Communication

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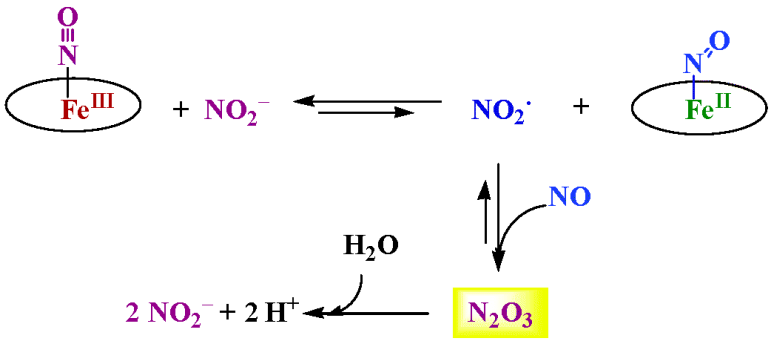
J. Am. Chem. Soc., **2003**, 125 (35), 10510-10511• DOI: 10.1021/ja036693b • Publication Date (Web): 12 August 2003



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Published on Web 08/12/2003

Nitrite Catalyzes Ferriheme Protein Reductive Nitrosylation

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Nitric oxide (nitrogen monoxide) serves important roles in mammalian biology including cardiovascular regulation, and its activity in various functions is often attributed to reactions with metalloproteins.[1] In the presence of nucleophiles, NO reactions with higher valent metal centers may lead to reduction of the metal and transfer of a NO^+ equivalent to the nucleophile. Such "reductive nitrosylation" has been demonstrated for several ferriheme proteins,[2,3] for cupric centers in cytochrome *c* oxidase and laccase,[4] and for Cu(II)[5] and Fe(III)[6,7] model systems. Reductive nitrosylation also received recent attention as a possible route to formation of S-nitrosylated β-cys-93 hemoglobin (SNO-Hb), a proposed NO carrier in mammalian blood,[8] in homogeneous reactions of NO with met-hemoglobin[9] (metHb), and in heterogeneous reactions with red blood cells.[10]

NO reduces certain ferriheme proteins including metHb via a multistep mechanism in buffered aqueous solution. Kinetics studies have demonstrated that this involves the equilibrium formation of the Fe(III) nitrosyl complex, followed by pH-dependent and -independent pathways ($k' = k_1 + k_{OH}[OH^-]$) to give the Fe(II) analogue (Scheme 1; P = porphyrin).[3] Here, we report that NO reduction of the ferriheme center of met-hemoglobin is also catalyzed by nitrite ion and suggest such catalysis might provide an alternative pathway for the cysteine nitrosation to form SNO-Hb.

Recently, we found that the water-soluble ferriheme model Fe^{III}-(TPPS)$(H_2O)_2$ (TPPS = tetra(4-sulfonato-phenyl)porphinato) undergoes slow reductive nitrosylation in weakly acidic buffered solutions (pH 3−6).[6] In analogy to the ferriheme proteins, the measured rates increased with [NO] in a manner consistent with equilibrium formation of the Fe^{III}−NO complex ($K_{NO} = 1.3 \times 10^3$ M^{-1}). At these pH's, the rates were pH independent but were modestly dependent on buffer concentrations indicating general base catalysis. More surprising was the observation that nitrite ion catalyzed this reaction with a rate constant ($k_{nitrite} = 3.1 \pm 0.1$ M^{-1} s^{-1}) several orders of magnitude larger than those measured for the buffers.[6] Because NO_2^- is a product, the reaction is autocatalytic.

These observations have led us to reexamine the NO reductions of the ferriheme proteins metHb and met-myoglobin (metMb) studied previously by Hoshino et al.[3] to probe for possible catalysis by NO_2^-. In this context, the kinetics of these reactions in 298 K, pH 7.0 aqueous phosphate buffer at low constant ionic strength were evaluated at various $NaNO_2$ concentrations (0−20 mM for metHb, 0−80 mM for metMb) and protein concentrations of 7×10^{-6} M (metHb) and 2.7×10^{-5} M (metMb). As previously demonstrated,[3] such metHb or metMb solutions reacted rapidly with NO ([NO] = 1.8 mM) to generate an equilibrium mixture of the ferriheme protein and its nitrosyl complex. This was followed by slow spontaneous reduction to the ferrous analogues Hb(NO) and Mb(NO). Typical spectral changes are illustrated by Figure 1. Without added nitrite, this occurred with lifetimes of about 10^3 and 10^4 s, respectively. Adding $NaNO_2$ led to markedly increased rates as illustrated by the linear dependence of k_{obs} on the concentration of added nitrite (e.g., Figure 2). The slopes of these plots give the catalytic rate constants $k_{nitrite} = 0.14 \pm 0.01$ M^{-1} s^{-1} for metHb and $(1.1 \pm 0.1) \times 10^{-2}$ M^{-1} s^{-1} for metMb. Although NO_2^- is known to bind weakly to metHb and metMb,[11] in the absence of NO there was no reduction of either metHb or metMb by added nitrite alone.

Scheme 1

$$Fe^{III}(P) + NO \underset{}{\overset{K_{NO}}{\rightleftharpoons}} Fe^{III}(P)(NO)$$

$$Fe^{III}(P)(NO) + H_2O \xrightarrow{k'} Fe^{II}(P) + NO_2^- + 2\,H^+$$

$$Fe^{II}(P) + NO \xrightarrow{\text{fast}} Fe^{II}(P)(NO)$$

Scheme 2 illustrates a possible inner sphere mechanism for nitrite catalysis in which NO_2^- is acting as the primary nucleophile toward the Fe^{III} coordinated nitrosyl to give an $Fe^{II}-N_2O_3$ complex as an intermediate. Dissociation of the latter releases N_2O_3 (which undergoes hydrolysis to nitrous acid) and the ferroheme protein (which is trapped by NO to produce the final nitrosyl complex). However, while this follows a pathway consistent with that seen

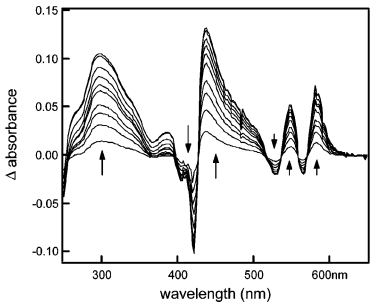


Figure 1. Optical absorbance difference spectrum following metHb(NO) reduction to Hb(NO) in pH 7.0 NaH_2PO_4/Na_2HPO_4 buffered aqueous solution at 298 K with $\mu = 0.15$ M and [NO] = 1.8 mM. The first spectrum (showing the smallest differences) was recorded 150 s after mixing, and the next three were recorded at 150 s intervals, the next four at 300 s intervals, and the final scans after 600 s intervals. (The initial and final spectra can be seen in the Supporting Information as Figure S1.)

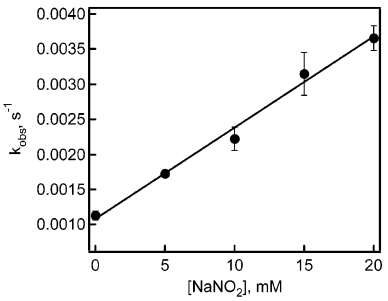
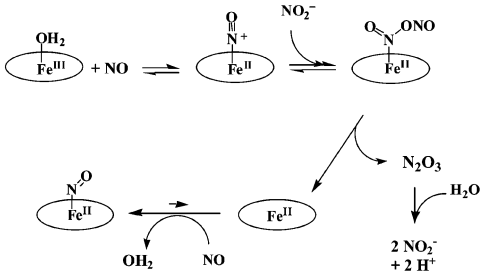


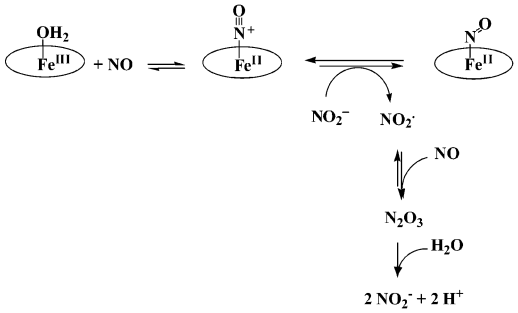
Figure 2. A plot of k_{obs} for the reduction of metHb by NO versus the concentration of added $NaNO_2$ in 41 mM NaH_2PO_4/Na_2HPO_4 buffer at pH 7.0 with $\mu = 0.15$ M and [NO] = 1.8 mM. The slope ($k_{nitrite}$) is 0.14 ± 0.01 M^{-1} s^{-1}.

10.1021/ja036693b CCC: $25.00

Scheme 2. Inner Sphere Pathway for Nitrite Catalysis of Ferriheme Reduction by NO



Scheme 3. Outer Sphere Mechanism for Nitrite Catalysis of Ferriheme Reduction by NO



for the activation of nitrosyl complexes by other nucleophiles, aqueous NO_2^- would not appear to have the unusual nucleophilicity necessary for such a catalytic mechanism.

A markedly different mechanism leading to the same products would be direct reduction of the ferriheme nitrosyl to the ferroheme analogue by outer sphere electron transfer from NO_2^- (Scheme 3). This would give NO_2 as the second product that would then be rapidly scavenged by reaction with excess NO ($k = 1.1 \times 10^9$ M^{-1} s^{-1})[12] to give N_2O_3, the same intermediate proposed for the inner sphere mechanism. Although the electron transfer is operating against an unfavorable potential ($\Delta E = -0.31$ V), evaluation according to the Marcus cross relation suggested that this may be a viable rate-limiting step for P = TPPS.[6] Furthermore, this step is followed by fast and favorable reactions (NO trapping and N_2O_3 hydrolysis) to deplete any NO_2 produced.

To our knowledge, direct measurements of the metHb(NO)/Hb(NO) and metMb(NO)/Mb(NO) half cell reduction potentials have not been reported; however, estimates of 0.49−0.57 and 0.47 V (vs NHE) can be generated from known redox potentials and equilibrium constants using Born−Haber type cycles.[13] Both values are smaller than that determined for the $Fe^{III/II}$(TPPS)(NO) half cell potential (0.59 V)[14] and correlate with the $k_{nitrite}$ trend: Fe^{III}(TPPS) > $metHb^T$ > metMb. Analysis of the reductive nitrosylation of another heme model Fe^{III}(TMPyP) (TMPyP = *meso*-tetrakis(*N*-methyl-4-pyridyl)porphyrinato) gives a much larger $k_{nitrite}$ value (85 ± 5 M^{-1} s^{-1})[15] consistent with the more favorable reduction potential of Fe^{III}(TMPyP)(NO) (0.79 V).[16] This is in accord with the expectation that outer sphere electron-transfer rates will follow the $Fe^{III/II}$(NO) reduction potential.[17]

In summary, nitrite ion catalysis of reductive nitrosylation demonstrated previously for Fe^{III}(TPPS)[6] carries over to the ferriheme proteins metHb and metMb. The marked sensitivity of the kinetics to the Fe^{III}(NO) reduction potential is consistent with the behavior expected for an outer sphere electron-transfer mechanism. Nitrite is the product of NO autoxidation in aqueous solution[18] and must be a ubiquitous component of experiments where aqueous NO is added to an aerobic system to study biological effects and should not be assumed to be innocuous. Consider, for example, the reactions of NO with red blood cells or with metHb reported to give SNO-Hb.[9,10] Nitrite may affect both the kinetics and the products, because both proposed catalysis mechanisms invoke the intermediacy of N_2O_3. If N_2O_3 were formed at a heme site, subsequent reactions of this strong oxidant and nitrosating agent could easily lead to protein modification, such as β-cys-93 nitrosation, in competition with hydrolysis to nitrite. The unexpected catalysis pathway described here emphasizes the potentially important roles of NO_x intermediates in biological transformations sometimes attributed to NO alone.

Acknowledgment. This work was supported by the U.S. National Foundation (CHE0095144).

Supporting Information Available: First and final spectra of the reaction of metHb with NO are illustrated in Figure S1 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA036693B