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Review

Mechanistic studies of nitrite reactions with metalloproteins and models relevant to mammalian physiology

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Contents

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ABSTRACT

This review provides a summary of reaction mechanisms involving the interactions of nitrite ion with metal centers relevant to physiology. The majority of the systems that have been investigated are heme proteins and models, where nitrite reacts with the central metal ions to generate important iron-NO_x intermediates and subsequent NO_x products. We also discuss reactions with other potentially relevant systems. Nitrite is formed as a product of NO autoxidation in aqueous media and can be formed by the bacterial reduction of ingested nitrate as well. It is now generally accepted that under certain conditions nitrite, which is present in mammalian fluids and tissue at micromolar concentrations, can serve as a biological reserve of the bioregulatory agent nitric oxide. However, it is possible that nitrite serves other functions as well. The goal of this review is to evaluate the present state of understanding regarding these pathways and the delicate interplay between nitrite and the various NO_x species of biological relevance.

1. Introduction

Nitric oxide (NO) is now well known to be an endogenous bioregulatory molecule of multiple mammalian functions including vasodilation and neurotransmission where it is effective at nanomolar (nM) levels [1]. NO is also generated in higher concentrations during incidents of immune response to infection. These properties have led to considerable interest, especially in the biomedical field, in the biochemistry not only of NO, but also other nitrogen oxide derivatives including metal nitrosyls, nitroxyl (HNO) [2], nitrogen dioxide (NO_2) [3–5], nitrite (NO_2^-) [6], peroxynitrite ($ONOO^-$) [7], and various nitrosoamines (R_2NNO) and nitrosothiols (RSNO) [8]. The interest in nitrite biology alone has led to a biennial conference (International Meeting on the Role of Nitrite in Physiology, Pathophysiology, and Therapeutics) convening for the third time in 2009.

Abbreviations: cGMP, cyclic guanylyl monophosphate; CysSH, reduced cysteine; DFT, density functional theory; DHA, dehydroxyascorbic acid; DMP, 2,9-dimethyl-1,10-phenanthroline; DMS, dimethyl sulfide; eNOS, endothelial nitric oxide synthase; GSH, glutathione; GSNO, *S*-nitrosoglutathione; Hb, hemoglobin; I/R, ischemia/reperfusion; iNOS, inducible nitric oxide synthase; Mb, myoglobin; metHb, met-hemoglobin; metMb, met-myoglobin; NEM, N-ethylmaleimide; NiR, nitrite reductase; NMR, nuclear magnetic resonance; NOS, nitric oxide synthase; OAT, oxygen atom transfer; OEP, octaethyl-porphyrinato; Por^{2-}, porphyrinato dianion; $PPIX^{2-}$, protoporphyrin IX dianion; RBC(s), red blood cell(s); sGC, soluble-guanylyl cyclase; TpivPP, picket fence porphyrin, *meso*-tetrakis(*o*-pivalamidophenyl)porphyrinato; TPP, *meso*-tetraphenylporphyrinato; TPPS, meso-tetrakis(4-sulfonatophenyl)-porphyrinato anion; TPPTS, water-soluble sulfonated triphenylphosphine; TTP, tetra(p-tolyl)porphyrinato; XOR, xanthine oxioreductase.

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The fundamental chemistry of the small molecular and ionic NO_x species must be relatively simple, although the biological consequences are not. Furthermore, much of the biochemistry of the NO_x species involves interactions with metal centers, principally heme, non-heme iron, and copper proteins. This review summarizes and discusses recent studies directed toward understanding the interplay between nitrite and the various NO_x species as mediated by metalloproteins and models and by other metal centers with an overall goal of unraveling the role(s) of nitrite in mammalian biology. One must keep in mind, however, that models are often studied under different conditions than living systems, so one consider these differences when extrapolating the insight gained to physiology.

It has been established quantitatively by Feelisch et al. that steady state species found in mammalian tissue and fluids include ionic nitrate (NO_3^-) and nitrite, RSNOs such as S-nitrosoglutathione and S-nitroso proteins, nitrosoamine derivatives, and nitrosylhemes [9,10]. The concentration of nitrite in the plasma and tissue is remarkably high, up to 500 nM and 10 μM, respectively. Nitrite has also been shown to induce vasodilation [11] and to reduce damage to organs during ischemia/reperfusion (I/R) injury [12,13]. Although the mechanisms of these biological functions have not been conclusively established, the role of nitrite may be to generate NO under hypoxic conditions [14]. Nitrite is also an intermediate in the denitrification processes by bacteria and fungi, whereby nitrate is converted to N_2 [15].

Endogenous mammalian NO is formed from arginine oxidation by various nitric oxide synthases (NOS), and possibly in part from nitrite as noted above. Continuous production by the constitutive enzyme eNOS in the endothelial cells lining blood vessels gives low steady state NO concentrations in the cardiovascular system, where a principal target is the heme protein soluble guanylyl cyclase (sGC). The purpose is to maintain blood pressure through control of smooth muscle tissue (NO thus serves as a vasodilator). The sGC is activated by surprisingly low NO concentrations. While early studies suggested sGC activation occurs at high nanomolar concentrations, more recent studies indicate that 100 pM–5 nm is sufficient [16]. However, NO concentrations in the micromolar (μM) range are locally produced by the inducible isoforms of nitric oxide synthase (iNOS) or expressed by macrophages and neutrophils during immune response to pathogen invasion [17].

What are the sources of ionic nitrite in plasma and tissues? This topic has not, to our knowledge, been quantitatively addressed in the literature. Autoxidation of endogenous NO is certainly one likely source of nitrite as this aqueous reaction leads principally to nitrite as the product (Eq. (1)) [18]. Consistent with this view, local inhibition of NOS activity in humans resulted in a 58% decrease in plasma nitrite [19]. NO autoxidation is a third order reaction, first order in $[O_2]$ and second order in [NO] (Eq. (2), $k_{aq} \sim 2 \times 10^6\ M^{-2}\ s^{-1}$) [20]. So, although autoxidation is a possible sink for NO in oxygenated media, the third order kinetics dictate that the rate is highly dependent on the conditions, especially the NO concentration, which with the exception of immune response incidents is generally quite low. It should also be noted that various cytotoxic intermediates are also generated during NO autoxidation [20]. Notably, during sepsis, serum levels of nitrate and nitrite are significantly increased [21].

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (1)$$

$$\frac{-d[NO_2^-]}{dt} = 4k_{aq}[NO]^2[O_2] \quad (2)$$

In non-protic media such as organic solvents or cellular membranes, NO autoxidation produces nitrogen dioxide (Eq. (3)) [22], which disproportionates to nitrite and nitrate when exposed to water. In this context, it is important to consider the heterogeneous nature of biological organisms, even at the cellular level. Lancaster et al. [23] have proposed that since NO and O_2 are significantly more soluble in hydrophobic media, these species will concentrate in the lipid membranes of a cell. Thus, partitioning of NO and O_2 between cellular hydrophobic and hydrophilic regions coupled with the third order rate law may mean a disproportionately large fraction of NO autoxidation occurs in hydrophobic regions to give NO_2. It should be emphasized that regardless of cell heterogeneity, the low concentrations of NO formed for bioregulatory purposes such as blood pressure control are too small to favor a significant role for autoxidation as a NO sink. This situation could, however, be very different during immune response.

$$2NO + O_2 \rightarrow 2NO_2 \quad (3)$$

The primary sink for NO in the cardiovascular system has been long thought to be oxidation by oxyhemoglobin (HbO_2) to give nitrate plus the ferriheme met-hemoglobin (metHb) (Eq. (4)) in red blood cells (RBCs) [24]. In buffered aqueous solutions, the large second order rate constants for the reactions of NO with HbO_2 is $9 \times 10^7\ M^{-1}\ s^{-1}$ [25]. Another suggested pathway for NO removal from plasma is oxidation by ceruloplasmin, a copper protein with NO oxidase properties (Eq. (5), $Cu^{II}P$ is a cupric protein) [26]. Ferriheme protein oxidation of NO (sometimes called the "autoreduction" of ferrihemes by NO) [27] is also a potential source of nitrite in tissues (Eq. (6), Fe^{III}(Por) is a ferriheme protein or model), although to our knowledge this pathway has not yet been demonstrated to be a source of endogenous nitrite.

$$NO + HbO_2 \rightarrow metHb + NO_3^- \quad (4)$$

$$Cu^{II}P + NO + H_2O \rightarrow Cu^{I}P + NO_2^- + 2H^+ \quad (5)$$

$$Fe^{III}(Por) + NO + H_2O \rightarrow Fe^{II}(Por) + NO_2^- + 2H^+ \quad (6)$$

Endogenous nitrite could also be formed from the reduction of ionic nitrate, which as an end product of nitrogen oxidation is present in physiological heme containing fluids and tissues at much higher concentrations than either NO or nitrite [28]. Although no mammalian enzymes having nitrate reductase activity as the sole function have been identified, certain mammalian enzymes such as xanthine oxidoreductase have been shown to reduce nitrate [29]. Nonetheless, such activity has not yet been shown to play a major role in human nitrogen cycles. Indeed, excessive dietary nitrate has generally been considered undesirable, although recent discoveries that eating certain high-nitrate foods such as beets and green leafy vegetables may lead to lowered blood pressure have generated some reevaluations of those concerns [30–32]. One explanation of the positive effect of high-nitrate vegetables is that nitrate in circulating fluids is transported to the mouth where it tends to be concentrated in the saliva. Bacteria living in the mouth have nitrate reductase activity that converts NO_3^- to NO_2^-, and the nitrite so generated is swallowed and converted to NO in the stomach. Intestinal bacteria have been reported to have nitrate reductase activity, so this may also be a potential physiological source of nitrite [33].

2. Some solution chemistry of NO_2^-, N_2O_3, NO^+ and NO_2

Nitrite is the conjugate base of nitrous acid (Eq. (7)) for which a recent reevaluation gave the pK_a as 3.16 at 25 °C (3.11 at 37 °C) [34]. Therefore, at physiological pH nitrite is ~99.99% in the ionic form. At lower pH, elevated HNO_2 formation, is particularly significant in the context of nitrous acid dehydration (Eq. (8)) [35]. The equilibrium constant for the latter reaction has been reported to be $3 \times 10^{-3}\ M^{-1}$ in dilute acidic aqueous solutions (0.1 M HCl at 23 °C) [36].

$$HNO_2 \rightleftharpoons H^+ + NO_2^- \quad (7)$$

$$2HNO_2 \rightleftharpoons H_2O + N_2O_3 \quad (8)$$

The aqueous redox chemistry of NO_2^- is highly pH dependent. For example, the one-electron reduction of nitrite to NO requires two equivalents of acid. Hence, nitrous acid is relatively oxidizing under acidic conditions (Eq. (9)), but the reduction potential of nitrite to NO drops to 0.37 V (vs. the normal hydrogen electrode, NHE) at pH 7.0 and is even less favorable in 1 M base (Eq. (10)) [37].

$$HNO_2 + H^+ + e^- \rightarrow NO + H_2O, \quad E^\circ = 0.99\,V(vs.NHE) \tag{9}$$

$$NO_2^- + H_2O + e^- \rightarrow NO + 2OH^-, \quad E^\circ = -0.46\,V(vs.NHE) \tag{10}$$

Nitrogen dioxide is a powerful oxidant with a one-electron reduction potential (to nitrite) of +0.895 V in aqueous solution (Eq. (11)). As noted above, NO_2 may be formed by NO autoxidation in hydrophobic membranes as well as by peroxynitrite decomposition (see below), so it is important to consider its possible physiological roles. While NO_2 can react directly with tyrosine, reaction with the tyrosyl radical to form 3-nitrotyrosine is much more rapid. The latter product has been associated with certain disease states [38]. NO_2 has also been shown to mediate meso hydroxylation of an Fe(III) porphyrin pi-cation radical. It was proposed that NO_2 binds to the meso-carbon followed by isomerization to the nitrito isomer and subsequent hydrolysis [39].

$$NO_2 + e^- \rightarrow NO_2^-, \quad E^\circ = +0.895\,V(vs.NHE) \tag{11}$$

N_2O_3, the anhydride of nitrous acid, can dissociate to NO and NO_2 (Eq. (12), $K_{12} = 2 \times 10^{-5}$ M [40]). Thus, the combination of Eqs. (7), (8) and (12) is the acid promoted disproportionation of nitrite. This is a viable pathway toward NO formation from nitrite, especially in the acidic fluids of the stomach [41]. Nitrite disproportionation may also occur during ischemic events where the metabolic activity under reduced blood flow leads to the tissues becoming more acidic, as evidenced by skeletal muscle tissue pH decreasing to 6.3 under ischemia. [14,42,43]. Given the second order nature of Eq. (8), NO production and concentration in equilibrium with nitrite is a complex function of pH and nitrite concentration. For example, at equilibrium, a solution 1.0 μM in $NaNO_2$ would contain ~0.2 pM NO and an equal concentration of NO_2 at pH 7.4 and ~11 pM NO at pH 6, if there were no other pathways depleting one or the other of these species. (These concentrations obviously will be proportionally higher for higher $[NO_2^-]$.) Thus, when considering the potential biological impact of HNO_2 disproportionation on NO concentrations, one must consider the equilibria and dynamics of the individual processes that deplete or enhance the concentrations of the key species involved.

$$N_2O_3 \rightleftharpoons NO + NO_2 \tag{12}$$

"Nitrosation" describes the reaction where (formally) NO^+ is added to a nucleophile or replaces an H^+ (e.g., Eq. (13)) [35]. There was considerable interest in this reaction long before the bioregulatory roles of NO were discovered owing to concerns about the formation of potentially carcinogenic N-nitrosoamines by nitrites in preserved meats [44,45]. Acidic nitrite solutions are well known to be nitrosating agents; indeed the nitrosonium ion (NO^+) itself is formed in highly concentrated acid solutions (e.g. 60% sulfuric acid). In more dilute acid, however, nitrosation of nucleophiles by nitrite can be largely attributed to the intermediacy of N_2O_3, which, notably, is a likely intermediate in NO autoxidation. Thus, it is not surprising that the reactive nitrogen species formed during autoxidation can lead to products attributed to nitrosation. Hence, under conditions (relatively high [NO]) where autoxidation may be a significant NO sink, the generation of N_2O_3 and its subsequent reactions, such as the S-nitrosation of thiols and the N-nitrosation of amines, are potentially relevant. N-nitrosation can also be mutagenic via DNA deamination (Eq. (13b)) [46] and would be most likely under conditions of immune response.

$$RNH_2 + NO^+ \rightarrow RNH(NO) + H^+ \tag{13a}$$

$$RNH_2 + NO^+ \rightarrow RNH_2(NO)^+ \xrightarrow{-H_2O} RN_2^+ \underset{-H^+}{\xrightarrow{H_2O}} ROH + N_2 \tag{13b}$$

3. The coordination chemistry of nitrite

Three linkage isomers can result from nitrite binding to metals in mononuclear complexes (Fig. 1). When NO_2^- is coordinated via the nitrogen atom, the resulting M-NO_2 species is termed a "nitro" complex, while when it is coordinated via a single oxygen, the M-ONO species is termed a "nitrito" complex. Alternatively, nitrite can be coordinated via both oxygens in a bidentate fashion. From examining the isomers depicted in Fig. 1, one can easily envision that the chemical reactivities of these species are likely to differ markedly. Additional modes of coordination are found in polynuclear complexes where the nitrite ligand may bridge two metal centers, two of which are illustrated in Fig. 2. Specific examples of nitrite complexes in mononuclear and polynuclear complexes are described in the textbook by Greenwood and Ernshaw [47].

The nitrito and nitro isomers are the most common forms in mononuclear complexes. When a crystal structure is not available, the isomers are most commonly differentiated by their IR spectra. For nitrito complexes, the two NO stretching frequencies ν(N=O) and ν(N–O) are well separated falling in the ranges 1510–1400 cm^{-1} and 1100–900 cm^{-1}. The separation is much smaller for the nitro-complexes, which typically exhibit $\nu_a(NO_2)$ and $\nu_s(NO_2)$ bands in the ranges 1470–1370 cm^{-1} and 1340–1290 cm^{-1} respectively [48,49].

Metal centers coordinated to nitrite form specific linkage isomers depending upon a variety of factors. For example, the oxophilic chromium(III) center in *trans*-Cr(cyclam)$(ONO)_2^+$ binds both nitrite ions as the nitrito isomers [50] while softer metal centers such as the cobalt(III) of $Co(NH_3)_5(NO_2)^{2+}$ give the nitro-complexes. The metastable nitrito isomer $Co(NH_3)_5(ONO)^{2+}$ can be prepared by photolysis, but this spontaneously reverts to the thermodynamically favored nitro form by a unimolecular pathway [51].

With regard to hemes, the large majority of crystallographically characterized nitrite complexes both of synthetic ferrous and ferric porphyrinato complexes [52] and of heme proteins [53–55] are nitro isomers. An exception is the "picket fence" porphyrin anion of the salt [K(222)][Fe(TpivPP)(NO_2)(NO)] (TpivPP = *meso*-tetrakis(*o*-pivalamido-phenyl)porphyrinato dianion) prepared by the reaction of Fe(TpivPP) with Kryptofix-222 solubilized KNO_2 followed by reaction with NO [56]. The product has two crystalline forms. One displays the nitro-nitrosyl structure Fe(TpivPP)$(NO_2)(NO)^-$, while

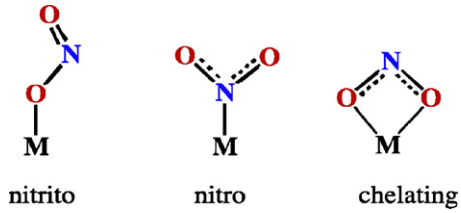


Fig. 1. Nitrite ligand bonding modes in mononuclear complexes.

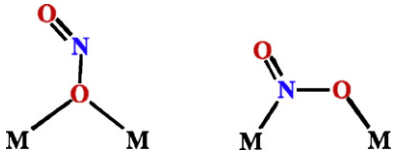


Fig. 2. Examples of bridging nitrite bonding modes in dinuclear complexes.

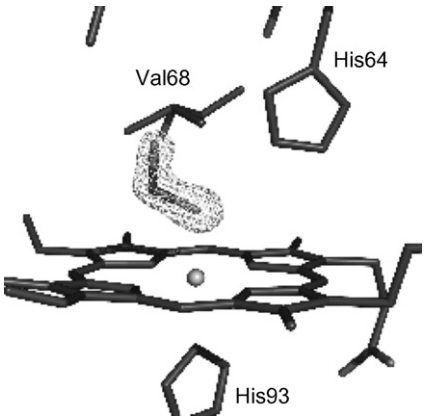


Fig. 3. The heme site of the nitrito complex of horse heart met-myoglobin [61] (figure provided by Prof. G. Richter-Addo).

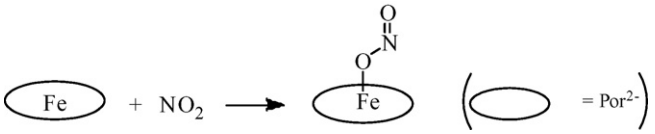
the other has two independent anions in the asymmetric unit. One of these is disordered, apparently because both linkage isomers were present. Structural [57] and computational [58] analyses of Fe(TPivPP)(NO_2)(Py) suggest that weak hydrogen bonding between the O-atoms of the nitro group and picket fence NH groups stabilize this linkage isomer.

There are now several other examples of nitrito heme complexes. For example, photolysis of Fe(TPP)(NO_2)(NO) in a low temperature KBr pellet leads to a metastable compound that was concluded to be the nitrito nitrosyl complex Fe(TPP)(η^1-ONO)(NO). This assignment was based on the N-isotope sensitive ν(N=O) and ν(N–O) IR bands characteristic of a Fe–O–N=O structure at 1507 and 934 cm^{-1} and on extensive DFT calculations [59,60]. More significant to the theme of the present review are the observations by Richter-Addo and coworkers [61], who soaked crystals of horse heart ferric myoglobin (metMb) in $NaNO_2$ solution. The crystal structure of the resulting protein clearly shows the nitrite ligand coordinated as the O-bound nitrito isomer (Fig. 3). The same structures could be crystallized from solution.

The same laboratory was able to prepare nitrite ion complexes of metHb in a similar fashion. However, for this protein, the ferriheme centers in the α- and β-subunits show significant differences in their coordination of nitrite ion [62]. For each subunit, the nitrite ligand is coordinated as the nitrito isomer, but the crystal structures show considerable differences in the mode of coordination. In the α-subunit the coordination environment is quite similar to that in the metMb analog with the Fe–O–N–O moiety oriented in a *trans* configuration, but in the β-subunit the Fe–O–N–O moiety is oriented in a distorted cis arrangement (Fig. 4).

The formation and reactivity of metalloporphyrin nitrite complexes have also been explored by Kurtikyan and coworkers using techniques that first isolate M(Por) complexes (M = Fe(II) or other M(II), Por = *meso*-tetraarylporphyrinato dianion) by sublimation onto a liquid nitrogen cooled KBr or CaF_2 substrate [63]. This method gives amorphous, microporous layers of M(Por) that can react with various volatile reagents introduced into the cryostat at different layer temperatures (77–400 K), while FTIR and optical spectra are recorded. Examining the spectra of species prepared in this manner allows one to identify the structures initially formed and to characterize subsequent chemical transformations. For reactions with nitrogen oxides, the use of isotopically labeled gases (^{15}N or ^{18}O) helps to confirm IR band assignments, and following an earlier analysis of 5- and 6-coordinate Fe(TPP) complexes by Nakamoto and coworkers [64], the positions of certain porphyrin vibrational bands were used to assign metal spin and oxidation states.

This technique was used to examine the reaction of NO_2 with sublimed layers of Fe^{II}(Por) (Por = TPP, or *meso*-tetra-p-tolylporphyrinato dianion, TTP) at 77 K. Notably, the product (formally Fe^{III}(Por)(nitrite)) is the same as would have been formed from the reaction of NO_2^- and the ferric species Fe^{III}(Por). The products were characterized spectroscopically as the 5-coordinate ferric O-coordinated nitrito complexes Fe^{III}(Por)(η^1-ONO) (Eq. (14)) on the basis of the FTIR spectra, which displayed new bands at 1525, 900 and 750 cm^{-1} corresponding to the {ν(N=O)}{ν(N–O)}{δ(ONO)} of O-coordinated nitrite, respectively. These bands shifted to lower frequencies when $^{15}NO_2$ was used. The optical spectra and frequencies of spin and oxidation state sensitive porphyrin vibrations supported the conclusion that the metal center was high spin Fe(III) [65]. The compounds formed in this way are stable even at ambient temperature.



(14)

DFT computations at the B3LYP/LACVP* level suggest [66] that the linkage isomers Fe(P)(ONO) and Fe(P)(NO_2) (P = the unsubstituted porphinato dianion) have similar energies, with the nitrito species being but slightly more stable. Despite this, the experimental studies showed only the nitrito isomer to be present as the product formed by the reaction of NO_2 with Fe(TPP) or Fe(TTP) in layered solids. One rationalization is that the nitro isomer may be destabilized by the *meso* aromatic substituents of TPP and TTP or by other interactions in the polarizable condensed phase not taken into account by the calculations.

The room temperature reaction of NO with sublimed layers containing pre-formed Fe(Por)(ONO) (Por = TPP or TTP) leads to rapid formation of the known nitro-nitrosyl complexes Fe(Por)(NO_2)(NO) [66]. However, at low temperature (130 K), the reaction initially forms the nitrito linkage isomers Fe(Por)(η^1-ONO)(NO) identified from isotope sensitive nitrosyl and nitrito bands in the vicinity of 1890 {ν(NO)}, 1500 {ν(N=O)} and 935 cm^{-1} {ν(N–O)}. Warming the sample from 130 to 220 K results in nitrite → nitro isomerization (Scheme 1) as evidenced by disappearance of the nitrito bands and

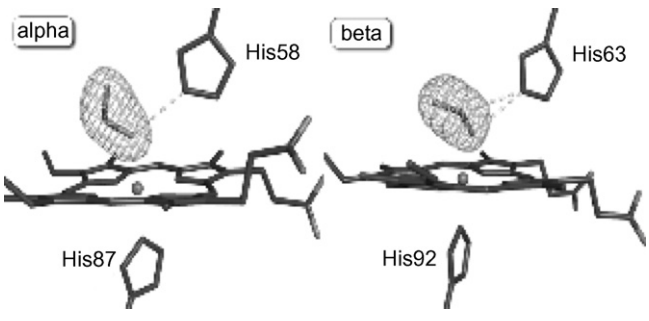
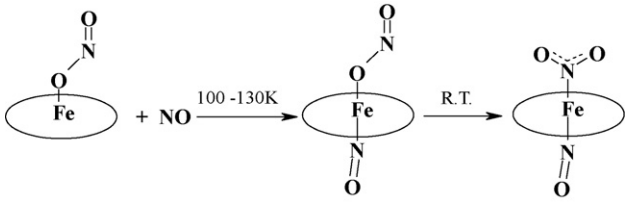
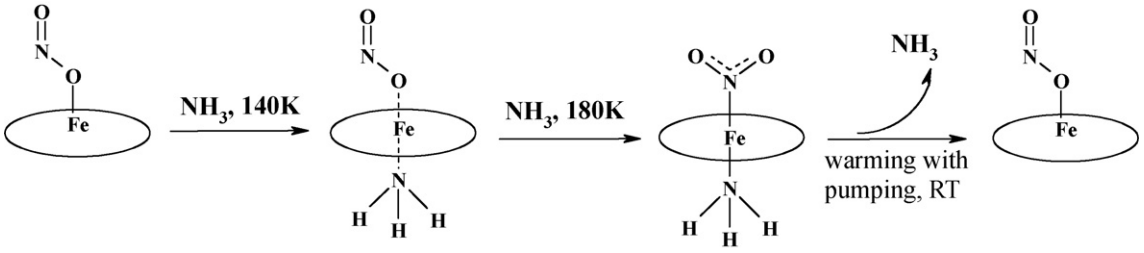


Fig. 4. The heme sites of the nitrito complex of α- and β-subunits of hemoglobin [62] (figure provided by Prof. G. Richter-Addo).



Scheme 1. Formation of Fe(Por)(ONO)(NO) and its isomerization to Fe(Por)(NO_2)(NO).



Scheme 2. The reaction of Fe(Por)(ONO) with NH_3.

appearance of new bands at 1456 and 1293 cm^{-1} characteristic of a nitro-complex. In contrast to the pentacoordinate Fe(Por)(ONO) complexes, both isomeric forms of the hexacoordinate NO adducts are low-spin.

By adding ^{15}NO to the layered Fe(Por)(ONO), or by adding NO to Fe(Por)($O^{15}NO$), it was possible to obtain the specifically labeled nitrogen oxide complexes Fe(Por)(ONO)(^{15}NO) and Fe(Por)($O^{15}NO$)(NO), respectively [66]. From the reactions of these labeled compounds, it was shown that isomerization of the nitrito-nitrosyl complexes into their nitro-nitrosyl analogs in the sublimed solids does not proceed via NO_2 dissociation, but instead involves an intramolecular mechanism.

When the Fe(Por)(NO_2)(NO) prepared as a solid layer in the cryostat was subjected to intense high vacuum pumping, both Fe(Por)(NO) and Fe(Por)(ONO) were formed, indicating that Fe(Por)(NO_2)(NO) is labile toward competitive dissociation of both NO and NO_2. Although NO loss would initially give the 5-coordinate nitro-complex Fe(Por)(NO_2), this apparently isomerizes to the more stable nitrito form. Spontaneous NO dissociation from Fe(Por)(NO_2)(NO) has also been observed in ambient temperature toluene solutions [67].

Exposing Fe(Por)(ONO) in room temperature solids to vapors of various Lewis bases (L = ammonia, pyridine or 1-methylimidazole) leads to immediate formation of the nitro-complexes Fe(Por)(L)(NO_2) [68]. These hexacoordinate species reveal different thermal stabilities depending on the nature of L. At 130 K, reaction with NH_3 gives the nitrito ammonia complex Fe(Por)(NH_3)(ONO) which then isomerizes to the nitro analog upon warming (Scheme 2). The latter is stable only when excess NH_3 (>20 Torr) is present. According to the positions of spin-sensitive FTIR bands all these 6-coordinate compounds are in the low-spin state.

4. Nitrite catalysis of reductive nitrosylation

It has long been known that NO reduces ferriheme models and proteins as well as certain cupric ion complexes, including cupric proteins [27,69–73]. For example, Fe(TPP)(NO) has been synthesized from Fe^{III}(TPP)(Cl) by reaction with NO in methanol (Eq. (15)) [74] as well as by reaction of sodium nitrite in the presence of a reducing agent [75]. Notably, the other product of the reaction shown in Eq. (15) is methyl nitrite, the product of methanol nitration. Reactions of this sort are often called reductive nitrosylation [27], although the term "autoreduction" is also used. In aqueous solution, reactions analogous to Eq. (15) would produce nitrous acid instead of MeONO.

$$Fe^{III}(TPP)(Cl) + 2NO + MeOH \rightarrow Fe^{II}(TPP)(NO) + HCl + MeONO \quad (15)$$

The water-soluble ferrous porphyrin complex Fe^{II}(TPPS)(NO) (TPPS = sulfonated tetraphenylporphyinato ligand) is formed via an analogous reductive nitrosylation of the ferric complex Fe^{III}(TPPS) in aqueous solution under excess NO [76,77]. Similar reactions have been studied quantitatively for ferri-cytochrome c, metMb and metHb by Hoshino et al. [78]. For such Fe^{III}(Por), initial formation of the nitrosyl complex (Eq. (16), $K = 1.32 \times 10^3\ M^{-1}$ for Fe^{III}(TPPS) [79,80]) gives a species that is often represented as having considerable Fe^{II}(Por)(NO^+) character. Such charge transfer to the metal center activates the NO toward nucleophilic attack by solvent H_2O (or OH^-) leading to net reduction of the metal center and oxidation of NO to nitrite ion (Eq. (17)). The Fe^{II}(Por) initially formed is trapped by the very fast reaction with the excess NO (Eq. (18)) and the driving force for the overall transformation is the very large equilibrium constant for Fe^{II}(Por)(NO) formation ($K > 10^{12}\ M^{-1}$ for Fe^{II}(TPPS)) [81]. In moderately acidic solution (pH 4–6), the reductive nitrosylation of aqueous Fe^{III}(TPPS) occurs via a slow, pH independent pathway ($k_{H_2O} = 2.7 \times 10^{-4}\ s^{-1}$ at 298 K). This reaction is also subject to general base catalysis by the buffer [77].

$$Fe^{III}(Por) + NO \rightleftharpoons Fe^{III}(Por)(NO) \quad (16)$$

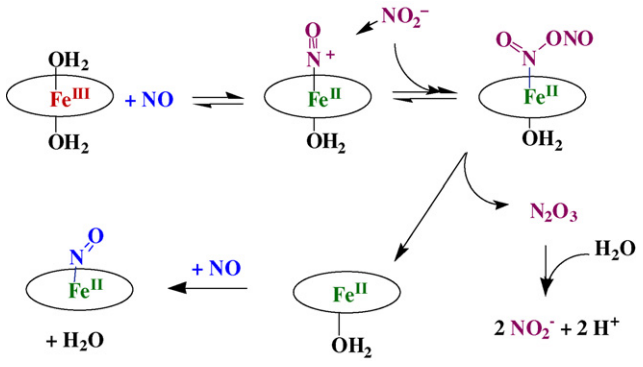
$$Fe^{III}(Por)(NO) + H_2O \rightleftharpoons Fe^{II}(Por) + NO_2^- + 2H^+ \quad (17)$$

$$Fe^{II}(Por) + NO \rightleftharpoons Fe^{II}(Por)(NO) \quad (18)$$

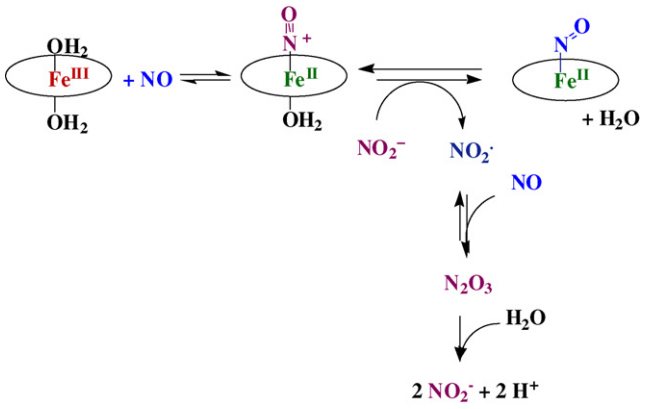
A particularly interesting development was the discovery that nitrite ion is not only the reaction product but also catalyzes the reductive nitrosylation of Fe^{III}(TPPS) in aqueous solution [82]. Nitrite catalysis has now been demonstrated for the reductive nitrosylation of metHb and metMb as well as for other ferriheme models [76,83].

Two mechanistic explanations for the nitrite catalysis of ferriheme reductive nitrosylation have been offered [77]. The first is the inner sphere pathway described in Scheme 3 where nucleophilic attack of nitrite on the $Fe^{II}(NO^+)$ moiety of the ferriheme complex occurs in a manner analogous to the apparent reaction with other nucleophiles such as water and hydroxide.

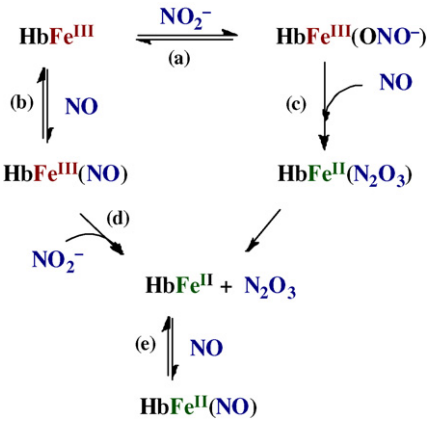
An alternative would be an outer sphere electron transfer mechanism in the manner suggested by Scheme 4. Although the depicted oxidation of nitrite to NO_2 by the ferric nitrosyl complex is energetically uphill, any NO_2 formed would be rapidly trapped by excess NO to give N_2O_3 and, ultimately, nitrous acid in aqueous media. An



Scheme 3. Inner sphere pathway for nitrite catalysis of reductive nitrosylation [77].



Scheme 4. Proposed electron transfer pathway for ferriheme reductive nitrosylation [77].



Scheme 5. Combined model for the reduction of met-hemoglobin by NO and nitrite.

observation consistent with this latter proposal is that the rate constant for nitrite catalysis of ferriheme reductive nitrosation tends to track with the reduction potential for the ferriheme nitrosyl complexes [77]. Although the authors favor a mechanism involving nucleophilic attack like that depicted in Scheme 3, the mechanism for nitrite-catalyzed reductive nitrosation has yet to be unequivocally resolved.

Notably both hypothetical mechanisms (Schemes 3 and 4) show N_2O_3 to be formed as an intermediate. If a nitrosating agent such as N_2O_3 were generated in the hydrophobic pocket of a protein, there could be biological consequences. Formation of N_2O_3 in this matter might be one source of nitrosated proteins such as SNO-Hb, which has a S-nitroso functionality at cysteine-93 of the Hb β-subunit, a protein modification that has been proposed to play a role in vasodilation under conditions of hypoxia [84].

Yet another mechanism has been proposed by Basu et al. from their studies of the analogous reaction of the ferriheme protein met-hemoglobin with NO and NO_2^- [85]. In work carried out at very low NO concentrations but with added excess nitrite (up to 5 mM), they found that the apparent rate constant k_{obs} for Fe(III) reduction to the ferrous state increased markedly at very low [NO] (<100 μM). On this basis (and others), they proposed that the nitrite facilitated NO reduction of metHb ($HbFe^{III}$ in the scheme) not only occurred via the reaction of NO_2^- with Fe(III) coordinated NO but also by the reaction of free NO with a nitrite already coordinated to the Fe(III) center, in the manner illustrated in Eq. (19). These workers [85] concluded that, at higher [NO], there is competitive inhibition of the NO_2^- binding to the Fe(III) center owing to the higher affinity of NO for metHb ($K_{(b)} = 1.3 \times 10^4\ M^{-1}$ at 298 K [78]). As a result, the apparent reactivity (as reflected by the higher k_{obs} values) increases at very low [NO] owing to the faster reduction via this alternative pathway. This observation contrasts to the pattern of the rate dependence on [NO] seen for the model compound Fe^{III}(TPPS) at higher NO concentrations, which was consistent with a preequilbrium to form a reactive Fe^{III}(TPPS)(NO) species with the NO activated toward nucleophilic attack.

$$\text{metHb} + NO_2^- \rightleftharpoons \text{metHb}(NO_2^-) \xrightarrow{NO} \text{Hb} + N_2O_3 \qquad (19)$$

Scheme 5 combines this pathway (right side of cycle) with the nitrite catalysis pathway described by Fernandez et al. (left side of the cycle) and with the known very high affinity of ferrous Hb for NO (see below). Basu et al. [85] concluded that the ferriheme reduction via Scheme 3 or 4 still functions, but is slower than that shown in Eq. (19). Both pathways are predicted to form intermediate N_2O_3, which in an aqueous environment would be expected to hydrolyze to nitrous acid or to react with nucleophiles such as cysteine or glutathione to form S-nitrosothiols. It was proposed [85] that either the N_2O_3 itself or an N-nitrosothiol derivative, such as CysSNO generated by reactions of nitrite with metHb in red blood cells, would provide a mechanism for NO equivalents to escape the RBC into the blood plasma. However, although some NO generation has been noted upon reaction of nitrite salts with hypoxic RBCs, the quantities seen are small relative to that formed by other biological tissues, such as hypoxic liver homogenates [14].

Regardless of the global importance of nitrite processing in RBCs, the mechanism proposed in Scheme 5 is quite interesting, especially given the reports described above that complexes of metMb and of metHb are both nitrito (Fe-ONO) isomers [61,62]. Although the structure of the nitrite ion complex of Fe^{III}(TPPS) in aqueous solution is unknown, analogous ferriheme models are nitro isomers, so the same would be expected for Fe^{III}(TPPS)(H_2O)(NO_2). Therefore, in comparing various ferriheme proteins and models involving nitrite complexes, different quantitative reactivities with NO might be expected, if the modes of nitrite bonding are not the same. This view is reinforced by DFT calculations, both by Basu et al. [85] and by Iretiskii [86], showing that the radical character of the nitrite ligand in the heme model Fe^{III}(P)(ONO) (P = porphine) complex is greater than that in the Fe^{III}(P)(NO_2) analog. This can be interpreted as suggesting that reactivity of the free radical NO with nitrito complexes might also be greater than with the nitro analogs. In addition, when comparing reactivities of models with proteins, it is very likely that the structure of the protein pocket introduces specific steric or hydrogen bonding interactions that enhance or suppress specific pathways. This may be especially true for hemoglobin, since the four subunits (2α- and 2β-) of Hb and metHb introduce heterogeneity into the kinetics and equilibria for the NO and nitrite coordination as well as the likelihood of allosteric effects not expected for simpler heme proteins. For example, Fig. 4 indicates that configurations of nitrite bound to metHb are different for the α- and β-subunits. In this context, would not be surprising if the nitrite catalysis of the NO reduction of metHb were to show mechanistic differences from the analogous reactions of ferriheme models such as Fe^{III}(TPPS) or even from those of metMb.

Even given these possible differences, the observation that the rate constant k_{obs} for the disappearance of metHb species and, correspondingly, for formation of the ferrous Hb species increases as [NO] is decreased below 100 μM is difficult to explain based upon the model shown in Scheme 5. If, in the presence of both NO and NO_2^-, the dominant pathways for Fe(III) reduction are steps (c) and (d) as shown in Scheme 5, then one can write a rate law for the consumption of the metHb ($HbFe^{III}$ in the scheme) as illustrated by Eq.

(20), where the rate and equilibrium constants are labeled according to the scheme. This treatment also assumes that the equilibria represented by steps (a) and (b) are fast relative to the rate limiting steps represented by (c) and (d). The latter assumptions appear to be appropriate for the reaction conditions based on the rate studies of ferriheme protein NO on and off reactions [79–81] as well as for NO_2^- on and off reactions with metMb and metHb [87].

$$-\frac{d[\mathrm{KbFe^{III}}]}{dt} = \left(\frac{(k_{(C)}k_{(a)} + k_{(d)}k_{(b)})[\mathrm{NO}][\mathrm{NO_2^-}]}{1 + k_{(a)}[\mathrm{NO_2^-}] + k_{(b)}[\mathrm{NO}]}\right)[\mathrm{HbFe^{III}}]_{total} \quad (20)$$

For this relationship, there is no range of concentrations where the overall rate $-d[\mathrm{HbFe^{III}}]/dt = k_{obs}[\mathrm{HbFe^{III}}]_{total}$ is predicted to increase as [NO] decreases even for very low [NO] where $K_{(b)}[\mathrm{NO}] \ll K_{(a)}[\mathrm{NO_2^-}]$. However, the answer may lie in the fact that the key observations by Basu et al. were made for NO concentrations comparable to and below the initial concentration of $\mathrm{HbFe^{III}}$ (30 μM). Since two NO's are consumed for each metHb reduced (owing to the very high affinity of $\mathrm{HbFe^{II}}$ for NO, see below), under such conditions the NO becomes the limiting reagent and k_{obs} must be defined differently. Indeed, since the concentrations of NO and $\mathrm{HbFe^{III}}$ are comparable, the first order decays observed are somewhat surprising. Perhaps the answer lies in an allosteric effect being triggered at very low [NO]. At this stage in the discussion the following quote from Basu et al. [85] is relevant: "Although this simple kinetic analysis perhaps does not do justice to the underlying complexity of the reactions, it illustrates that a hitherto unappreciated second reaction with faster kinetics than nitrite-catalyzed reductive nitrosylation is occurring in the initial stages of this reaction, and this faster reaction dominates as NO concentration is lowered."

5. Nitrite reductase reactions

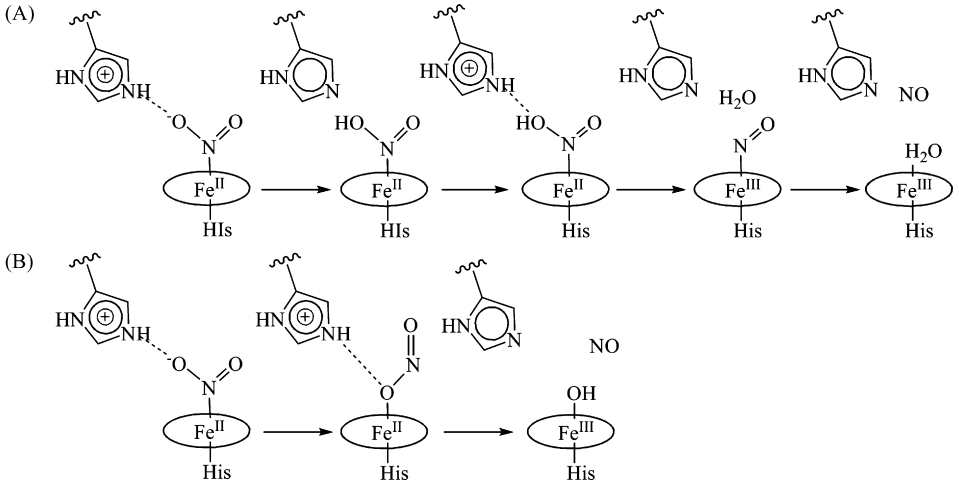
Nitrite reduction to NO by bacterial and fungal enzymes is a key step in the global nitrogen cycle, and there has long been an interest in the mechanisms of such nitrite reductase (NiR) reactions [15]. These NiR are generally either copper or heme iron proteins. Although initially thought to be inactive as a vasodilator under physiological concentrations [88], reinvestigation led to the conclusion that nitrite induces vasodilation in humans [11,89]. To explain this, it was proposed that there is NiR activity occurring with the ferriheme proteins Hb and Mb in mammalian blood and tissue.

The NiR reaction of Hb was first characterized by Brooks in 1937, who showed that nitrous acid reacts with deoxygenated Hb to form metHb and NO (Eq. (21)) [90]. The NO produced in this system is rapidly trapped by the remaining deoxygenated Hb (Eq. (22)) ($k_{on} = 2.6 \times 10^7\ \mathrm{M^{-1}\ s^{-1}}$) with a large equilibrium constant ($K = 9 \times 10^9\ \mathrm{M^{-1}}$ T state, $1 \times 10^{11}\ \mathrm{M^{-1}}$ R state) [91]. Subsequent kinetics studies by Doyle and Hoekstra described this reduction as a first order reaction giving product ratios of 70:30% metHb:HbNO [25]. Product ratios have more recently been experimentally redetermined by Gladwin et al. and found to be 50:50 when oxygen was scrupulously eliminated from the system, so that less than 3% of the Hb was in the oxy form [89,92]. Furthermore, the reaction did not display simple first order kinetics as previously described, but instead showed behavior consistent with the different forms of the protein defined by the allosteric effects. The rate of nitrite reduction was found to be slower with the T state tetramer, as evidenced by an initial lag phase. The products stabilized the R state of Hb, which in turn accelerated the reaction. Under pseudo first order conditions (50 μM heme and 10 mM NO_2^-, 25 °C and pH 7.4), the second order rate constants $k_{nitrite}$ for the T and R state were determined to be 0.12 $\mathrm{M^{-1}\ s^{-1}}$ and 6 $\mathrm{M^{-1}\ s^{-1}}$, respectively.

$$\mathrm{Hb} + \mathrm{NO_2^-} + 2\mathrm{H^+} \rightarrow \mathrm{metHb} + \mathrm{NO} + \mathrm{H_2O} \quad (21)$$

$$\mathrm{Hb} + \mathrm{NO} \rightleftharpoons \mathrm{HbNO} \quad (22)$$

Although the NiR activity of mammalian ferroheme proteins such as Hb has been known for some time, the quantitative mechanism has recently drawn renewed attention. For example, one might assume that the reaction sequence begins with the formation of a ferroheme nitrite complex, but this intermediate species has not been well characterized. DFT calculations examining nitrite binding to the ferrous heme d_1 in bacterial nitrite reductases have concluded that there is a 4.5 kcal/mol energy difference favoring the N-bound nitro isomer over the O-bound nitrito isomer [93] in that system. Recently Perissinotti et al. [94] used hybrid quantum mechanical/molecular dynamics simulations to compare the hypothetical mechanisms for reductions via the nitrito and nitro intermediates. The favored mechanism for nitrite reduction by deoxyMb involves nitrite coordination as the nitro isomer, followed by two stepwise proton transfers from the distal histidine to the nitrite. The first proton transfer would give a nitrous acid complex, followed by reprotonation of the histidine as illustrated in Scheme 6. Experimentally, the proton transfer is not rate limiting in bacterial heme NiRs. However, the



Scheme 6. Possible reaction mechanisms for nitrite reduction by Hb from either nitro (A) or nitrito (B) coordination. Calculations show that the nitro isomer (A) was more favorable even with two proton transfers (adapted from Ref. [94]).

energy barrier for NO dissociation from metMb could slow the rate of reaction, given the k_{off} value of 24 s^{-1} (25 °C) and $\Delta H^{\ddagger}_{off}$ of 15.5 kcal/mol [95].

Nitrite reduction originating from the O-coordinated nitrito complex (Scheme 6B) cannot be ruled out. Proton transfer was calculated to be almost barrierless and linkage isomerism could be slow enough for NO dissociation to occur after protonation. In this context, it is particularly notable that Richter-Addo and coworkers [96] have shown that crystals of metHb and metMb form the nitrito isomers when soaked in nitrite salt solutions as do the Co and Mn substituted myoglobins (see above). Preference for M-ONO coordination mode is likely due to hydrogen bonding from the distal histidine, H64, which stabilizes the nitrito isomer [96]. Notably, a hydrogen bond is energetically comparable to the calculated difference between the nitro and nitrito isomers noted above for ferrous hemes [93].

Recently, Rifkind et al. [97] in NiR experiments using millimolar concentrations of Hb have reported data based on EPR and optical spectra that were interpreted as indicating the presence of two, relatively stable, intermediates in the Hb + NO_2^- to metHb + NO pathway. One of these intermediates was argued to be the expected Hb(NO_2^-) complex; the other was described as a stabilized metHbNO species different from the one that releases NO. The latter was described as being a "delocalized" $Fe^{II}(NO^+)$ species somehow different from the $Fe^{III}(NO)$ complex that is the precursor to NO dissociation. They also reported that after adding NO to metHb, 62.6% of the heme protein could not be accounted for as metHb, metHbNO or HbNO and argued that the difference was this delocalized intermediate that could also be trapped with CO to give the ferrous hemoglobin carbonyl HbCO. The proposed delocalized $Fe^{II}(NO^+)$ species is problematic, given that this is simply a resonance form of $Fe^{III}(NO)$, unless it also involves a protein conformational change that gives a more stable species, perhaps involving a donor-acceptor interaction of an amino acid nucleophile with the cationic nitrosyl. In that case, the reaction with CO might give ferrous carbonyl concomitant with net nitrosation of the associated nucleophile. Further investigations of these phenomena are warranted.

Given that the reaction of nitrite with deoxyHb produces NO, a key issue is how this product might escape the RBC once NO is produced from NiR activity under hypoxic conditions. The NO will rapidly react with Hb (Eq. (22)) [98] to form the very stable (and not very labile) ferrous HbNO complex and with any oxyHb present (Eq. (4), $k_{ox} = 9 \times 10^7$ M^{-1} s^{-1}) [27]. The reported half life for HbNO at 37 °C in aerobic solution is 79 min, whereas in human subjects it was shown by EPR experiments to decrease to 40 min [99]. Some postulated mechanisms for escape include cooperation between oxyHb/deoxyHb [100], formation of nitrosothiols [84,101] and release by reductants such as dihydroxyascorbic acid (DHA)[102].

It has been proposed that NO can be readily released from the ferrous iron nitrosyl complex via a redox mechanism whereby an oxidant such as NO_2, formed from nitrite and oxyHb in the cell at the sub-membrane, oxidizes HbNO to metHbNO [27]. The latter has a relatively low binding constant resulting in NO release. The metHb is then recycled to deoxyHb by met-hemoglobin reductase. However, it's not clear how this pathway would have a major impact on NO release, since any NO thus formed still has to diffuse out of the RBC before being trapped by deoxyHb or oxyHb.

Another possible pathway for NO escape from the RBC would be formation of a hydrophobic molecule such as N_2O_3 or a nitrosothiol RSNO that might be less reactive with hemoglobin derivatives in the RBCs and that might be transported across the membrane. For example, it is known that $Fe^{III}(NO)$ complexes are susceptible to nucleophilic attack leading to reductive nitrosylation (see Section 4) [27]. In this manner, the reaction with nitrite would lead to N_2O_3 [77], while the reaction of either $Fe^{III}(NO)$ or N_2O_3 with a thiol RSH would lead to formation of RSNO species (Eq. (23)). As described in the previous section Basu et al. [85] have argued that this is a possible mechanism for NO release from RBCs.

$$\text{metHbNO} + \text{RSH} \rightarrow \text{Hb} + \text{RSNO} \quad (23)$$

Addition of dihydroascorbic acid has been shown to enhance the release of NO from human RBCs [102]. Under aerobic conditions, the second order rate constant for the DHA mediated release of NO from HbNO is 0.071 M^{-1} s^{-1}. Both DHA and the ascorbate free radical enhanced oxidation of NO specifically to nitrite, which might suggest a role for DHA and ascorbic acid in NO release and consumption.Nitrite reduction is also effected by the ferroheme protein Mb. The $k_{nitrite}$ of 6 M^{-1} s^{-1} is the same as for R state Hb, presumably because of the similar redox potentials ($E_{½}$ (Mb) = +0.046 V, ($E_{1/2}$ (Hb_R) = +0.045 V) [103,104]. This reaction may play a critical biological role, since tissue nitrite levels can be almost 100-fold higher than corresponding levels in plasma [12]. For example, mitochondrial respiration has been shown to be inhibited by the NO released upon deoxyMb reduction of nitrite to NO [105]. Shiva et al. found that having equimolar concentrations of deoxyMb and nitrite (25 μM) led to the greatest respiratory inhibition [105], presumably since a large excess of Mb would outcompete cytochrome c oxidase for NO, given the respective k_{on} values of 1.7×10^7 and 1.0×10^7 M^{-1} s^{-1} [98,106]. The idea that nitrite is involved in a multitude of biological processes was reinforced by using myoglobin knockout mice where the myocardial infarction protective effects of nitrite were no longer evident [107].

Both xanthine oxidoreductase (XOR) and eNOS have been shown to be present in the cellular membranes of RBCs [108–110]. The selective inhibition of these enzymes decreases the conversion of NO_2^- to NO. Inhibition of XOR in RBCs showed a two-third decrease in NO production, suggesting an important role for this enzyme in RBC mediated nitrite reduction [110]. Also noteworthy is that the presence of XOR on the endothelium where it could provide nitrite derived NO more efficiently to the blood vessel smooth muscle tissue than from the RBCs [110].

Mammalian NiR activity has also been shown to occur in blood free tissue, and to this activity has been attributed the role of nitrite in protecting organs during incidents of ischemia/reperfusion (I/R). For example, homogenates prepared from blood free whole-livers of male Wistar rats are quite active in reducing added nitrite to NO under hypoxic conditions [14]. Kinetic observations for whole-liver homogenates show first order kinetics with respect to nitrite and, to a lesser extent, tissue protein concentration, but the reactions are largely quenched under normoxic conditions. Addition either of a free thiol inhibitor such as N-ethylmaleimide (NEM) or of heme inhibitors such as cyanide to liver homogenates decreased the hypoxic nitrite conversion to NO, indicating that both free thiols and heme proteins are important to this process. When the tissues were fractionated into microsomes, cytosol, and mitochondria, the dominant reductase activity was in the microsomal fraction, where the cytochrome P450 proteins are located. However, the mitochondria and cytosol still showed significant activity, so one can conclude that such tissues have several complementary mechanisms for nitrite reduction [14].

There are two known classes of bacterial nitrite reductases, those containing two hemes, heme c and heme d_1, and those having a copper center at the active site. Heme c, d_1 NiRs are 60 kDa dimeric enzymes present in a majority of denitrifying bacteria [111]. Each monomer contains two domains, the larger having a heme d_1, the smaller a heme c, covalently bound to the peptide chain (Fig. 5) [112–114]. The copper NiRs, isolated from both bacteria and fungi, are 106 kDa homotrimers, each subunit having two different copper sites [115,116]. In the resting state, the type-1 Cu center is coor-

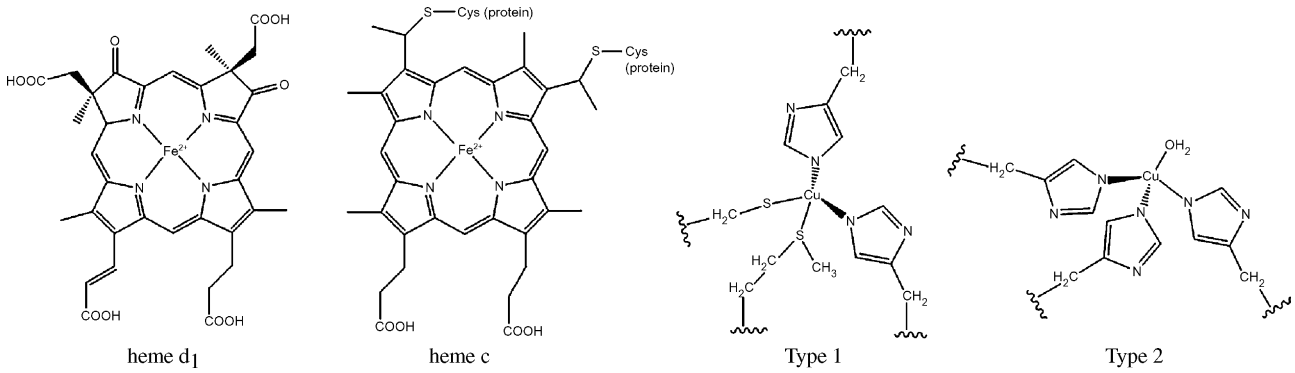


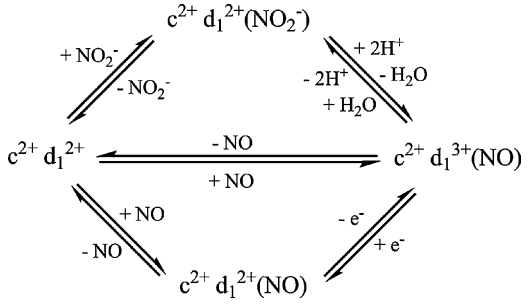
Fig. 5. Structures of the heme c and heme d_1 present in heme nitrite reductases and the two copper centers present in Cu containing nitrite reductases.

dinated to two histidine nitrogens, and two sulfurs, one from a cysteine, the other from a methionine, while the type-2 Cu centers coordinate to three histidines and a water as seen in Fig. 5.

The accepted mechanism for heme c, d_1 NiRs involves initial nitrite coordination to give a nitro-complex of the heme d_1 Fe^{II} followed by formation of a ferriheme nitrosyl intermediate. After the NO dissociation, electron transfer from the heme c restores the enzyme resting state as shown in the top half of the cycle in Scheme 7 [15]. While formation of a ferrous-nitrosyl would usually be considered a dead end given the typical low lability of such species, for *P. aeruginosa* c, d_1 NIR, the fully reduced state at pH 7 showed biphasic NO release with k_{off} values of 35 s^{-1} and 6 s^{-1} [117]. These off rates are >10^3 times faster than most Fe(II) nitrosyls, and may also be involved in NO release (lower cycle of Scheme 7).

The mechanism for the Cu containing NiR is less well characterized. Electron transfer to the type-1 Cu center is followed by a second electron transfer via a 12.5 Å cys-his bridge to the type-2 Cu where the nitrite reduction is thought to occur [118]. It is particularly interesting to note that after reduction, crystal structures indicate that the NO is bound to the Cu side-on [119].

As stated in Section 1, the plasma protein ceruloplasmin may serve as a NO oxidase to balance NO/NO_2^- ratios in the blood (Eq. (5)) [26]. There are several reports that copper containing NiRs are bidirectional catalysts, depending on the reactions conditions [73,113]. For example, the copper NiR from *A. faecalis* S-6 was found to catalyze the oxidation of NO to nitrite, the reverse of its physiological reaction at higher pH. The equilibrium constant is a function of the pH dependent reduction potentials of the physiological electron donor pseudoazurin and the NO_2^-/NO, couple. Above pH 6.2 the NiR substrates (nitrite and reduced pseudoazurin) are favored over the products (NO and oxidized pseudoazurin), and it has long been proposed that this occurs via a Cu(I)-NO^+ intermediate that reacts readily with nucleophiles such as water [116]. Similar chemistry was demonstrated by Tran et al. [120] using the Cu(II) complex $Cu(dmp)_2^{2+}$ (dmp = 2,9-dimethyl-1,10-phenanthroline) as a model



Scheme 7. Proposed catalytic cycle for heme cd_1 NiR shown (upper cycle) with a possible contribution from NO dissociation by Fe(II) (lower cycle) [15,117].

compound. The $Cu(dmp)_2^{2+}$ ion reacts with NO in aqueous solution to produce the $Cu(dmp)_2^+$ and NO_2^- (Eq. (24)) via kinetics that are dependent on base concentration. The direction of this reaction could be reversed by lowering the pH or by replacing the dmp ligands with 1,10-phenanthroline.

$$Cu(dmp)^{2+} + NO + H_2O \rightarrow Cu(dmp)^+ + NO_2^- + 2H^+ \quad (24)$$

6. Heme mediated nitrite oxidation

Under oxygenated conditions, the reactions of nitrite with deoxyHb and oxyHb occur in parallel with each other. Nitrite oxidizes deoxyHb to produce NO, whereas the reaction with oxyHb occurs through a series of complicated steps to produce nitrate (Eq. (25)) [121,122]. The maximum efficiency of NO production by these simultaneous reactions is reported to occur at the half saturation point of the Hb plus O_2 association [100]. Several groups have probed the mechanism(s) of the oxyHb/nitrite reaction experimentally as well as using kinetic simulations to resolve the intermediates [121–125]. It is thought that the reaction occurs through a complex series of steps involving the formation of a heme ferryl Fe^{IV}(Por)(O), H_2O_2, NO_2, and protein free radicals. Herold and Shivashankar have observed the peroxynitrito intermediate, Fe^{III}(OONO), that rapidly decays to Fe^{III}(Por) and nitrate [126]. However Yukl et al. [127] have recently concluded that this is a Fe^{III}(Por) nitrato complex using rapid freeze quench techniques and Raman spectroscopy (Eq. (26)). In an attempt to decipher the roles of H_2O_2 and NO_2 during the reaction sequence, Keszler et al. used kinetic simulations [128]. H_2O_2 was found to play a role only in the initiation steps of the reaction, and the oxidation of oxyHb by nitrite was shown to occur through an autocatalytic mechanism relying on NO_2 as the free radical propagation species.

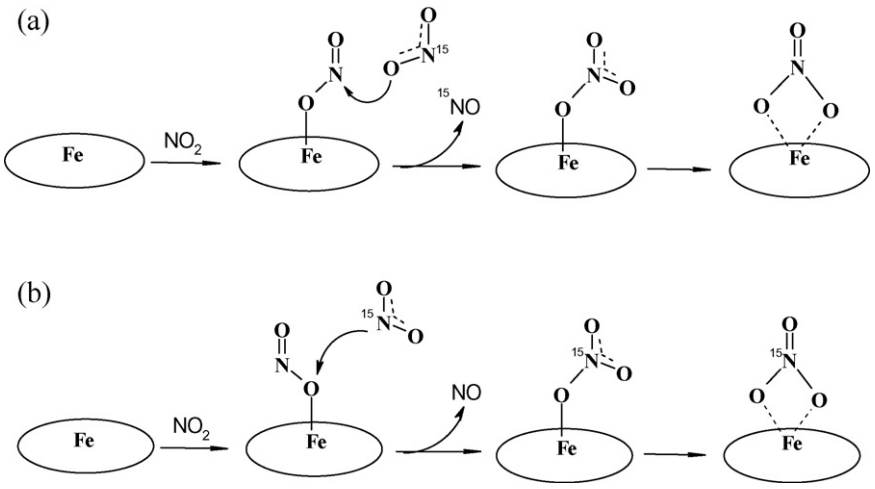
$$HbO_2 + NO \rightarrow metHb(OONO^-) \rightarrow metHb + NO_3^- \quad (25)$$

$$HbO_2 + NO \rightarrow metHb(NO_3^-) \rightarrow metHb + NO_3^- \quad (26)$$

The production of NO_2 from nitrite has also been proposed for the reaction with from the ferryl form Mb^{IV}(O) of myoglobin with nitrite [129]. Mb^{IV}(O), which has the ability to oxidize lipids, is generated from the reaction of H_2O_2 with either metMb or MbO_2 [130]. The reaction between the ferryl species and nitrite produces NO_2 and metMb with a second order rate constant of 13.9 M^{-1} s^{-1}. The rate determining step is thought to include intramolecular electron transfer to form an Fe^{III} protein radical (Eq. (27)).

$$Mb^{IV}(O) \rightarrow {}^+\cdot metMb{-}O^- \quad (27)$$

Kurtikyan et al. have described the oxidation of iron coordinated nitrite in the layered solids of Fe^{III}(Por)(NO_2) prepared by sublimation of Fe^{II}(Por) onto a low temperature substrate followed by reaction with gaseous NO_2 [66] (see above). Incremental addition



Scheme 8. Prospective mechanisms for Fe(Por)(η^2-O_2NO) formation by reaction of free NO_2 with Fe(Por)(ONO).

of more NO_2 to the layers resulted in a new species that was shown by optical and FTIR spectroscopy to be the high spin nitrato complex $Fe^{III}(Por)(O_2NO)$ accompanied by formation of NO (Eq. (28)). The nitrato complex had previously been characterized by X-ray crystallography as having an asymmetrically bound nitrate ligand [131],

$$Fe(TPP)(ONO) + NO_2 \rightarrow Fe(TPP)(\eta^2\text{-}O_2NO) + NO \quad (28)$$

Two potential scenarios for this transformation are depicted below. The most straight-forward would be simple oxygen atom transfer from the incoming NO_2 to the nitrogen atom of the nitrito ligand to give a coordinated nitrate as shown in Scheme 8a. An alternative pathway (Scheme 8b) would involve bond formation between the nitrogen atom of the incoming NO_2 and the coordinated O of the nitrito ligand followed by NO release from the originally coordinated nitrite.

Both scenarios would give a monodentate nitrato complex as the initial product but differ in the origin of the nitrogen in the eventual nitrato ligand. In principle, the two could be differentiated by isotope labeling experiments. For the first, the reaction of unlabeled $Fe^{III}(Por)(ONO)$ with $^{15}NO_2$ would give an unlabeled nitrato complex. For the second, the nitrato nitrogen would have originated with the gaseous $^{15}NO_2$ giving $Fe^{III}(Por)(\eta^2\text{-}O_2{}^{15}NO)$. It was observed that reaction of $^{15}NO_2$ with unlabeled $Fe^{III}(Por)(ONO)$ gives initially some unlabeled $Fe^{III}(Por)(\eta^2\text{-}O_2NO)$ product in agreement with the first mechanism. However, this result was complicated by exchange between the $^{15}NO_2$ and the coordinated NO_2 of $Fe^{III}(Por)(ONO)$ at a rate competitive with that of the oxygen atom transfer [66].

7. Oxygen atom transfer and other transformations of coordinated nitrite

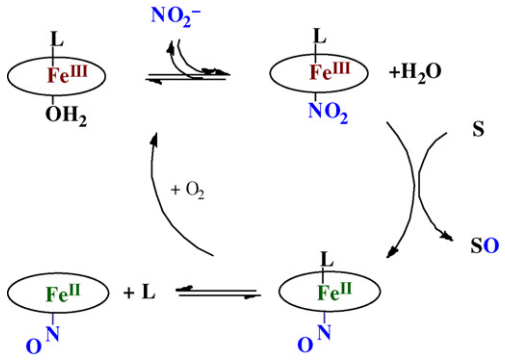
Another relevant redox reaction of nitrite is the Fe(III) mediated oxygen atom transfer (OAT) depicted in Eq. (29) (S = substrate). Although such reactivity has not been a significant part of the discussions concerned with the bioactivity of nitrite in mammalian systems, OAT is potentially a significant route to the formation of physiological nitric oxide.

$$Fe^{III}(Por)(NO_2{}^-) + S \rightarrow Fe^{II}(Por)(NO) + SO \quad (29)$$

Oxygen atom transfers involving coordinated nitrite have been reported for a number of iron complexes in nonaqueous media [58,132–138], including both heme [58,136–138] and non-heme [133–136] systems. Particularly relevant to the present review are the reports by Castro et al. [137,138] that stoichiometric OAT to various substrates can be accomplished in solutions of 18-C-6 crown ether solubilized nitrite salt [K(18-C-6)]NO_2 and Fe(OEP)Cl (OEP = octaethyl-porphyrinato dianion) in acetic acid/N-methylpyrrolidone (NMP) solutions. A ferric nitro-complex $Fe^{III}(OEP)(NO_2{}^-)$ was postulated to be a key reactive intermediate in analogy to OAT reactions from similar cobalt(III) complexes [139,140]. Given the donor solvent used, it may be assumed that $Fe^{III}(OEP)(NO_2{}^-)$, if present, is six coordinate, and in the context of the studies by Kurtikyan described above [66], would be the nitro isomer. Concerted oxygen transfer from a nitro-complex to give a very stable ferrous-nitrosyl $Fe^{II}(NO)$ would appear considerably more favorable than from the nitrito isomer, which would give initially an unstable isonitrosyl species $Fe^{II}(ON)$. Subsequently, Conradie and Ghosh [58] used DFT to probe the hypothetical mechanism of OAT from $Fe^{III}(Porphine)(NO_2)$ and from $Fe^{III}(Porphine)(Py)(NO_2)$ to dimethylsulfide. Concerted OAT from either nitro-complex was a viable process, although it was concluded that the former reaction would have the lower activation energy by ~0.32 eV. Calculations were not carried out for the nitrito analogs.

Although there are considerable precedents and subsequent examples of OAT from nitrite ion coordinated to redox active metal centers, there is one qualification that should be noted for the studies with [K(18-C-6)]NO_2 and Fe(OEP)Cl described above. These reactions were carried out in acidic media [137,138], and under such conditions, the redox chemistry might be affected by formation of NO_2 generated by the acid disproportionation of nitrite described in Section 2.

There is one published investigation of heme iron mediated OAT reaction from nitrite in near-neutral, aqueous solutions. This system is more applicable to physiological conditions and consists of $NaNO_2$, the water-soluble porphyrin complex $Fe^{III}(TPPS)(H_2O)_2$ [141], and various substrates in phosphate buffer. The species oxidized included water-soluble sulfonated triphenylphosphine (TPPTS), dimethylsulfide (DMS) and the biological thiols cysteine (CysSH) and glutathione (GSH). For TPPPS and DMS, the products were shown to be the respective monoxides TPPTSO and DMSO, however, the disulfides CysSSCys and GSSG were the products formed from the latter two substrates. Although such disulfides might be the result of one-electron reaction of the thiols (RSH) to give radicals which would then dimerize, is it known that sulfenic acids, the expected OAT products, react with excess thiol to form disulfides (Eq. (30)) [142]. Upon mixing dilute deaerated solutions of $Fe^{III}(TPPS)(H_2O)_2$ and $NaNO_2$, there was little spectral change,



Scheme 9. Cycle showing OAT from ferriheme coordinated nitrite and reoxidation by air.

in part because the equilibrium constant for formation of the nitrite complex $Fe^{III}(TPPS)(H_2O)(NO_2^-)$ is quite small ($K \sim 3\,M^{-1}$) [58]. However, when deaerated solutions of substrate were added, there was a rapid change of the optical spectrum to that of the ferrous-nitrosyl as expected for the transformation depicted in Eq. (29).

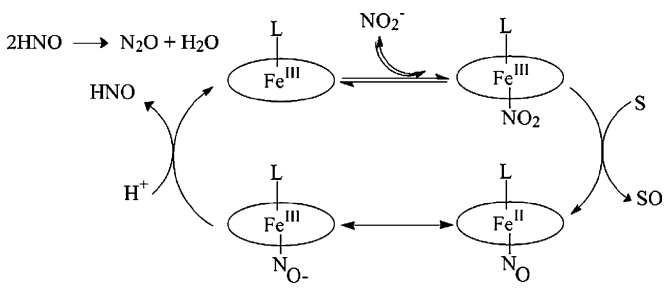
$$RS(O)H + RSH \rightarrow RSSR + H_2O \quad (30)$$

Deaerated solutions of $Fe^{II}(TPPS)(H_2O)(NO)$ formed in this manner persisted for a long time when excess substrate was present [141]. However, the introduction of small quantities of air to the reaction media regenerated the spectrum of the initial ferric complexes. This cycle is illustrated in Scheme 9, but it has not yet been determined whether the coordinated NO was also oxidized, and if so, to what product?

More surprising was the observation by Khin et al. [141] that the reduced system undergoes a slow spontaneous regeneration of the ferric complexes under anoxic conditions as well. Analysis of the gas phase of the system utilizing TPPTS as the substrate indicated stoichiometric formation of nitrous oxide, Thus, the Fe(TPPS) acts as a catalyst for oxidation of the substrate by nitrite (Eq. (31)).

$$2S + 2NO_2^- + 2H^+ \xrightarrow{Fe(TPPS)} 2SO + N_2O + H_2O \quad (31)$$

Scheme 10 illustrates a mechanism proposed [141] for this reaction, proceeding first through the Fe^{III} nitro-complex followed by OAT to the substrate. The relatively stable ferrous-nitrosyl $Fe^{II}(TPPS)(NO)$ would be the resting state of the system, so long as there is excess reductant, but the system eventually returns to the ferric state once the reductant is expended. Initial rate studies show that the relatively fast transformation of $Fe^{III}(TPPS)$ species to the ferrous-nitrosyl $Fe^{II}(TPPS)(NO)$ is approximately first order in substrate, in nitrite and in the ferric complex. This behavior is consistent with the OAT step following a preequilibrium as shown in Schemes 8 and 9. The slow regeneration of the ferric complex is pH sensitive, reacting faster in more acidic media, and this observation is consistent with the view that this slow transformation may involve the unprecedented protonation of the ferrous-nitrosyl, followed by dissociation to $Fe^{III}(TPPS)$ plus HNO. The latter species is very reactive and would undergo rapid dehydrative dimerization to N_2O [2]. However, it should be emphasized that the premises undergirding this mechanism need to be more critically interrogated experimentally.



Scheme 10. The proposed catalytic cycle for the OAT from $Fe^{III}NO_2^-$ to substrate with formation of HNO and consequently N_2O.

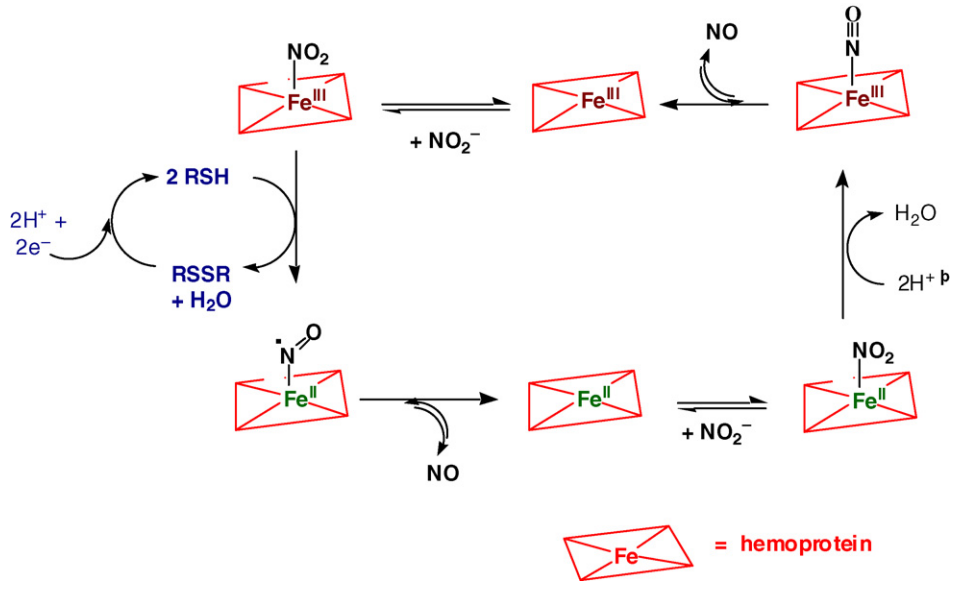
8. Summary

In this article we have provided an overview of known potentially physiologically relevant reactions involving inorganic nitrite and its metal complexes. Endogenous nitrite is largely the consequence of nitric oxide production by various isoforms of the enzyme NOS, but may also be formed by nitrate reductase action, especially by the oral bacteria. The concentrations of nitrite in plasma (50–300 nM) and tissues (0.5–25 μM) [14] are considerably larger than that of NO, which is much more reactive. Although once thought to be either relatively inert or physiologically deleterious by contributing to amine nitrosation, there is now a growing body of evidence that endogenous nitrite may play roles in vasodilitation under hypoxic conditions and in cytoprotection during incidents of ischemia/reperfusion. Furthermore, various authors have proposed potential therapeutic opportunities for nitrite, leading to considerable change in perceptions regarding physiological nitrite.

Interpretations of the beneficial roles of nitrite have focused on nitrite serving as a reservoir relatively easily converted to the known bioregulator NO via various mechanisms. However, the metal center mediated reactions of nitrite may also be a source of HNO (Section 7), for which there has been considerable interest in potential physiological roles [2], and of other nitrosylated products such as S-nitrosothiols that may have key functions in signaling. It has been shown that ferroheme proteins such as Hb and Mb can act as nitrite reductases to generate NO, but there are certain conditions where similar ferriheme proteins and models are NO oxidases to form nitrite. Analogously, copper proteins might act either as nitrite reductases or nitric oxide oxidases depending on the conditions and the coordination environment of the Cu centers. Recent studies have mapped out the general landscape of such reactions, but further study is needed to provide the detailed mechanistic understanding necessary for elucidating the key roles in physiology.

Let us conclude with a speculative discussion of the results described briefly above for components of blood free rat liver tissue. Feelisch et al. [14] reported that substantial NO is generated when nitrite solutions are added to liver homogenates, but only under hypoxic conditions. The use of various inhibitors as well as studies on subcellular fractions led to the conclusions that the NiR activity involves multiple enzymatic systems, but that the most active of these is in the microsomal fraction and is dependent both on the presence of thiols and on cytochrome P450 proteins.

A catalytic sequence is proposed in Scheme 11. In the first step, the ferriheme protein coordinates with nitrite to form a Fe(III)-nitrite complex. Reduction of this to the corresponding ferrous-nitrosyl intermediate (Fe^{II}-NO) can be effected by OAT to a thiol (RSH) present in these hypoxic/anoxic systems consistent with the model studies by Khin et al. [141]. The next step would be NO release to give the ferrous P_{450}, which can be reoxidized by nitrite to regenerate the ferric P_{450} plus another NO in analogy to reports that ferroheme proteins may act as nitrite reductases. Although ferroheme nitrosyls are often very slow in releasing NO [90], examples of much greater lability of ferrous P_{450} nitrosyls have been reported [117,143]. Notably, NO production via this cycle would be directly dependent on the $Fe^{III}(NO_2^-)$ concentration, thus linearly dependent on nitrite concentration, as was observed [14]. Although



Scheme 11. .

not included in Scheme 11, S-nitrosothiols might be formed from nucleophilic attack by thiols (RSH) on the activated nitrosyl of the ferriheme NO complex [27,144] that is an intermediate in this sequence.

Although quite speculative as an explanation of the NiR activity of liver homogenates, all of the steps of this cycle have been separately demonstrated. So the key question is whether combining several such steps is necessary or whether a simpler explanation, such as reduction of the ferriheme protein to the ferrous state by reaction with the thiol followed by nitrite reduction by the ferrous state is sufficient. It will be interesting to see how these mammalian biochemical mechanisms will be resolved.

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