Pure Appl. Chem., Vol. 76, No. 2, pp. 335–350, 2004.

Probing fundamental mechanisms of nitric oxide reactions with metal centers*

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Abstract: Studies in this laboratory have been concerned with mapping the chemical properties and mechanisms of NO interactions with hemes and other metal centers. These are models relevant to the mammalian biology of nitric oxide, an important bioregulatory molecule. Presented here will be an overview of flash photolysis kinetics investigations of ferri- and ferro-heme nitrosyl formation in model complexes and several heme proteins. Also described will be ongoing studies of reductive nitrosylation mechanisms involving the reactions of NO with water-soluble Fe(III) porphyrins and ferri-heme proteins and of several Cu(II) model complexes.

INTRODUCTION

It is now well established that nitric oxide (a.k.a. nitrogen monoxide) plays fundamental roles in biochemical processes [1,2]. Natural physiological activities are now known to include roles in blood pressure control, neurotransmission, and immune response, and a number of disease states have been shown to be associated with NO imbalances [2,3]. Since the biological chemistry of NO is ultimately defined by its activity at the molecular level, there has been renewed interest in the fundamental solution phase chemistry of NO. Here, we will summarize aspects of ongoing studies at UCSB that have the goal of probing the mechanisms of model reactions that may have relevance to the physiological functions of this "simple" molecule.

NO is a stable free radical, and this is understandably a dominant theme in its chemistry. It reacts rapidly with other free radicals, for example, the reaction with superoxide ion O_2^- to form peroxynitrite ion $ONOO^-$ (eq. 1) occurs with a nearly diffusion-limited rate constant

$$NO + O_2^- \longrightarrow ONOO^- \tag{1}$$

($k_2 \sim 10^{10}$ M^{-1} s^{-1}) [4]. It also reacts readily with substitution labile, redox-active metals, but is not a strong one-electron oxidant or reductant. NO is readily diffusible, and its diffusion in cellular and vascular systems has been modeled quantitatively [5].

AUTOXIDATION REACTION

NO is known to react with dioxygen to give nitrogen dioxide in the gas phase and in nonaqueous media, but the autoxidation product in aqueous solution is nitrite (eq. 2) [6].

$$4NO + O_2 + 2H_2O \longrightarrow 4H^+ + 4NO_2^- \tag{2}$$

*Plenary lecture presented at the 2nd Santa María Workshop on Chemistry Devoted to Bioinorganic Chemistry, Santa María del Mar, Havana, Cuba, 7–11 July 2003. Other presentations are published in this issue, pp. 321–388.

One might ask how a species that is readily oxidized by aqueous O_2 is sufficiently long-lived to serve as an important bioregulator in the cardiovascular system. This is a superb example of the importance of the rate law to defining the lifetimes of reactive species. The answer lies in the kinetics of the NO/O_2 reaction. Although NO reacts very readily with other free radicals, processes requiring multiple electron changes, such as the reaction of NO with O_2, are generally much slower under physiological conditions. The explanation is drawn from the third-order rate law for the autoxidation of aqueous NO (eq. 3, where $4k_{aq} = 9 \times 10^6\ M^{-2}\ s^{-1}$) [7].

$$-\frac{d[NO]}{dt} = 4k_{aq}[NO]^2[O_2] \tag{3}$$

At the low [NO] relevant to bioregulatory processes, autoxidation is slow relative to other depletion pathways, and NO is sufficiently long-lived to allow for fast reactions with target proteins such as soluble guanylyl cyclase (sGC) that are in close proximity. However, when much higher NO levels are produced, e.g., by stimulated macrophages under immune response, autoxidation is faster and forms intermediates, like N_2O_3, that are responsible for oxidative and nitrosative reactions that contribute to cytotoxic and mutagenic activities under these conditions. Thus, the third-order kinetics behavior defines how this reactive molecule can play bioregulatory roles in oxygenated media, yet participate in cytotoxic action when generated at higher concentration.

The reaction of NO with O_2 in aqueous solution can be mediated by the presence of a metal center (such as a ferro-heme) that can participate in the redox chemistry. For example, the second-order reaction rates of NO with oxy-hemoglobin or oxy-myoglobin (eq. 4) are very fast

$$NO + Mb(O_2) \longrightarrow metMb + NO_3^- \tag{4}$$

(e.g., $k = 4 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.0 for MbO_2) [8], but the products include nitrate ion as well as the ferri-heme protein met-myoglobin (metMb). In contrast, the reaction of nitrosyl myoglobin, the pink color of cured meat, with O_2 to give the same products (eq. 5) is quite slow and

$$O_2 + Mb(NO) \longrightarrow metMb + NO_3^- \tag{5}$$

proceeds by a limiting first-order kinetic process (k_{obs} of $2.3 \times 10^{-4}\ s^{-1}$ in 298 K aqueous buffered solution) [9]. The rate of this reaction is similar to that of NO dissociation, so the rate-limiting step may be NO dissociation from Mb(NO). This would be followed by O_2 trapping of the Fe(II) center by O_2 to give a $Mb(O_2)$ type species which would indeed be very reactive with NO. However, further studies are needed to elucidate this mechanism.

COMPLEXES WITH METALS

The principal targets for NO under bioregulatory conditions are metal centers, primarily iron proteins. The best-characterized example is the ferro-heme enzyme sGC, which is activated by formation of the iron(II) nitrosyl complex [2]. Other reports describe roles of NO as an inhibitor for metalloenzymes such as cytochrome P450 [10], cytochrome oxidase [11], nitrile hydratase [12], and catalase [13], as a substrate for mammalian peroxidases [14], and as the vasodilator carried by a salivary ferri-heme protein of blood-sucking insects [15]. Heme centers are also involved in the in vivo generation of NO by oxidation of arginine catalyzed by nitric oxide synthase (NOS) enzymes [16].

NO concentrations generated for bioregulatory purposes are low, for example, submicromolar concentrations were reported in endothelium cells for blood pressure control [17]. The biological relevance of the formation ("on") and decay ("off") reactions of metal-NO complexes is emphasized by noting that activation of sGC involves an "on" reaction where the acceptor site is a Fe^{II}(PPIX) moiety [18]. Biological functions of NO such as catalase inhibition also apparently involve coordination at a heme iron, so delineation of the dynamics and mechanisms of the nitrosyl complex formation is essential to understanding the biochemistry of NO. The "off" reaction mechanism is equally important. For example, the NO release from ferric-heme nitrophorin proteins is the mechanism by which certain blood-sucking insects increase blood flow to the site of the bite.

The character of NO in a square pyramidal or hexacoordinate metal complex can range from that of a nitrosyl cation (NO^+), isoelectronic to CO with approximately linear M–N–O bonds to that of a nitroxyl anion (NO^-) for which a bond angle of ~120° is expected. The former case involves considerable charge transfer to the metal center, while in the latter, charge transfer is in the opposite direction. A generalized description of the metal–NO interaction by Feltham and Enemark [19], proposed the $\{MNO\}^n$ formulation, where n is the sum of the metal d-electrons and the nitrosyl $\pi*$ electrons. Walsh-type diagrams were used to predict the bond angles of this unit. Much less common are metastable complexes generated photochemically in low-temperature solids are η^1-NO coordinated at the oxygen atom or η^2-NO coordinated with the NO bond perpendicular to the metal–ligand axis [20].

FORMATION OF METAL-NITROSYL COMPLEXES

Ruthenium(III) complexes

An important reaction for any ligand is the formation of the ligand–metal bond. The question one might pose is whether the free-radical behavior of NO has serious impact on the mechanism for the substitution reactions of this ligand. Until recently, there had been little systematic study of the reaction mechanism(s) of metal–NO bond formation, but there is precedence for this view in studies by Taube and Armor of NO substitution into the coordination sphere of pentaammine and hexaamine Ru(III) complexes (eq. 6) [21],

$$Ru(NH_3)_6^{3+} + NO + H^+ \longrightarrow Ru(NH_3)_5(NO)^{3+} + NH_4^+ \tag{6}$$

These workers found the rate for eq. 6 to be much faster than NH_3 replacement by other ligands and proposed an associative mechanism whereby the paramagnetic d^5 Ru(III) center interacts with the odd electron of NO to give a seven-coordinate intermediate $Ru(NH_3)_6(NO)^{3+}$. This mechanism gains support from temperature effects studies [21b] that found a small activation enthalpy $\Delta H^‡$ (36 kJ mol^{-1}), but a large and negative activation entropy $\Delta S^‡$ (–138 J K^{-1} mol^{-1}) consistent with an associative pathway. Recent studies have used hydrostatic pressure effects to determine a negative activation volume ($\Delta V^‡$ –13.6 cm^3 mol^{-1}), as expected for this mechanism [22].

Kinetics studies in this laboratory of NO replacement of solvento ligands bound to several Ru(III) salen complexes (eq. 7) [X = Cl^-, ONO^-, H_2O; Sol = solvent; R-salen =

$$Ru^{III}(\text{R-salen})(\text{Sol})(X) + NO \longrightarrow Ru^{III}(\text{R-salen})(NO)(X) + \text{Sol} \tag{7}$$

derivatives of the *N,N′*-bis(ethylenediamine) dianion] demonstrated very different behavior [23]. The solvento complexes were generated by flash photolysis of the Ru^{III}(R-salen)(NO)(X) complexes in the presence of excess NO, and the kinetics of the subsequent back reactions (e.g., eq. 7) were studied in different solvents. The rates are dramatically dependent on the identity of Sol with k_{NO} (298 K, X = Cl^-) ranging from 5×10^{-4} M^{-1} s^{-1} in acetonitrile to 4×10^7 M^{-1} s^{-1} in toluene. Thus, Ru^{III}-Sol bond breaking is clearly important in the rate-limiting step, and a mechanism of dissociative nature is supported by the positive $\Delta V^‡$ (+22 cm^3 mol^{-1}) for the reaction of $Ru(^tBu_4salen)$(Cl)(toluene) with NO as well as the markedly difference in $\Delta H^‡$ for Sol = toluene (34 kJ mol^{-1}) vs. for Sol = acetonitrile (87 kJ mol^{-1}).

Ferri- and ferro-heme models

Ligand substitution kinetics with NO were first studied for metalloporphyrins and heme proteins years ago [24]; however, systematic mechanistic studies have been limited. These reactions are generally very fast, so flash photolysis techniques were used to measure the rates [24–27]. Photoexcitation labilizes NO from a M(Por)(NO) precursor (eq. 8), and subsequent relaxation of the resulting non-steady-state system back to equilibrium (eq. 9) is monitored spectroscopically (Fig. 1).

$$\mathrm{M(Por)(NO)} \underset{}{\overset{h\nu}{\rightleftarrows}} \mathrm{M(Por) + NO} \tag{8}$$

$$\mathrm{M(Por) + NO} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \mathrm{M(Por)NO} \tag{9}$$

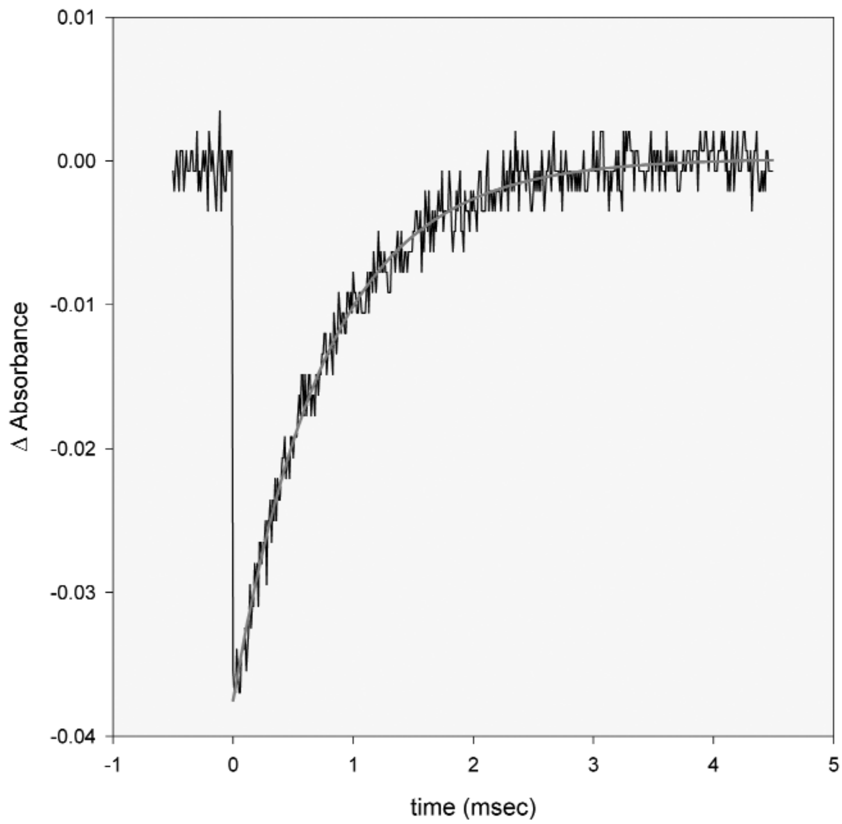


Fig. 1 Illustration of the temporal relaxation of the transient bleaching resulting from flash photolysis of an aqueous solution of Fe^{III}(TPPS) plus NO as monitored at the Soret band λ_{max} of the Fe^{III}(TPPS)(NO) complex.

Under excess NO, transient absorbances decay exponentially with a rate constant (k_{obs}) equal to

$$k_{obs} = k_{on}[\mathrm{NO}] + k_{off} \tag{10}$$

Consequently, plots of k_{obs} vs. [NO] should be linear with a slope equal to k_{on} and an intercept equal to k_{off} as illustrated in Fig. 2 for the relaxation dynamics of the Fe(III) heme model Fe^{III}(TPPS) under excess NO. For ferri-heme compounds, thermal ligand dissociation may be sufficiently fast to determine

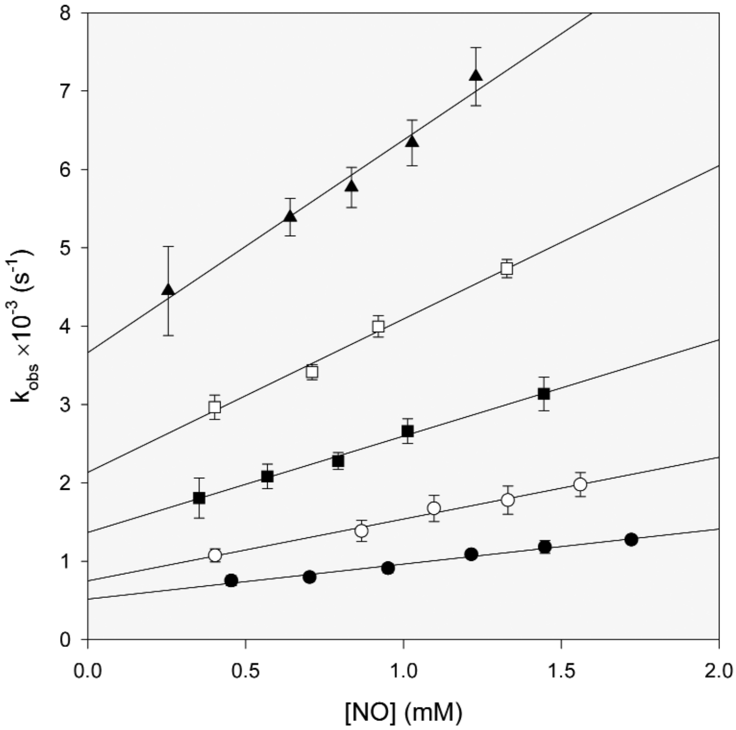


Fig. 2 Plots of k_{obs} vs. [NO] for the reaction of NO with Fe^{III}(TPPS) at different temperatures. (filled circles = 25 °C, open circles = 30 °C, filled squares = 35 °C, open squares = 40 °C, triangles = 45 °C).

an accurate value of the intercept (k_{off}). Consequently, the ratio k_{on}/k_{off} gives the equilibrium constant (*K*) for eq. 9 that can be confirmed by direct measurement of optical spectra changes as a function of [NO]. However, this is not the case for the ferro-heme complexes for which k_{off} is too small to determine with accuracy from the intercepts of plots such as Fig. 1 [25c,26].

In order to probe the mechanism(s) of eq. 9, Laverman et al. [25] determined the activation parameters for the aqueous solution reactions of NO with the Fe(II) and Fe(III) complexes of the water-soluble porphyrins TPPS and TMPS. These studies involved systematic measurements of k_{on} and k_{off} as functions of temperature (298–318 K, Fig. 2) and hydrostatic pressure (0.1–250 MPa, Fig. 3) to determine values of $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta V^{\ddagger}$ for the “on” and “off” reactions of the ferri-heme models and for the “on” reactions of the ferro-heme models (Table 1).

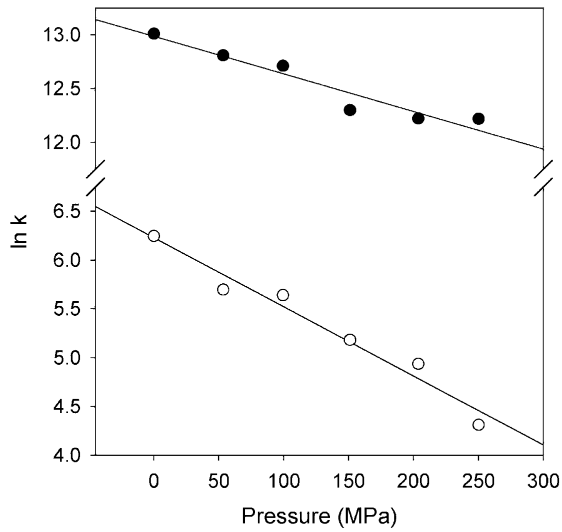


Fig. 3 Plots of $\ln(k_{on})$ (circles) and $\ln(k_{off})$ (squares) vs. hydrostatic pressure to determine activation volume values $\Delta V_{on}^{\ddagger}$ and $\Delta V_{off}^{\ddagger}$ for the reaction of NO with Fe^{III}(TPPS) in aqueous solution at 298 K.

Table 1 Activation parameters for the "on" and "off" reactions with several water soluble ferro- and ferri-hemes [25c,27].

Reaction	ΔH kJ mol^{-1}	$\Delta S^{\ddagger}$ J mol^{-1} K^{-1}	$\Delta V^{\ddagger}$ cm^3 mol^{-1}
"on" reactions			
Fe^{III}(TPPS) + NO	69 ± 3	95 ± 10	9 ± 1
Fe^{III}(TMPS) + NO	57 ± 3	69 ± 11	13 ± 1
metMb + NO	63 ± 2	55 ± 8	20 ± 6
Fe^{II}(TPPS) + NO	24 ± 3	12 ± 10	5 ± 1
Fe^{II}(TMPS) + NO	26 ± 6	16 ± 21	2 ± 2
"off" reactions			
Fe^{III}(TPPS)(NO)	76 ± 6	60 ± 11	18 ± 2
Fe^{III}(TMPS)(NO)	84 ± 3	94 ± 10	17 ± 3
metMb(NO)	68 ± 4	14 ± 13	18 ± 3

For the ferri-heme models, the large and positive $\Delta S^{\ddagger}$ and, particularly the large and positive $\Delta V^{\ddagger}$ measured for k_{on} and k_{off}, represent signatures for a substitution mechanism dominated by ligand dissociation, i.e.,

$$Fe^{III}(Por)(H_2O)_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe^{III}(Por)(H_2O) + H_2O \quad (11)$$

$$Fe^{III}(Por)(H_2O) + NO \underset{k_{-2}}{\overset{k_1}{\rightleftharpoons}} Fe^{III}(Por)(H_2O)(NO) \quad (12)$$

Making the steady-state approximation with regard to intermediate $Fe^{III}(Por)(H_2O)$ would give the following expression for the k_{obs}.

$$k_{obs} = \frac{k_1 k_2[NO] + k_{-1}k_{-2}[H_2O]}{k_{-1}[H_2O] + k_2[NO]} \quad (13)$$

It may be assumed that $k_{-1}[H_2O] >> k_2[NO]$, since $[H_2O] >> [NO]$. Accordingly, $k_{on} = k_1k_2/k_{-1}[H_2O]$ and $k_{off} = k_{-2}$, and the apparent activation parameters for k_{on} would be a summation of terms, e.g., $\Delta V^{\ddagger}_{on} = \Delta V^{\ddagger}_1 + \Delta V^{\ddagger}_2 - \Delta V^{\ddagger}_{-1}$. Since the k_2 and the k_{-1} steps represent similar reactions of the unsaturated intermediate $Fe^{III}(Por)(H_2O)$ with an incoming ligand (NO and H_2O, respectively), the differences in their activation parameters (e.g., $\Delta S^{\ddagger}_2 - \Delta S^{\ddagger}_{-1}$ and $\Delta V^{\ddagger}_2 - \Delta V^{\ddagger}_{-1}$) should be small. The principal contributor to $\Delta V^{\ddagger}_{on}$ would then be $\Delta V^{\ddagger}_1$, the activation volume for the H_2O dissociative step. The k_1 step should thus display a positive $\Delta H^{\ddagger}_1$ consistent with the energy necessary to break the Fe^{III}–OH_2 bond, a large, positive $\Delta S^{\ddagger}_1$ owing to formation of two species from one, and a substantially positive $\Delta V^{\ddagger}_1$ for the same reason. These conditions are met for the k_{on} activation parameters for the ferri-heme models.

Some years ago Hunt et al. [27] used NMR techniques to determine activation parameters for H_2O exchange on $Fe^{III}(TPPS)(H_2O)_2$. As predicted for the mechanism described by eqs. 11 and 12, this occurs at a first-order rate ($k_{ex} = 1.4 \times 10^7$ s^{-1} in 298 K water) far exceeding the k_{obs} values measured at any [NO]. Furthermore, $\Delta H^{\ddagger}_{ex}$ (57 kJ mol^{-1}) and $\Delta S^{\ddagger}_{ex}$ (+84 J K^{-1} mol^{-1}) are very similar to the respective k_{on} activation parameters measured in this laboratory for the reaction with NO (Table 1). A recent reexamination of the thermal exchange using variable temperature/pressure NMR [28] reported $\Delta H^{\ddagger}_{ex} = 67$ kJ mol^{-1}, $\Delta S^{\ddagger}_{ex} = 99$ J mol^{-1} K^{-1}, and $\Delta V^{\ddagger}_{ex} = 7.9$ cm^3 mol^{-1}, for $Fe^{III}(TPPS)(H_2O)_2$, in even better agreement with those measured by flash photolysis for k_{on} (eq. 9) [25]. Thus, the factors that determine the exchange kinetics for $Fe^{III}(TPPS)(H_2O)_2$ with solvent H_2O dominate the NO reaction with the same species and the k_{on} activation parameters for this Fe(III) heme model appear to be largely defined by a dissociative mechanism.

The principle of microscopic reversibility argues that iron nitrosyl bond breakage (k_{-2}) would be the energetically dominant step of the "off" reaction. Since coordination of NO to $Fe^{III}(Por)$ is accompanied by considerable charge transfer, the activation parameters of the "off" reaction must reflect the intrinsic entropy and volume changes associated both with the spin change and with the solvent reorganization as the charge localizes on the metal as NO dissociates.

Activation parameters for the NO reaction with the ferri-heme protein metMb according to eq. 14 demonstrate marked similarities [29] to those determined for $Fe^{III}(TPPS)(H_2O)_2$ and $Fe^{III}(TMPS)(H_2O)_2$. For example, the k_{on} step appears to be defined largely by the H_2O lability of $metMb(H_2O)$, although it is clear that the diffusion through protein channels, the distal residues and the proximal histidine binding to the Fe(III) center must all influence the NO binding kinetics.

$$\mathrm{metMb(H_2O) + NO} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{Mb(NO) + H_2O} \tag{14}$$

The ferro-heme models $Fe^{II}(TPPS)$ and $Fe^{II}(TMPS)$ are about three orders of magnitude more reactive with NO than are the Fe(III) analogs and display much lower values of $\Delta H^{\ddagger}_{on}$ and $\Delta S^{\ddagger}_{on}$. The magnitude of the latter is consistent with rates largely defined by diffusional factors, although the k_{on} values reported are about an order of magnitude less than diffusion limits in aqueous solutions. In general, high-spin $Fe^{II}(Por)$ complexes are considerably more labile than the $Fe^{III}(Por)$ analogs for most heme proteins as well. This is likely due to the ferro-heme center being 5-coordinate. In such cases, formation of a metal–NO bond would not require displacement of another ligand and would not be limited by ligand labilization rates.

For NO to act as an intracellular signaling agent at submicromolar concentrations, it must be generated near the target, and the reactions with ferro-hemes must be very fast to compete with other chemical and physiological processes leading to NO depletion. The above study is consistent with the intuitive notion that the fast reactions of ferro-heme proteins with NO are due to a vacant or exceedingly labile coordination site.

The slow "off" reactions for the Fe(II) model complexes such as Fe^{II}(TPPS)(NO) could not be measured by the flash photolysis technique, since the experimental uncertainties in the extrapolated intercepts of k_{obs} vs. [NO] plots were larger than the values of the intercepts themselves. Trapping methods were used to evaluate NO labilization rates from Fe^{II}(TPPS)(NO) by using $Ru(edta)^-$ as a NO scavenger. The small k_{off} values (Table 1) obtained in this manner are consistent with the behavior seen for the ferro-heme proteins discussed above.

NO REDUCTIONS OF METAL CENTERS, THE REDUCTIVE NITROSYLATION REACTION

As noted above, when NO coordinates to a metal, there often is charge transfer in one direction or another. If the metal center is in a higher oxidation state, it is likely that such charge transfer is from the nitric oxide to the metal, leaving (formally) a NO^+ ligand, isoelectronic to CO. An NO^+ species would be susceptible to nucleophilic attack, and such chemistry has been observed for a number of metal complexes. Although iron is the most important metal target for nitric oxide in mammalian biology, other metal centers might also react with NO. Cobalt (in the form of cobalamin) [30,31] and copper (in the form of different types of copper proteins) [32] have been identified as potential NO targets. In addition, certain bacterial nitrite reductases (which catalyze reduction of NO_2^- to NO) are copper enzymes [33]. The interactions of NO with such metal centers remains a rich area of research.

In this context, Dat Tran et. al investigated the reaction of NO with the Cu(II) complex $Cu(dmp)_2(H_2O)^{2+}$ (dmp = 2,9-dimethyl-1,10-phenanthroline) to give $Cu(dmp)_2^+$ plus nitrite (eq. 15) in aqueous solution and mixed solvents [34]. The reduction potential for $Cu(dmp)_2(H_2O)^{2+}$

$$Cu(dmp)_2(H_2O)^{2+} + NO + ROH \longrightarrow Cu(dmp)_2^+ + RONO + H^+ + H_2O \quad (15)$$

(0.58 V vs. NHE in water) [35] is more positive than most other cupric complexes owing to steric repulsion between the 2,9-methyls that provides bias toward Cu(I) tetrahedral coordination over the trigonal pyramidal structure of Cu(II). The less-crowded 1,10-phenanthroline complex $Cu(phen)_2(H_2O)^{2+}$ is a weaker oxidant (0.18 V). In methanol, the product of the $Cu(dmp)_2(H_2O)^{2+}$ oxidation of NO is CH_3ONO; in water, it is NO_2^-. The reaction did not occur in CH_2Cl_2 unless methanol was added.

The kinetics of this reaction were followed by tracking appearance of the 455 nm metal-to-ligand charge transfer (MLCT) absorption band of $Cu(dmp)_2^+$ using a stopped-flow kinetics spectrometer. At a fixed pH, the kinetics in aqueous solution followed the rate law.

$$\frac{d\left[Cu(dmp)_2^+\right]}{dt} = k_{NO}[NO]\left[Cu(dmp)_2^{2+}\right] \quad (16)$$

Addition of $NaNO_2$ (50 μM) had no effect, and no reaction was observed when NO was absent. However, at higher concentrations, anions, such as the conjugate bases of various buffers slowed the reaction. This was attributed to the competition for the labile fifth coordination site of $Cu(dmp)_2(H_2O)^{2+}$.

These results were analyzed in the context of two different mechanisms. The first would be simple outer-sphere electron transfer followed by rapid hydrolysis of NO^+ (eqs. 17 and 18),

$$Cu(dmp)_2^{2+} + NO \underset{k_{-OS}}{\overset{k_{OS}}{\rightleftharpoons}} Cu(dmp)_2^+ + NO^+ \quad (17)$$

$$NO^+ + H_2O \xrightarrow{k_{hyd}} H^+ + HNO_2 \quad (18)$$

For this sequence, reversible equilibrium followed by rate-limiting hydrolysis of the nitrosonium ion gives $k_{NO} = K_{OS}\, k_{hyd}/[Cu(dmp)_2{}^+]$ where $K_{OS} = k_{OS}/k_{-OS}$. Alternatively, k_{OS} would be rate-limiting ($k_{NO} = k_{OS}$), and electron transfer is effectively irreversible owing to rapid hydrolysis of NO^+. For either, k_{OS} is the maximum rate constant by which NO reduction of Cu(II) would occur. This can be estimated from the Marcus cross-relation, i.e., $k_{OS} \sim (k_{11}\, k_{ex}\, K_{OS})$, where k_{11} is the rate constant for $Cu(dmp)_2{}^{2+}/Cu(dmp)_2{}^+$ self exchange and k_{ex} is that for NO^+/NO self exchange. This treatment gave an estimate for k_{OS} five orders of magnitude smaller than the k_{NO} measured at lower pHs, and on this basis, the outer-sphere reaction mechanism was concluded to be unlikely [34].

Another possibility would be the mechanism illustrated in Scheme 1. The key difference is step (i), the reversible displacement of solvent (H_2O or ROH) by NO to form a Cu(II) nitric oxide complex, which is subject to nucleophilic attack by ROH (step ii). Dissociation of the RONO complex (step iii) would be rapid owing to the preference of cuprous complexes for tetrahedral coordination. The inner-sphere pathway parallels the reductive nitrosylation mechanisms discussed below with the exception that the Cu^{II}–NO complex is formed with a very low K_{NO}. Attempts to observe the putative inner sphere complex $[Cu(dmp)_2(NO)]^{2+}$ have been unsuccessful.

$$L_2Cu^{II}(ROH)^{2+} + NO \xrightleftharpoons{K_{NO}} [L_2Cu^{I}\text{-}NO^{+}]^{2+} + ROH \qquad \text{(i)}$$

$$[L_2Cu^{I}\text{-}NO^{+}]^{2+} + ROH \xrightarrow{k_{ROH}[ROH]} [L_2Cu^{I}\text{-}\overset{\displaystyle OR}{\overset{|}{N}}{=}O]^{+} + H^{+} \qquad \text{(ii)}$$

$$[L_2Cu^{I}\text{-}(N(O)OR)]^{+} \xrightarrow{\text{fast}} L_2Cu^{+} + RONO \qquad \text{(iii)}$$

$$\text{rate} = k_{ROH}K_{NO}[Cu(dmp)_2^{2+}][NO][ROH]$$

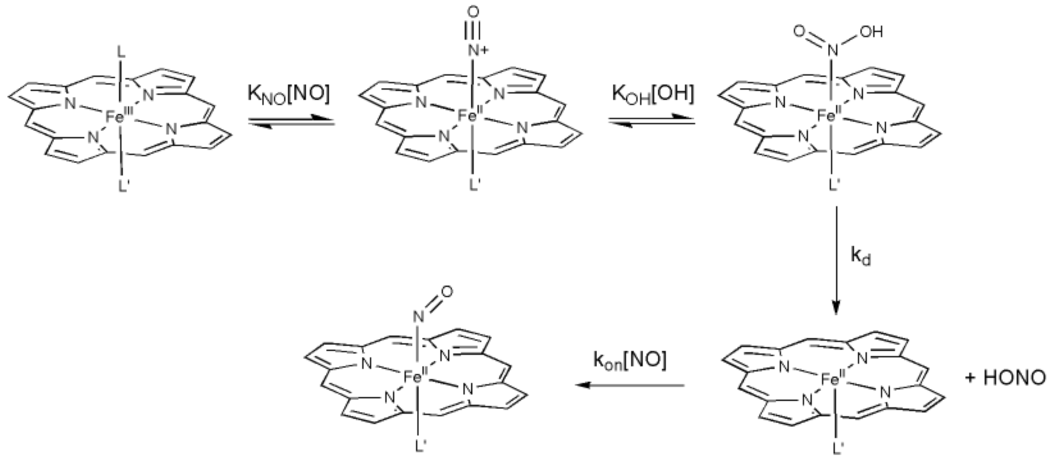
Scheme 1

Nucleophilic reactions with coordinated NO finds analogy in the reversible reaction of hydroxide with the nitrosyl ligand of the nitroprusside ion (NP) to give the nitro analog $Fe(CN)_5(\underline{N}O_2)^{4-}$ (eq. 19). Similar reactions are seen with the ruthenium and osmium analogs, as well with numerous other coordination compounds of NO [37].

$$Fe(CN)_5(NO)^{2-} + 2OH^- \rightleftharpoons Fe(CN)_5(\underline{N}O_2)^{4-} + H_2O \qquad (19)$$

Ferric porphyrins and other redox-active metal centers have long been known to undergo reduction in the presence of excess NO [38]. For example, Fe^{III}(TPP)(Cl) (TPP = tetraphenylporphyrin) reacts with NO in toluene containing a small amount of methanol to give Fe^{II}(TPP)(NO). Analogously, when aqueous ferri-hemoglobin, (metHb) is exposed to NO, the product is the ferro-hemoglobin NO adduct, Hb(NO) [39]. The kinetics of this reaction and analogous reactions with two other ferri-heme proteins metMb and Cyt^{III} have been described by Hoshino et al. (see below) [40]. 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 addition, reaction of excess NO with metMb in pH 7.4 phosphate buffer with the antioxidant glutathione GSH was reported [41] to give Mb(NO) as one product and nitrosoglutathione (GSNO) as the other product.

Mechanistic insight into the reductive nitrosylation of ferri-heme proteins was drawn from kinetics studies carried out on aqueous solutions of Cyt^{III}, metMb, and metHb at various pHs [42]. For example, Cyt^{III} undergoes reduction by NO to Cyt^{II} in aqueous solutions at pH values > 6.5 at pH and [NO] dependent rates: $k_{obs} = k_{OH} \times K_{NO}[NO][OH^-]/(1 + K_{NO}[NO])$ at low pH (where $k_{OH} = k_d \times K_{OH}$) and $k_{obs} = k_{OH}[OH^-]$ at high [NO]. A hypothetical reaction mechanism is shown in Scheme 2.



Scheme 2 Model for reductive nitrosylation of ferri-heme center (porphyrin substituents not shown).

The rate law predicted for this scheme (eq. 20) is consistent with the observed kinetics

$$\frac{d\left[\mathrm{Fe^{II}}\right]}{dt} = k_d\left[\mathrm{Fe^{III}(Por)}\right]\left(\frac{K_{\mathrm{NO}}[\mathrm{NO}]}{1+K_{\mathrm{NO}}[\mathrm{NO}]}\right)\left(\frac{K_{\mathrm{OH}}\left[\mathrm{OH^-}\right]}{1+K_{\mathrm{OH}}[\mathrm{OH}]}\right) \tag{20}$$

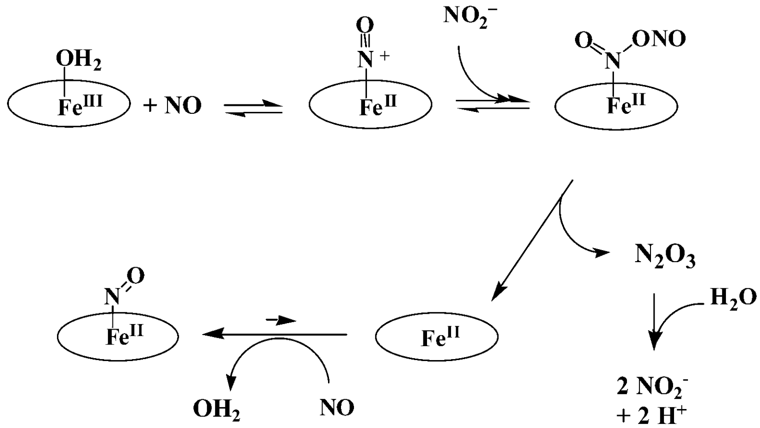
No evidence for the N-bound nitrous acid complex $Fe^{II}[\underline{N}(O)OH]$ was found for the three ferri-heme proteins studied. Thus, either formation of this intermediate is rate-limiting, or K_{OH} is very small. However, since the reaction of NO with Cyt^{II} to form $Cyt^{II}(NO)$ is very slow, formation of Cyt^{II} could be observed directly.

Unlike Cyt^{III} or metMb, reductive nitrosylation of metHb also occurs at lower pHs (<6) implying that metHb(NO) reacts with not only OH^-, but also with H_2O, perhaps under the influence of general base catalysis. This observation led to studies by Fernandez et al. in this laboratory of the comparable reaction using the water-soluble ferri-heme Fe^{III}(TPPS) as a model [43]. In analogy to the ferri-heme 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}). This species also undergoes reductive nitrosylation in moderately acidic (pH 4–6) solution (eq. 21), and the rate is dependent on the concentration and nature of the buffer in a manner consistent with general base catalysis.

$$\mathrm{Fe^{III}(TPPS) + H_2O + 2NO \xrightarrow{k_{H_2O}} Fe^{III}(TPPS)(NO) + ONO^- + 2H^+} \tag{21}$$

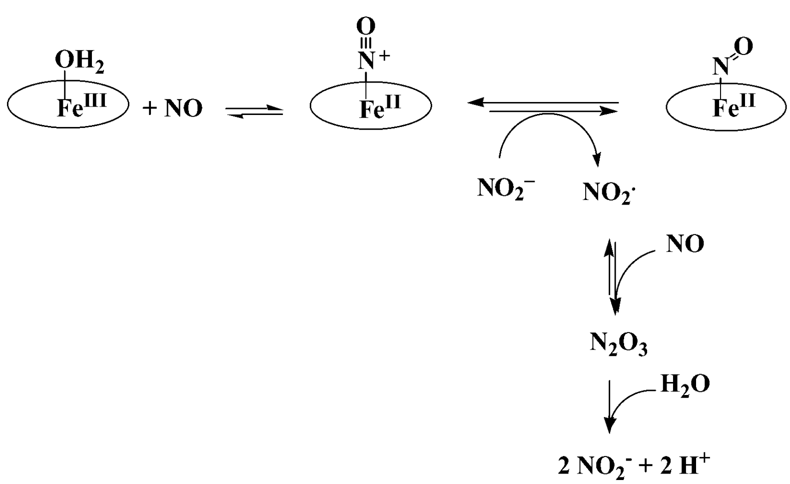
More surprising was the observation that nitrite ion catalyzed this reaction with a rate constant ($k_{nitrite} = 3.1 \pm 0.1$ M^{-1} s^{-1} in 298 K) several orders of magnitude larger than those measured for the buffers. Since nitrite is a product of the reductive nitrosylation reaction in aqueous solution, the system is, in principle, autocatalytic.

There are two mechanisms that could explain the nitrite catalysis. The first is an inner-sphere pathway in which nitrite acts as a nucleophile toward the $\{Fe^{II}NO^+\}$ moiety (Scheme 3) to form a Fe(II) coordinated N_2O_3 which dissociates then is hydrolyzed to give nitrite. The Fe^{II}(TPPS) generated would be rapidly trapped by NO to give the ferro-heme nitrosyl. This follows a pathway consistent with that seen for the activation of nitrosyl complexes by other nucleophiles, but aqueous NO_2^- would not appear to have the unusual nucleophilicity necessary for such a catalytic mechanism.



Scheme 3 Inner-sphere pathway for nitrite catalysis of ferri-heme reduction by NO.

An alternative but markedly different mechanism would be an outer-sphere electron transfer in which nitrite is oxidized to NO_2 (Scheme 4). The NO_2 so generated would then be rapidly scavenged by reaction with excess NO ($k = 1.1 \times 10^9$ M^{-1} s^{-1}) [44] to give N_2O_3, the same intermediate proposed for the inner-sphere mechanism. Although the electron transfer would be operating against an unfavorable potential (ΔE = –0.31 V), this step is followed by fast and favorable reactions (NO trapping and N_2O_3 hydrolysis) to deplete any NO_2 produced.



Scheme 4 Outer-sphere mechanism for nitrite catalysis of ferri-heme reduction by NO.

These observations led us to reexamine the NO reductions of metHb and metMb to probe for possible catalysis by NO_2^- [45]. Rates were measured 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 low protein concentrations. As previously demonstrated [26], these metHb or metMb solutions reacted rapidly with NO (1.8 mM) to generate an equilibrium mixture of the ferri-heme protein and its nitrosyl complex. Without added NO_2^-, spontaneous reduction occurred with lifetimes of about 10^3 s and 10^4 s, respectively. Adding $NaNO_2$ led to markedly increased rates and plots of k_{obs} vs. $[NO_2^-]$ (e.g., Fig. 4) are linear. The slopes gave 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. In the absence of NO, there was no reduction of either metHb or metMb by added nitrite alone.

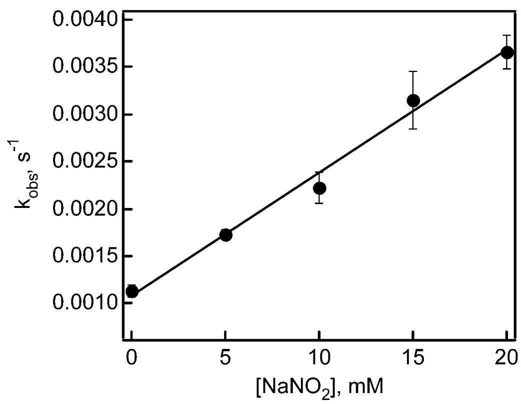


Fig. 4 Plot of k_{obs} for the reduction of metHb by NO vs. [$NaNO_2$] (41 mM phosphate buffer at pH 7.0 with μ = 0.15 M and [NO] = 1.8 mM).

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 V and 0.47 V (vs. NHE), respectively, can be generated from known redox potentials and equilibrium constants using Born–Haber-type cycles [45]. Both values are smaller than the $Fe^{III/II}$(TPPS)(NO) half-cell potential (0.59 V) and correlate with the $k_{nitrite}$ trend: Fe^{III}(TPPS) > $metHb^T$ > metMb. 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}) consistent with the more favorable reduction potential of Fe^{III}(TMPyP)(NO) (0.79 V) [46] (Fig. 5). 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 pathway [47].

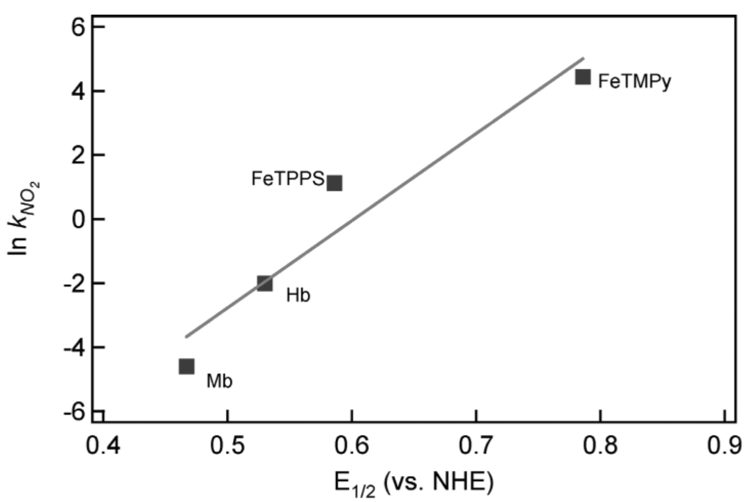


Fig. 5 Plot of the nitrite catalysis rate constant for reductive nitrosylation vs. the reduction potentials of the ferriheme complexes.

Nitrite is the product of NO autoxidation in aqueous solution [6] and is a ubiquitous component of experiments where aqueous NO is added to an aerobic system to study biological effects. The above observations indicate that such nitrite impurities 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 [48]. Nitrite may affect both the kinetics and the products, since the catalysis mechanisms proposed in Schemes 3 and 4 both invoke the intermediacy of N_2O_3. If N_2O_3 was 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 nitrosylation, 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.

SUMMARY

This article has provided an overview of recent mechanistic studies at University of California, Santa Barbara concerned with the interaction of NO with transition-metal centers with the goal of providing insight into how these substitution and redox reactions may be relevant to biological functions of NO. Despite the daunting volume of published research regarding the biochemistry and pathobiology of NO, the fundamental chemistry of NO is the key to systematizing and understanding this information. Certain features are immediately apparent. NO as a stable free radical participates very readily in one-electron events such as coupling to other free radicals and in reactions with redox-active and/or labile metal ion centers. These generally display kinetic rate laws first-order in [NO]. This behavior contrasts to reactions where two-electron changes are necessary, for example, the direct autoxidation of NO, which is a third-order kinetic process, second-order in [NO]. Thus, NO autoxidation and related third-order processes are slow under the conditions of bioregulation by this species. However, autoxidation and the accompanying formation of highly reactive oxidizing and nitrosative species such as N_2O_3 are likely to be important in immune response to pathogen infection, where higher [NO] are the norm. Although effective in fighting a localized infection, the generation of these and related reactive species such as peroxynitrite may have long-term deleterious effects on the host, especially if such immune response is against a chronic problem.

With respect to bioregulatory roles, the principal action centers on reactions with metal centers to form nitrosyl complexes, primarily the activation of sGC. Given the low NO concentrations generated for such functions, the "on" reaction must be very fast in order to provide the appropriate response to stimuli, and the target metal center must have a vacant coordination site or be very labile. The "off" reaction of metal nitrosyls may be equally important as this is a likely mechanism for deactivation of sGC. There is also considerable biological relevance with regard to NO reactions with ligands coordinated to a redox-active metal and to the reactivity of coordinated NO. For example, NO trapping by $Mb(O_2)$ or $Hb(O_2)$ is very fast and is mechanistically distinct from NO autoxidation. Coordination may serve to activate NO toward nucleophilic or electrophilic attack depending on the nature of the metal center. Of particular interest are the reactions with nucleophiles since this may well be a mechanism for biological thionitrosyl formation. Lastly, investigations of nitric oxide roles in biology and medicine need to consider the potential chemical consequences of NO_x impurities. Reactive species such as N_2O_3 and NO_2^- will be formed when air is present, especially if the manner of assembling the system components leads to high localized NO concentrations. Experimental demonstrations of chemical or biological NO mechanisms need to be supported by careful control studies to assess the impacts of NO_x impurities.

ACKNOWLEDGMENTS

Studies related to NO reaction mechanisms in this laboratory were supported by grants from the U.S. National Science Foundation, a Collaborative UC/Los Alamos Research grant, a grant from the U.S. Japan Cooperative Research Program (NSF INT 9116346), and a grant from the ACS Petroleum Research Fund. I acknowledge the students and postdoctoral fellows at UC Santa Barbara who participated in this research and collaborations with Dr. David Wink (National Cancer Institute, Bethesda MD, USA), Dr. Mikio Hoshino (RIKEN, Wako-shi, Japan), and Dr. Jon Schoonover (Los Alamos National Laboratory).

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