophilicity (alpha-effect), and greater one-electron reducing capabilities (via H-atom donation, RSSH → RSS· + H·, or one-electron donation, RSS⁻ → RSS· + e⁻) and are likely to be a better metal ligand (related to its increased nucleophilicity). In biological systems, thiols are known to be strong nucleophiles, metal ligands, and reducing agents. Indeed, much of the biological utility of thiols relies on these chemical properties. It is not difficult, therefore, to imagine that hydropersulfides can be hyperactivated congeners of thiols since all of the important chemical properties of thiols are seemingly enhanced in hydropersulfides.¹²⁰ Figure 13 schematically depicts the types of enhanced chemistry possibly associated with persulfide generation from thiols.

Whether the proposed enhanced reactivities of persulfides, compared to thiols, depicted in Figure 13 are relevant to the biological activity of H₂S remains to be determined.

■ INTEGRATED BIOLOGICAL CHEMISTRY OF O₂, NO, CO, H₂S, AND THEIR DERIVATIVES

The discussions above focused on the biological chemistry associated with, for the most part, the individual chemical species O₂, NO, CO, H₂S, and their derived species (although numerous examples of chemical interactions between these molecules have already been mentioned). As stated earlier, the

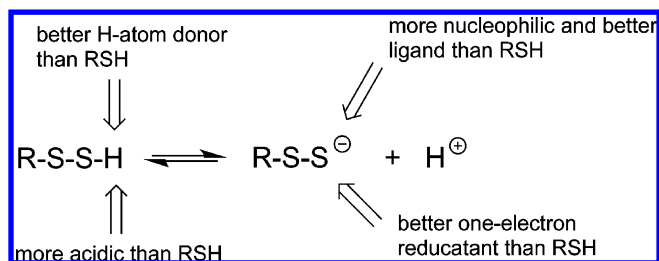
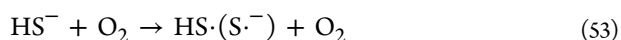


Figure 13. Enhanced chemical properties of persulfides compared to thiols.

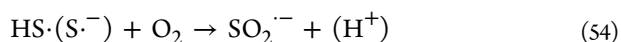
tenet of this review is that all of these small molecule signaling agents have integrated physiology based on their integrated chemistry. That is, these species (or derivatives thereof) have the ability to (1) react with each other, leading to the cessation of activity of one (or both) of the individual species, (2) react with each other to (re)activate an inhibited state, (3) react with each other to generate another species with different activity, (4) compete with each other at specific biological targets leading to regulation of that target, (5) enhance or inhibit each other's actions via chemical modification at diverse sites on the same proteins or signaling system, or (6) possess similar activity but have distinct lifetimes/stabilities under certain biological states/conditions. Although other possibilities exist for meaningful interactions between these species, the focus herein will be on these.

Interactions of O_2 and Derived Species with NO, CO, and H_2S . The only small molecule species that has the potential to chemically interact with all molecules discussed herein is O_2 (and derived species). As mentioned above, O_2 reacts with NO to give a series of reactive nitrogen oxides such as NO_2 , N_2O_3 , and NO_2^- . The kinetics of the generation of these products indicate that this chemistry will only occur to a significant extent at elevated concentrations of NO (vide supra). The reaction of NO with O_2 leads to the formation of species with entirely different reactivity compared to the starting molecules. Neither NO or O_2 are good one-electron oxidants, whereas NO_2 is a reasonable one-electron oxidant. O_2 and NO are also not themselves reactive with nucleophiles (e.g., thiols), whereas N_2O_3 is very electrophilic and capable of modifying thiol nucleophiles (reaction 27).

O_2 will also react with H_2S . This chemistry is very complex and leads to the generation of oxidized sulfur species, including sulfite (SO_3^{2-}), thiosulfate ($\text{S}_2\text{O}_3^{2-}$), sulfate (SO_4^{2-}), and elemental sulfur (S^0), depending on the concentrations of the reactants, ionic strength, and pH.^{133,134} Many of the highly oxygenated products are a result of numerous parallel reactions occurring after an initial step that is first order in the anion HS^- and first order in O_2 . This first step is thought to be a one-electron oxidation by O_2 of the anion to give, initially, a sulfhydryl radical ($\text{HS}\cdot$) and superoxide (reaction 53).¹³³ Although the pK_a of $\text{HS}\cdot$ has not been accurately determined, it is generally thought to be fairly acidic (possibly as low as 3–4)¹¹¹ and therefore exists primarily as the radical anion (S^-) at pH 7.

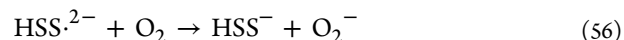


A series of subsequent reactions can then occur, leading to the many oxidized products. For example, it is reported that $\text{HS}\cdot(\text{S}^-)$ can react further with O_2 to give the sulfur dioxide radical anion ($\text{SO}_2^{\cdot-}$) in a very fast reaction ($k = (4\text{--}5) \times 10^9 \text{ M}^{-1}\text{s}^{-1}$) (reaction 54).¹¹¹



The sulfur dioxide radical anion, also known as the dithionite radical, is a good reductant ($\epsilon^0 = -0.31 \text{ V}$ at pH 2 and above) and can react quickly with O_2 to give SO_2 and $\text{O}_2^{\cdot-}$ ($k = 1.5 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ at pH 6.8).¹³⁵ Thus, $\text{HS}\cdot/\text{S}^-$, which is a reasonable oxidant (vide infra), can react with O_2 to generate a good reductant.

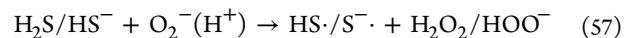
S^- can also react with HS^- to give $\text{HSSH}\cdot^-/\text{HSS}^{2-}$ with a rate constant of $4 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ at pH 7¹¹¹ and an association constant of $2.5 \times 10^4 \text{ M}^{-1}$ ¹³⁶ (reaction 55) and it is likely that the reaction of S^- with alkyl thiolates leads to similar products. $\text{HSSH}\cdot^-$ is acidic since it has been reported to exist primarily as the deprotonated dianion (HSS^{2-}) at neutral pH. This species is also a good reductant capable of rapidly reacting with O_2 to give $\text{O}_2^{\cdot-}$ with a rate constant of $4 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at pH 7¹¹¹ (reaction 56). Thus, similar to the situation described above, the reaction of the reasonable oxidant $\text{HS}\cdot$ with HS^- results in the generation of a reductant.



To be sure, the overall kinetics of H_2S autooxidation are fairly slow, and the likelihood of uncatalyzed H_2S autooxidation occurring in most biological systems appears to be low, especially considering other possible reactions. For example, the second order rate constants for the generation of either thiosulfate or sulfite from H_2S autooxidation are only around 0.1 to $0.5 \text{ M}^{-1}\text{min}^{-1}$.¹³⁴ However, this chemistry can be catalyzed by metals.^{137–139} Thus, there may be circumstances that allow the generation of $\text{HS}\cdot$ in biological systems via oxidation by O_2 , although this remains to be demonstrated.

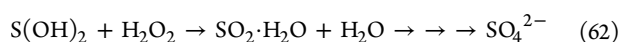
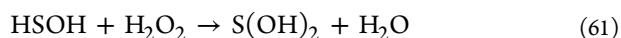
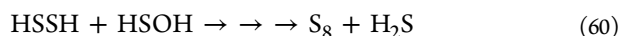
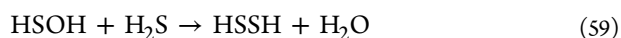
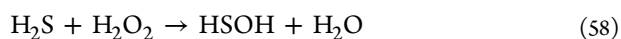
There is no direct chemical interaction between O_2 and CO under most biological conditions. However, they can affect the actions of each other via competition at a common target (i.e., a heme protein). These interactions are described below.

Interactions of O_2^- with NO, H_2S , and CO. As mentioned above, NO and O_2^- readily react, generating peroxynitrite, a much better overall oxidant than either precursor (reaction 23). This reaction has been touted as a mechanism for the endogenous generation of a potent oxidant with significant (patho)physiological implications,⁶¹ although this idea has been questioned.¹⁴⁰ Regardless, it is clear that O_2^- can remove NO quickly from solution, leading to a cessation of NO biological activity (and vice versa). The reaction of O_2^- and H_2S has been examined.^{141,142} Superoxide is capable of oxidizing $\text{H}_2\text{S}/\text{HS}^-$ by a single electron forming the corresponding thiyl radical ($\text{HS}\cdot/\text{S}^-$) (reaction 57).



These reports are consistent with the fact that the reported reduction potentials for O_2^-/H^+ and $\text{HS}\cdot$ are similar; thus it may be expected that O_2^- under acidic conditions can lead to the oxidation of H_2S ($\text{O}_2^-, \text{H}^+/\text{H}_2\text{O}_2$, $\epsilon^0 = 0.89$ and $\text{HS}\cdot, \text{H}^+/\text{H}_2\text{S}$, $\epsilon^0 = 0.9\text{--}1 \text{ V}$, vs NHE, pH 7). The rate of this reaction has been determined to be only $6.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ¹⁴¹ (although a higher rate constant ($1.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) has also been reported using a less reliable assay system for superoxide).¹⁴² There appears to be no significant chemical interactions between O_2^- and CO. Thus, to date the most prevalent reaction of O_2^- with the small molecule signaling species discussed herein appears to be the reaction with NO.

Interactions of H₂O₂ with NO, H₂S, and CO. As discussed previously, the primary reactivity of H₂O₂ in biological systems is as an oxidant and/or electrophile. Thus, its reaction partners are typically reductants and/or nucleophiles. For the most part, the nitrogen oxides discussed herein are themselves oxidants or electrophiles and, therefore, do not react directly with H₂O₂ under biological conditions. Being relatively inert, CO does not react with H₂O₂. However, as a possible reductant and nucleophile, H₂S is capable of direct interaction with H₂O₂. The reaction of H₂S with H₂O₂ is complex and ultimately leads to the formation of oxidized sulfur species such as S⁰ and SO₄²⁻, depending on reaction conditions.¹²⁹ The initial reaction is the attack of the nucleophilic sulfur of HS⁻ on an electrophilic oxygen atom of H₂O₂ leading to the sulfenic acid (HSOH) and H₂O (reaction 58). Further reaction with H₂S leads to the formation of higher order sulfides and, eventually, a stable form of elemental sulfur (S₈) (reactions 59 and 60). In the presence of excess H₂O₂, the sulfenic acid can further react with H₂O₂ leading to higher oxides of sulfur such as SO₄²⁻ (reactions 61 and 62).



Interactions of H₂S with Nitrogen Oxides. Most of the reactions of H₂S with nitrogen oxide species can be considered as analogous to the reactions of typical biological thiols. For example, due to the nucleophilicity of H₂S/HS⁻ it has the capability to react with electrophilic species. The reaction of H₂S/HS⁻ with electrophiles such as H₂O₂ or disulfides (RSSR) has already been discussed. Recently, it has been reported that H₂S can also react with S-nitrosothiols resulting in NO generation.¹⁴³ The chemistry responsible for this observation has not yet been delineated. However, the analogous reaction of an alkyl thiol with a nitrosothiol has been examined. As discussed previously, Wong and co-workers propose that the reaction between a thiol and a nitrosothiol can result in either a trans-nitrosation reaction (the transfer nitrosonium (NO⁺) from one sulfur to the other) (reaction 43) or the generation of the corresponding disulfide and HNO (reaction 42).⁸⁵ Considering that HNO can be converted to NO via a simple oxidation (vide supra), HNO intermediacy in the reaction of H₂S with nitrosothiols appears possible (although speculative at this time). It is likely that H₂S can also react with other electrophilic or oxidizing nitrogen oxides such as N₂O₃, NO₂, or ONOOH¹⁴⁴ since all of these species are known to react readily with biological thiols.^{67,145} The products/intermediates in these reactions would be analogous to those found with biological alkyl thiols, namely, thiol radicals, sulfenic acid, and nitrosated species. It needs to be mentioned, however, that normal cells contain very high levels of thiols besides H₂S (e.g., glutathione), and specific reactions of H₂S seem unlikely unless its relatively small size allows access to reaction sites not available to larger thiol species.

Common Targets: Interactions at Metals. All of the parent species mentioned herein have the ability to react with a variety of biological metal centers. Taking, for example, heme proteins as representative metalloprotein targets, many O₂, NO,

CO, and HS⁻ complexes have been characterized. Indeed, the biological utility of O₂ and NO as well as the toxicology of CO and H₂S are, in part, attributed to reactions with heme proteins. The fundamental coordination chemistry of O₂, NO, and CO has already been discussed, and as mentioned above, all of these species are capable of binding to ferrous heme proteins such as deoxymyoglobin or deoxyhemoglobin. With respect to the ability of these diatomics to serve as signaling agents, one of the most important factors associated with their chemical biology is how metalloproteins achieve selectivity for one species over another (if indeed they do). That is, how can a metalloprotein discriminate among, for example, NO, O₂, and CO since all of these species have the potential to bind to ferrous heme proteins with an available coordination site? With free ferrous protoheme (no protein) the affinity of CO binding is approximately 20,000 times greater than that of O₂. Thus, in the absence of any protein interactions, CO has much stronger binding to the ferrous ion compared to that of O₂. However, the affinity of CO for the ferrous heme in myoglobin is only 25–100 times greater, indicating a significant effect by the protein to alter the relative affinities of CO versus O₂ for the ferrous heme component. As mentioned above, NO, CO, and O₂ all bind ferrous heme proteins utilizing primarily two types of bonding interactions, σ -donation via a lone pair of electrons from the diatomic ligand and π -back-donation, which is a result of the overlap of filled d-orbitals on the metal with π^* antibonding orbitals on the diatomic ligand. With O₂, the electrons that form the σ -bond with the metal are in an sp² hybridized orbital indicating that O₂ prefers to bind in a bent fashion (that is, the metal–O–O geometry is bent, vide supra). However, the σ -bond-forming electrons in CO are in an sp hybridized orbital and, therefore, favoring CO binding with a linear geometry (that is, the metal–C–O atoms are linear). Interestingly, NO can bind in either fashion (i.e., like CO or O₂) depending on whether it binds as NO⁺ (which is isoelectronic with CO and therefore binds in a linear fashion) or as NO⁻ (which is isoelectronic with O₂ and therefore binds in a bent fashion).¹⁴⁶ The binding of NO to, for example, Fe³⁺-hemes results in a linear (or near linear) complex since the NO ligand has significant NO⁺ character. In biological systems, NO appears to bind ferrous heme proteins in primarily a bent fashion (i.e., O₂-like). Thus, it is conceivable that one way a protein can discriminate between, for example, O₂ (and NO) and CO is to place a steric restriction to one of the binding geometries (Figure 14).¹⁴⁷ That is, a steric restriction to the preferred linear binding mode of CO may inhibit its binding without significantly affecting the bent geometries of O₂ and NO binding.

The idea that discrimination between the simple diatomic ligands can be a result of unfavorable steric interactions is an attractive idea but recent reports indicate that it is likely that this effect plays only a minor role in the overall binding energetics.¹⁰⁴ A much more important effect that allows significant discrimination between O₂ and CO involves specific electrostatic interactions. As discussed above, the degree of backbonding present in the ferrous heme complexes of the diatomics discussed herein is in the following order: CO < NO < O₂. Indeed, the Fe²⁺-O₂ complex is often depicted as a Fe³⁺-O₂⁻ complex (a ferric-superoxide complex) to reflect the significant transfer of charge from the metal to the coordinated O₂. Thus, ferrous-bound O₂ is a much better hydrogen bond acceptor than CO. Using ferrous myoglobin as an example, it has been found that H-bond formation between bound O₂ and a distal histidine is an important aspect of the overall energetics of O₂ binding and

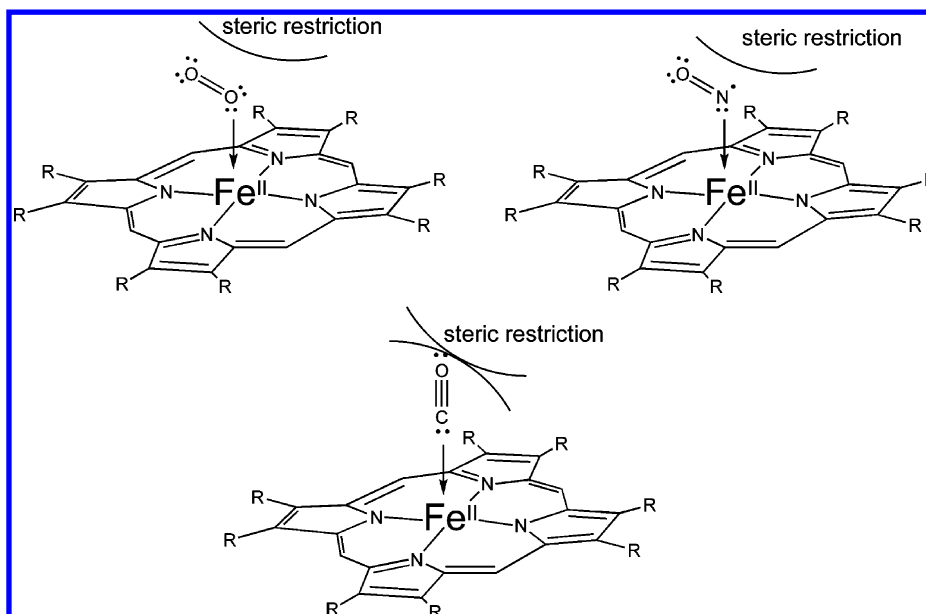


Figure 14. Binding geometries of O_2 , NO, and CO to a ferrous heme protein. Preferred linear binding of CO causes steric crowding that is thought to inhibit binding.

is responsible for most of the discrimination between O_2 and CO. That is, the ability for myoglobin to exhibit a greater discrimination between O_2 and CO is due primarily to an increased affinity of ferrous myoglobin for O_2 (due to hydrogen bond formation) rather than a decreased affinity for CO (as the steric model might suggest) (Figure 15).

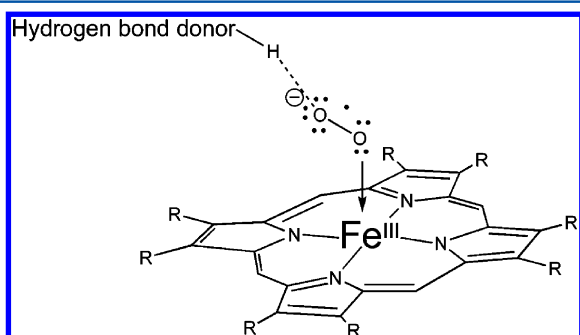


Figure 15. Hydrogen bond stabilization of the heme $Fe^{3+}-O_2^-$ complex.

The ability of hemeproteins to discriminate between O_2 and CO can be at least partially rationalized using the arguments above. Discriminating between NO and O_2 is also an important factor in some heme proteins. As mentioned above, the affinity of CO over O_2 for free ferrous protoheme (protein free) is approximately 20,000-fold, and NO has an even greater affinity for protoheme than CO (approximately 20-fold greater than CO).¹⁴⁸ Thus, NO appears to have a particularly high natural affinity for ferrous hemes. However, signaling levels of NO are reported to be in the low nanomolar range (i.e., 1–10 nM)⁷⁰ while intracellular O_2 levels can be significantly higher (μM range). Thus, in spite of the higher normal affinity of NO versus O_2 , there is still the need for NO-sensing proteins to select for NO over O_2 . For example, a primary receptor for NO is the heme protein soluble guanylate cyclase (sGC). The ferrous form of this enzyme binds to NO resulting in an increase in enzyme activity.¹⁴⁹ Interestingly, sGC is capable of

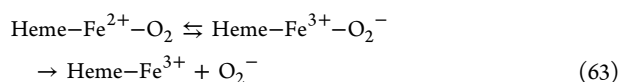
discriminating between NO and O_2 as it does not appear to bind O_2 . Although the factors that allow sGC to discriminate between NO and O_2 have not been unequivocally established, it has been postulated that proteins analogous to sGC that bind O_2 possess a distal tyrosine residue in the heme pocket that, like the histidine in myoglobin, H-bonds to the coordinated O_2 . Thus, the lack of a distal tyrosine in a protein like sGC will decrease O_2 affinity and, possibly, allow for significant discrimination between O_2 and NO.^{150,151} Thus, in this case, the discrimination between NO and O_2 is due to a lack of stabilization of the O_2 adduct as opposed to a special stabilization of the NO complex. It is worth remembering that the coordination of NO to the ferrous heme is distinct from O_2 in that NO ligation typically results in a weakening of the proximal ligand bond and the generation of a 5-coordinate complex (vide supra). Whether the change in the coordination number/geometry between the NO and O_2 complexes play a role in ligand discrimination has not been established.

As mentioned earlier, H_2S (or HS^- , S^{2-}) binds heme proteins. However, unlike O_2 and CO, H_2S prefers to bind (likely as HS^-) the higher oxidation state ferric (Fe^{3+}) ion. Thus, there is not likely to be any direct competition between CO and O_2 with H_2S at a ferrous heme site. However, unlike O_2 and CO, NO will bind to ferric hemes, and therefore, there is the possibility of an interaction between NO and H_2S at a ferric heme protein, although this has not been reported.

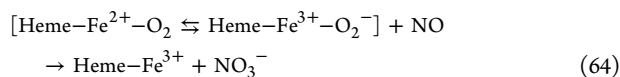
It is clear that heme proteins have evolved to be able to select one small molecule signaling species over others. That is, proteins have managed to fine tune the relative affinities for these potential ligands in order to achieve signaling/biochemical specificity, and the competitiveness of the ligands under (patho)physiological conditions can vary from protein to protein. For example, one of the H_2S biosynthesis enzymes cystathionine β -synthase (CBS) is a heme protein capable of coordinating CO and NO. Catalysis by CBS is not directly dependent on heme chemistry; thus, the heme group is thought to be purely regulatory.¹⁵² CBS can be inhibited by CO binding to the ferrous heme with a K_i of $5.6 \pm 1.9 \mu M$. This relatively low K_i indicates that CO can be an endogenously generated regulator of CBS activity. However, CBS binds NO with

an extremely high K_i of $320 \pm 60 \mu\text{M}$ and is therefore thought to be physiologically irrelevant.¹⁵³ CBS does not appear to bind O_2 . However, O_2 will oxidize the ferrous heme to the ferric species resulting in an increase in activity¹⁵⁴ (vide infra). However, the previously mentioned enzyme sGC, which also contains a regulatory heme, is regulated by NO at low nanomolar levels and does not appear to be regulated by (or bind to) CO at presumed physiological levels.¹⁴⁹ These two examples illustrate how heme proteins are capable of responding differently to these signaling species by, at one level, altering the relative affinities for the ligands. However, this is not to say that significant interactions cannot exist between the various signaling agents at, for example, heme centers. It is conceivable that competition between these ligands at heme centers exists at (patho)physiological levels and is regulatory. A possible example of this is the terminal, O_2 -binding component of respiration cytochrome *c* oxidase (CcOX). The normal substrate for CcOX is, of course, O_2 . However, it is postulated that endogenous generation of CO and NO may be important regulators of mitochondrial respiration via competitive binding to CcOX^{109,154} and, possibly, other chemistries.¹⁵⁵

The above discussion focused on ligand binding only. However, it must be considered that some ligands can react further after initial binding while others may not. As discussed earlier, NO is capable of reducing a ferric heme to the ferrous species via reaction 39. Thus, a ferric heme protein unable to bind/respond to O_2 or CO can be converted to the ferrous species by NO, which would then be capable of binding all three. (To be sure, reduction of ferric hemes can be accomplished in many other ways in biological systems that are unrelated to NO.) However, a ferrous heme– O_2 complex (as mentioned above) has significant $\text{Fe}^{3+}\text{--}\text{O}_2^-$ character, and a process referred to as autoxidation can occur whereby the O_2^- dissociates, resulting in an oxidized, ferric species (reaction 63). Because of the fact that O_2 is a poor one-electron oxidant (vide supra), the formal transfer of an electron from the ferrous ion to O_2 is thermodynamically unfavorable.¹⁵⁶ However, the presence of a proton (acidic conditions) or a nucleophile greatly accelerates this autoxidation process.



Thus, O_2 has the potential to oxidize NO- and CO (and O_2^-)-binding heme proteins to the corresponding ferric species, which will no longer bind CO and O_2 (and have significantly less affinity for NO). It is also worth noting that NO can also react with ferrous heme– O_2 complexes resulting in the ferric heme protein and nitrate ion (reaction 64).¹⁵⁷



This reaction is analogous to the previously discussed and facile reaction of NO with O_2^- (reaction 23), the only difference being that O_2^- is coordinated to the ferric ion prior to the reaction. Thus, NO can avidly bind to ferrous hemes, but in the presence of O_2 , the combination of NO/ O_2 can result in ferrous heme oxidation. For the most part, CO binding to ferrous hemes does not impart any further reactivity to either the metal or ligand. Thus, except for simple dissociation, the bound CO is thought to be inert. However, the binding of CO can protect ferrous species from the autoxidation associated with O_2 since CO will

prevent O_2 binding (assuming autoxidation occurs via an inner-sphere mechanism, i.e. occurs as a result of O_2 coordination).

Interactions at Thiols. Hopefully, it is clear that metal centers represent a common target for all of the signaling species discussed herein. Moreover, some metal centers may interact with several of the signaling molecules (or not) depending on the protein environment around the heme center. The other obvious biological targets for these species are thiols/thiol proteins. As discussed earlier, the reactions of thiols with nitrogen oxides (i.e., NO_2 , N_2O_3 , HNO , and HOONO), O_2 -derived species (i.e., O_2^- and H_2O_2), and H_2S are prevalent and potentially important biochemical occurrences. In general, the nitrogen oxide and O_2 -derived species are thought to oxidatively modify protein thiols, oftentimes leading to an alteration of protein function. However, H_2S appears capable of reducing some oxidized thiol species and may even generate a hyperactivated persulfide species (vide supra). Significantly, most of the nitrogen oxide species capable of modifying thiols are generated via O_2 -dependent chemistry (vide supra). Exceptions to this are HNO , which has not been determined to be physiologically relevant, and as discussed previously the possible oxidation of thiols (nitrosothiol formation) via a ferric-nitrosyl species (reaction 41). NO itself will not react directly with thiols under biological conditions (vide supra). O_2 itself also does not react directly with thiols and requires either reduction or reaction with NO before it generates a thiol-reactive species. Thus, NO/ O_2 -derived species can oxidize thiols, and H_2S can reduce at least some of the oxidized thiol species, allowing for all of these signaling molecules to be involved in the redox regulation of thiol protein activity.

With regard to possible points of integrated signaling associated with NO, O_2 , CO, and H_2S , of particular interest are metalloproteins (i.e., heme proteins) that also possess regulatory/redox active thiols. In these cases, the integrated signaling by these small molecules can be the result of actions at distinct regulatory sites on the same protein. Several proteins already mentioned herein are known to be regulated via heme co-ordination by specific small molecules as well as by protein thiol redox modification. sGC, the primary receptor for NO is also regulated by protein cysteine modification⁸⁷ allowing enzyme activity to be responsive to not only the presence of NO but also the thiol redox status of the cell (which can be based on the relative levels of all of the thiol reactive species present). The H_2S -generating CBS can also be regulated (inhibited) by CO¹⁵³ as well as protein thiol modification¹⁵⁸ and heme oxidation state.¹⁵⁹ Interestingly, S-nitrosothiol formation on crucial cysteine residues on CBS results in an increase in enzyme activity¹⁵⁸ alluding to the possibility that generation of NO-derived nitrosating species increases activity. Moreover, since generation of CO decreases activity via complexation to the regulatory heme and heme oxidation, possibly by O_2 -derived oxidants, can decrease activity, it may be that the levels and fluxes of all of these signaling species are important in the overall regulation of this enzyme. Finally, the possibility that H_2S can reverse the thiol oxidation (or even react with the ferric protein) alludes to the possibility that this protein is responsive to all the signaling species discussed herein. It is likely that many other examples of this type exist and that this type of interplay between these signaling species is prevalent.

■ REGULATION OF HIF1 α : AN EXAMPLE OF INTEGRATED SIGNALING

Although sGC and CBS (briefly mentioned above) can serve as examples of possible single target integrative signaling

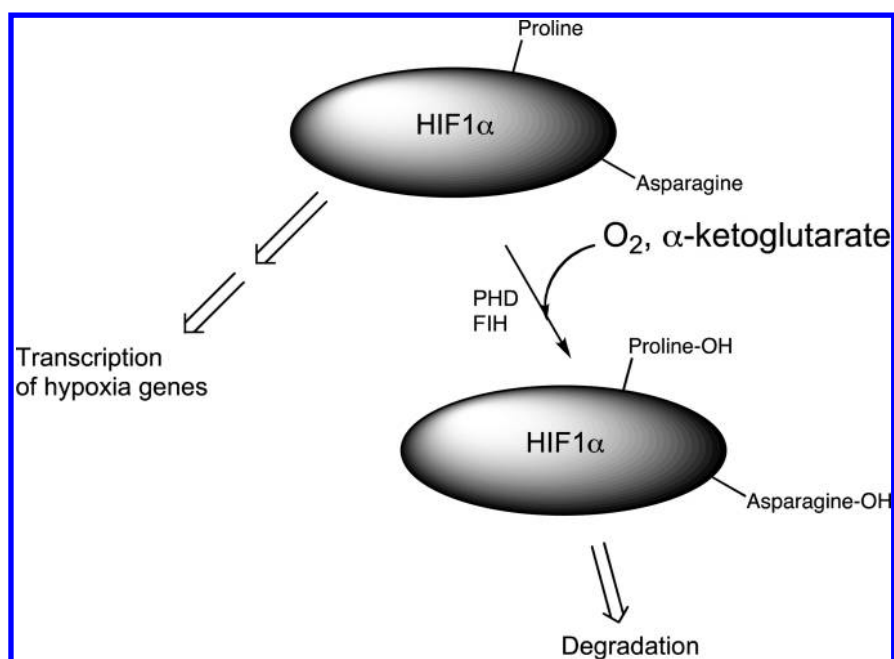


Figure 16. Simplistic scheme for HIF1 α regulation by O₂ via PHD and FIH.

associated with the small molecule agents NO, CO, O₂, and H₂S, another primary example of this is the signaling system associated with the biological response to hypoxia. HIF1 α (hypoxia inducible factor 1- α) is a transcription factor that is largely responsible for the biological response to low levels of O₂ (hypoxia). HIF1 α activity leads to an increase in a variety of gene products including erythropoietin, vascular endothelial growth factor (VEGF), and other proteins involved in an adaptive response to hypoxia.¹⁶⁰ The way this system “senses” O₂ is via enzymes that utilize O₂ to oxidatively modify HIF1 α at a proline, leading to degradation, or an asparagine, leading to an inhibition of the binding of coactivators. Thus, the prolyl (prolyl hydroxylase domain, PHD) and asparaginyl hydroxylases (factor inhibiting HIF, FIH) serve to deactivate or degrade HIF1 α and, therefore, are the major O₂-dependent regulators of HIF1 α activity. Under low O₂ conditions, the activity of these enzymes is decreased (due to the lack of the O₂ cosubstrate), and levels of HIF1 α increase, leading to gene expression (Figure 16).

Although the presentation of the hypoxia-sensing signaling system is greatly oversimplified (as it is much more extensive and complex than that described herein, involving many other factors and proteins), the important point here is that the prolyl and asparaginyl hydroxylases are proteins that represent possible targets for integrative signaling by the small molecule agents. For more extensive descriptions of the HIF1 α system, many other reviews are available.^{161,162}

PHD and FIH are members of a family of dioxygenase enzymes that utilize nonheme iron and α -ketoglutarate (also called 2-oxoglutarate) as a source of reducing equivalents to activate O₂ leading to the oxygenation of, in this case, proline. For the sake of brevity, the discussion herein will focus on PHD (of which there are three major isoforms) as a target for regulation by small molecule signaling agents. The K_m value for O₂ in PHDs is approximately 7–8 μ M, a range consistent with the idea that these enzymes are O₂ sensors.¹⁶³ The general mechanism of 2-oxoglutarate- and O₂-dependent hydroxylation of substrate is schematically depicted below (Figure 17).^{164,165}

Clearly with any O₂-binding protein there is the possibility of NO or CO binding as well, representing a possible site of interaction of these species with the HIF-1 α system. Moreover, since the resting state of the protein is ferrous (Fe^{II}), oxidation of the iron may represent a redox regulation since the ferric species will not perform the same chemistry (but can bind, for example, H₂S). Finally, the PHDs have been reported to have redox thiols that can interact with thiol-modifying species, leading to changes in activity (vide infra). Thus, it is evident that the PHD-HIF-1 α system is subject to possible regulation by all of the small-molecule signaling agents described herein (and their derived species) and represents an example of the potential integrative signaling of the small molecule agents. Below, evidence for this is presented.

As mentioned above, coordination of NO or CO to the nonheme ferrous iron of PHD would be expected to inhibit the oxygenation of proline by competing with O₂ and therefore mimicking hypoxia. Indeed, the NO-donor GSNO was found to inhibit PHD leading to HIF-1 α accumulation under normoxia.^{165,166} Inhibition of PHD by NO has been proposed to occur via an interaction between NO and the ferrous ion of PHD.^{167,168} A similar effect has been noted with CO as well, albeit at high, nonphysiological levels of CO.¹⁶⁹ A mechanistically distinct pathway of possible NO-mediated regulation involves NO-dependent S-nitrosation (via NO-derived species) of HIF-1 α leading to increased stabilization¹⁷⁰ and transcriptional activity.^{171,172} Thus, NO has the ability to increase HIF-1 α activity via multiple mechanisms that may be highly dependent on the levels of NO.¹⁷³

Interestingly, both NO and CO have also been reported to decrease HIF-1 α signaling as well¹⁷⁴ indicating a complex picture with respect to the small molecule signaling species and O₂ sensing. Indeed, numerous studies report decreased levels of HIF-1 α upon exposure to NO (especially under hypoxic conditions).¹⁶⁸ One possible mechanism for this is proposed by Hagen and co-workers who found that NO inhibits respiration (and therefore O₂ consumption) leading to a redistribution of O₂ to nonrespiratory pathways such as the PHDs, leading to

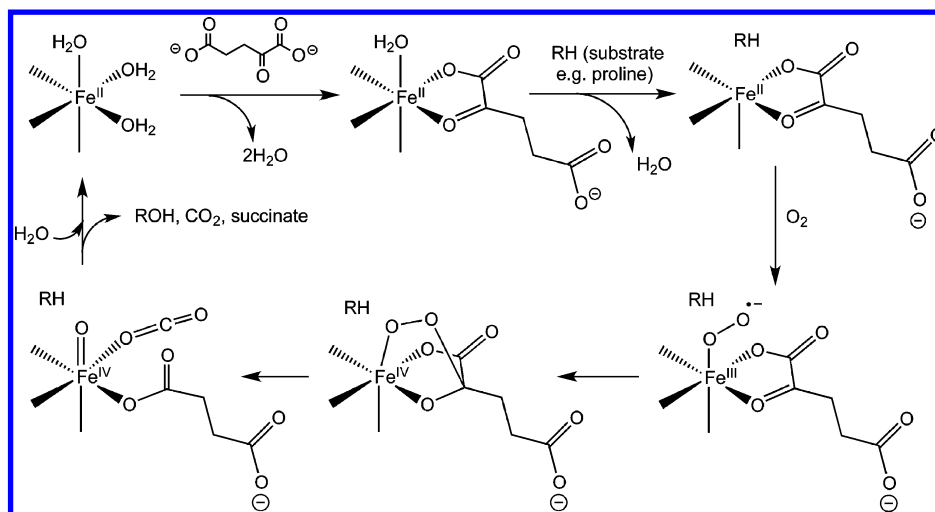


Figure 17. General mechanism for 2-oxoglutarate-dependent, nonheme iron hydroxylases.

the degradation of HIF-1 α .¹⁷⁵ This represents an indirect effect on the system (as opposed to the direct interactions described above for NO- and CO-mediated increases in HIF-1 α activity). Inhibition of respiration by CO under hypoxic conditions may also have a similar effect.¹⁷⁶

As shown in Figure 17, PHD requires ferrous iron for activity. Thus, small-molecule oxidants (O_2 or NO-derived species) may lead to oxidation of Fe(II) to Fe(III) and a decrease in PHD activity and subsequent increase in HIF-1 α levels/activity. Indeed, O_2 -derived oxidants (so-called ROS) have been reported to elicit an increase in HIF-1 α activity, possibly via PHD iron oxidation.¹⁷⁷ The picture becomes extremely complex when NO and, for example, O_2^- are present since either can affect PHD activity, and upon reaction with each other, a decrease in levels of both occurs,^{178,179} and the product ONOO $^-$ can have a distinct effect including possible utilization as an oxidizing equivalent in catalysis.¹⁸⁰ CO appears to be able to affect HIF-1 α levels indirectly since the exposure of macrophages to CO has been found to lead to a burst of ROS via interaction with mitochondria that leads to increased levels of HIF-1 α .¹⁸¹ Finally, several recent studies indicate a possible role for H_2S in controlling HIF-1 α levels. In *C. elegans*, H_2S is capable of increasing HIF-1 α activity.¹⁸² Although the mechanism by which H_2S increases HIF-1 α activity is not described, it remains possible that inhibition of respiration (or other electron transport system) and subsequent generation of O_2 -derived oxidants¹⁸³ inactivate PHD via iron oxidation (akin to an effect of NO described above). Finally, the pro-angiogenic effect of H_2S has been reported to be a result of an increase in HIF-1 α protein (and mRNA) levels in rat endothelial cells.¹⁸⁴ Again, the mechanism by which H_2S elicits this response is not established.

The above discussion of the interaction of various small molecule signaling agents, and derived species, with the HIF-1 α /PHD system serves to illustrate how these multiple agents may interact either directly or indirectly to elicit a variety of outcomes that can be dependent on the levels of the mediator/effector, the cell/cellular environment, and/or the presence of other factors/reactants. To be sure, this discussion is by no means comprehensive or complete, and clearly, other interactions exist that can have profound effects on hypoxic sensing. There are likely myriad signaling systems that are also subject to similar integrated interactions with these small molecule agents.

CONCLUSIONS

As increasing evidence mounts for important and diverse signaling functions associated with O_2 , NO, CO, and H_2S (and species derived from these agents), it is becoming increasingly important to understand the chemical biology of these agents and how this chemistry is integrated and regulated by Nature. As mentioned in the beginning of this review, the fundamental chemical properties of these species predict similar/overlapping biological targets, which include metals and thiol redox systems. Moreover, the striking similarities in the nature of the biological functions/targets that likely interact with most (and in some cases all) of these small molecule signaling agents strongly suggests a system by which multiple signaling agents are able to regulate fundamental signaling pathways. It was the intention of this review to begin to describe the basic chemistry of the relevant species as a prelude to an attempt to understand the intricate and integrated signaling web. Clearly, this discussion is only beginning, and other chemistries, biological targets, and signaling systems remain to be discovered and described. In any event, the chemical biology and physiology of integrated small molecule signaling agents is a growing, exciting, and important field of research endeavor that will occupy the efforts of many laboratories in many disciplines for many years to come.

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Notes

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ABBREVIATIONS

NHE, normal hydrogen electrode; BDE, bond dissociation energy; SOD, superoxide dismutase; SOMO, singly occupied molecular orbital; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CcOX, cytochrome *c* oxidase; HIF1 α , hypoxia inducible factor 1- α ; sGC, soluble guanylate cyclase; CBS, cystathionine β -synthase; PHD, prolyl hydroxylase domain; FIH, factor inhibiting HIF; VEGF, vascular endothelial growth factor.

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