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Reactions of HNO with heme proteins: new routes to HNO-heme complexes and insight into physiological effects

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The formation and interconversion of nitrogen oxides has been of interest in numerous contexts for decades. Early studies focused on gas phase reactions, particularly with regard to industrial and atmospheric environments, and on nitrogen fixation. Additionally, investigation of the coordination chemistry of nitric oxide (NO) with hemoglobin dates back nearly a century. With the discovery in the early 1980s that NO is biosynthesized as a molecular signaling agent, the literature has been focused on the biological effects of nitrogen oxides, but the original concerns remain relevant. For instance, hemoglobin has long been known to react with nitrite, but this reductase activity has recently been considered to be important to produce NO under hypoxic conditions. The association of nitrosyl hydride (HNO; also commonly referred to as nitroxyl) with heme proteins can also produce NO by reductive nitrosylation. Furthermore, HNO is considered to be an intermediate in bacterial denitrification, but conclusive identification has been elusive. The authors of this article have approached the bioinorganic chemistry of HNO from different perspectives, but have converged due to the fact that heme proteins are important biological targets of HNO.

HNO-heme adducts

Interest in HNO in the Farmer lab originated from efforts to model reductive heme-based catalysis involved in the global nitrogen cycle (Scheme 1).¹ The six-electron reduction of nitrite to ammonia can be driven by a single enzyme, as in the assimilatory nitrite reductases, or can occur stepwise via the dissimilatory enzymes, which take nitrite to NO, N₂O and N₂. Heme nitroxyl intermediates have been postulated in these NO_x reductions, as indicated by the dotted line in Scheme 1.

A common mechanistic question in enzymatic reduction of nitrogen oxides is whether a nitroxyl intermediate is generated by sequential electron transfers or by a two-electron process such as hydride transfer (Scheme 2).^{2, 3} The contention is that a ferrous nitrosyl intermediate (Fe^{II}-NO) if transiently formed would be stable and difficult to reduce, thus acting as a catalytic dead end. For example, the single electron reduction of NO-Fe^{II}Mb occurs at ca. -650 mV vs. NHE,⁴ which is at the edge of the biological reduction range.⁵

An authentic nitroxyl intermediate has been observed during turnover of the fungal NO reductase cytochrome P450nor.⁶ In its catalytic cycle, a ferric nitrosyl complex of P450nor

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Supporting Information Available: Additional experimental details and figures. This material is available free of charge via the internet at <http://pubs.acs.org>.

is reduced by NADH to generate an intermediate (λ_{max} at 444 nm) that subsequently reacts with NO to give ferric heme and nitrous oxide, which is the nitrogen product of the HNO self-consumption pathway.^{7, 8} Ulrich showed that the putative nitroxyl intermediate may be formed from reaction of NaBH₄ with the ferric nitrosyl adduct,⁹ suggesting that nature does indeed bypass the thermodynamically stable Fe^{II}-NO in forming a reactive nitroxyl intermediate. The electronic structure, basicity and possible sites of protonation during turnover of this formal {Fe^{II}-NO}⁸ species has been investigated recently.^{10, 11} Correspondingly to the free species,¹²⁻¹⁴ Lehnert *et al.* found an energetic preference for the Fe-N(H)O tautomer over that of Fe-NOH in the monoprotonated form.¹¹ The axial thiolate ligand to the P450 heme promoted a second protonation, yielding a catalytically active intermediate described as Fe^{IV}-NHOH.¹¹

In a recent series of papers, we have shown that an HNO adduct of ferrous myoglobin (HNO-Fe^{II}Mb) can be formed by reduction of NO-Fe^{II}Mb or by trapping of free HNO by deoxymyoglobin (Fe^{II}-Mb).^{15, 16} The HNO-Fe^{II}Mb adduct is relatively stable, and as a unique structural model for heme nitroxyl intermediates, it has been structurally characterized by ¹H NMR,¹⁷ Resonance Raman and XAS/XANES analysis.¹⁸ The ¹H NMR spectrum is particularly diagnostic, with characteristic signals for HNO at 14.9 ppm and for the methyl group of Val68 at -2.5 ppm.

In the Farmer lab,¹⁹ NO-Fe^{II}Mb is generally synthesized by the common technique of mixing metmyoglobin (Fe^{III}Mb), nitrite and dithionite (Eq. 1).²⁰ This reaction also has physiological relevance for biosynthesis of NO.²¹ Ulrich's work⁹ prompted us to change the reductant from NaNO₂ to NaBH₄, in the hope of obtaining the HNO-Fe^{II} species. Indeed, the reaction of Fe^{III}Mb with nitrite and borohydride results in a product mixture with absorbance spectra that is consistent with production of HNO-Fe^{II}Mb in >96% yield (S2-3). Similarly, sequential addition of nitrite and borohydride to hemoglobin yields HNO-Fe^{II}Hb solutions of ca. 65% yield (S4-6).

In contrast, independently prepared and purified NO-Fe^{II}Mb does not undergo reaction with borohydride (S8, Eqs. 1, 3). This lends credence to the supposition that NO-Fe^{II} is avoided during catalytic turnover in P450_{nor}. This also implies that free HNO, or an HNO-releasing species, is generated upon reduction of nitrite by borohydride in aqueous solution.

A complication is that the ¹H NMR spectrum of HNO-Fe^{II}Mb prepared by the nitrite/borohydride method has a doubling of the proton resonances within the heme pocket (Figure 1), suggesting a diastereomeric mixture. Neither photolysis nor heating to 60°C affects this doubling. However, if an HNO-Fe^{II}Mb sample prepared by this method is air oxidized to Fe^{III}Mb, and the HNO-adduct is then regenerated using the trapping methodology, only single resonances are observed in the two characteristic regions. A similar doubling of resonances was previously perceived for reconstituted metmyoglobin adducts and was

attributed to heme orientational disorder;²²⁻²⁴ the vinylic side groups of the heme are asymmetrical, and thus flipping of the heme within a protein pocket will produce two different diastereomers. To examine this hypothesis, a sample of apomyoglobin was reconstituted with the iron complex of symmetrical 2,4-dimethyl deuteroporphyrin (Scheme 3), and its HNO adduct was prepared by the nitrite/borohydride method. The resulting ¹H NMR spectrum of the low concentration product solution yields single HNO and valine resonances, consistent with the heme orientational hypothesis. Further 2D NMR studies are underway to better characterize the structural differences of these diastereomeric forms.

To better understand how HNO adducts are formed in the nitrite/borohydride procedure, the three reactants were combined sequentially in several ways (S1a). HNO-Fe^{II}Mb was formed when either met or deoxymyoglobin was reacted with nitrite and borohydride in any addition order. One plausible reaction of hydride with nitrite is the generation of free HNO, which might then be trapped by Fe^{II}-Mb (Eqs. 4 and 5).

The possible generation of free HNO was examined with the known scavengers nickel(II)tetracyanate (Ni(CN)₄²⁻)²⁵ and iron(II) N-methyl-D-glucamine dithiocarbamate (Fe^{II}MGD)²⁶ (Eqs. 6, 7). The characteristic 498 nm absorbance band of Ni(CN)₃NO³⁻ was observed when Ni(CN)₄²⁻ was reacted with nitrite/borohydride (Figure 2), but was not apparent in the analogous reaction of Ni(CN)₄²⁻ with nitrite/dithionite. Likewise, the reaction of Fe^{II}MGD with nitrite/dithionite yielded a product solution whose EPR spectrum at 77 K matched that of the reported NO-Fe^{II}MGD complex (Figure 2). Substitution of dithionite by borohydride yielded a product solution with a similar signal but at less than 5% of the intensity, suggesting that the majority of product was the diamagnetic NO⁻-Fe^{II}MGD species. Both reactions clearly suggest that HNO or an HNO-releasing species is formed in the presence of nitrite/borohydride.

The versatility of using borohydride to generate HNO adducts in aqueous solutions was investigated with nitroprusside ([Fe(CN)₅NO]²⁻), which was recently shown to undergo two sequential reductions at high pH to generate an HNO adduct ([Fe(CN)₅HNO]³⁻) with characteristic absorbance and ¹H NMR spectra.²⁷ Reaction of borohydride with [Fe(CN)₅NO]²⁻ at pH 9 (Eq. 8) yields a product solution with the 450 nm absorbance band and 20.2 ppm resonance in the ¹H NMR (Figure 3) that are consistent with the reported [Fe(CN)₅HNO]³⁻ complex. Thus, use of borohydride provides a convenient route to HNO complexes in a one-flask reaction using simple chemical reagents, rather than the laborious stepwise procedures previously utilized.

Acidity/Basicity of HNO-metal adducts

In 1970, the pK_a of HNO in solution was estimated at 4.7, but more recent work showed the value to be ~ 11.5 .^{28, 29} Coordination to a cationic metal ion should lower the pK_a of HNO, but this has been difficult to characterize in known small molecule complexes. As mentioned above, Olabe and coworkers reported generation of an HNO adduct of nitroprusside ($[\text{Fe}^{\text{II}}(\text{CN})_5(\text{HNO})]^{3-}$) by two sequential reductions of $[\text{Fe}^{\text{II}}(\text{CN})_5(\text{NO})]^{2-}$ using dithionite at high pH.²⁷ The adduct is unstable at pH 10, however lowering the pH to 7 increases stability such that an HNO resonance can be observed at 20.02 ppm (Figure 3). Analysis of the ^1H NMR signal during pH titration indicated a pK_a value of 7.7.

For HNO- $\text{Fe}^{\text{II}}\text{Mb}$, a change in the HNO resonance in the ^1H NMR spectrum was not observed from pH 6.5–10, suggesting that the pK_a is well above the range of the well-known acid-alkaline transition for $\text{Fe}^{\text{III}}\text{Mb}$. Protein samples at higher pH are unstable over time and unsuitable for NMR studies.³⁰ However, electronic absorption spectra of dilute samples of HNO- $\text{Fe}^{\text{II}}\text{Mb}$ at high pH do show characterizable changes. As seen in Figure 4, the high energy band blue shifts at pHs above 11, whereas changes were not evident in the spectra of NO- $\text{Fe}^{\text{II}}\text{Mb}$ or $\text{Fe}^{\text{II}}\text{-Mb}$. This suggests that the pK_a of the HNO adduct is above 10 and likely close to 11.³¹

For heme-based oxidoreductases and other metalloproteins, the influence of hydrogen bonding residues within an active site is often crucial to the mechanism of action. A recent DFT analysis of Resonance Raman data on a variety of NO adducts of heme proteins led Spiro to postulate a differential effect of hydrogen bonding to the nitrogen or oxygen atoms of the coordinated nitrosyl.³² Whereas hydrogen bonding to the oxygen atom strengthens back bonding with the metal, hydrogen bonding to the nitrogen atom weakens both the Fe-N and N-O bonds and primes the nitrosyl adduct for reduction to the HNO- $\text{Fe}^{\text{II}}\text{Mb}$ state. Such hydrogen bonding interactions would likely play an important role in NO_x reducing enzymes, such as several heme-based nitrite reductases and the P450 and binuclear iron nitric oxide reductases.³³

Evidence for such interactions is observed by analysis of deuterium exchange in HNO- $\text{Fe}^{\text{II}}\text{Mb}$, which can be quantified by integration of the HNO peak at 14.9 ppm against that of the methyl group of Val68 at -2.5 ppm in the ^1H -NMR (Figure 5). The rate of H/D exchange of HNO in HNO- $\text{Fe}^{\text{II}}\text{Mb}$ is also quite distinctive. The exchange rate is slow at physiological pH ($t_{1/2} \sim 5.5$ h at pH 8), but increases significantly under more alkaline conditions ($t^{1/2}$ is ~ 16 or 9 min at pH 9 or 10, respectively). This behavior may be linked to changes in hydrogen bonding interactions with the distal His64, which hydrogen bonds to the oxygen of the HNO adduct.

Previous NMR analysis of HNO- $\text{Fe}^{\text{II}}\text{Mb}$ found few differences between spectra collected at pH 7 or 10; notable exceptions were resonances assigned to N-H protons on the proximal His93, which shifted from 9.32 to 9.68 ppm, and that of distal His64, which at 8.11 ppm at pH 8.5 but is not observed at pH 10. Loss of this distal His64 hydrogen bonding interaction with the HNO adduct would explain the abrupt change in H/D exchange in the same pH region. As illustrated in Scheme 4, the His64 hydrogen bonding interaction promotes charge buildup on the HNO moiety via back bonding with the electron-rich Fe^{II} . This may be considered as stabilization of a resonance form with full charge delocalization onto the ligand, i.e., an $\text{Fe}^{\text{III}}\text{-HNO}^-$ form for which the N-bound proton would be more tightly held. A similar doubly protonated form was suggested as an $\text{Fe}^{\text{IV}}\text{-NHOH}^-$ intermediate in the P450_{nor} cycle.¹¹ Loss of this charge stabilizing interaction at the oxygen atom may result in a lowering of the pK_a of the nitrogen-bound proton and thus an increase in H/D exchange. A reviewer suggests that deprotonation of His64 may open access to the distal pocket, thus facilitating H/D exchange.³⁴

HNO as an O₂ analogue

HNO is the simplest analogue of alkyl nitroso compounds (RNO), which have long been known to bind to ferrous heme proteins.³⁵⁻³⁶ Mansuy and coworkers were the first to describe the binding of RNO compounds to Mb and Hb,³⁷ as well as to make the analogy of RNO binding to that of dioxygen.³⁸ Although quite rare, a small number of well-characterized organometallic HNO complexes have been identified. Several routes to HNO-metal complexes have been reported, including direct reduction of a metal nitrosyl, two-electron oxidation of a metal-hydroxylamine adduct,³⁹⁻⁴⁰ insertion of NO into a metal hydride bond,⁴¹⁻⁴² and addition of hydride to a metal bound NO.⁴³⁻⁴⁵ Similarly to O₂-Fe^{II}Mb, all known HNO complexes are low spin *d*₆ and diamagnetic, and all have a characteristic HNO resonance significantly downfield in the ¹H NMR.⁴⁶

Until this year, only Fe^{II}Mb had been shown to directly complex HNO in solution to form an identifiable HNO complex. Recently,⁴⁷ other oxygen-binding proteins such as hemoglobin (Hb), leghemoglobin (legHb) and an H₂S-binding hemoglobin from the clam *L. pectinata* were shown to readily trap free HNO in solution to form HNO adducts in good yield as characterized by peaks at ca. 15 ppm in the ¹H NMR spectra. Peptidic protons in strong hydrogen bonds may also have downfield resonances, as shown in Figure 6 for the HNO adduct of hemoglobin, which has distinctive downfield resonances at ca. 12-13 ppm due to hydrogen bonds at the α and β subunit interface. Therefore, a key proof is to generate a labeled H¹⁵NO adduct, as the resulting HNO resonance will be split into a doublet by the ¹⁵N nuclear spin. ¹⁵N-labeled samples also allow Heteronuclear Single Quantum Coherence (HSQC) spectra to be readily obtained, which provide characterization of the ¹⁵N chemical shifts, as demonstrated in Figure 6.

The affinity of the oxygen-binding heme proteins for both HNO and O₂ also suggests that HNO might bind and/or inhibit non-heme oxygenases. For instance, HNO-precursors inhibit the pigmentation of melanogenic cells,⁴⁸ which depend on the activity of tyrosinase, an oxygenase which binds O₂ between two copper centers.⁴⁹

HNO in mammals

Interest in HNO in mammalian systems dates to the early 1980s when vasodilation was determined to be actively mediated by an unidentified species⁵⁰ designated the endothelium-derived relaxing factor (EDRF)⁵¹. The EDRF was subsequently determined to be NO, but the identification process led to comparisons of the effects of NO and HNO donors in vasoactive assays. Such experiments were the genesis of the current expanding interest in the pharmacological effects of HNO donors. Consequently, analysis of the aqueous chemistry as well as the biochemistry of HNO has arisen in order to identify the chemical origins of the pharmacological effects. The chemical reactions of HNO under physiological conditions and their consequences in mammalian biology are beyond the scope of this review and have been presented recently elsewhere.⁴⁶⁻⁵²⁻⁵⁴ Other recent research has focused on identification of mechanisms and markers of HNO biosynthesis and on production of novel HNO donors with properties tailored for clinical use. Donor compounds are necessary not only for facile or controlled delivery but also due to the self-consumption of HNO via irreversible dehydration of the dimer.⁵⁵⁻⁵⁶ Donors of HNO have also been recently reviewed.⁵⁷⁻⁵⁸

About the same time that the vasoactivity of HNO was observed, Nagasawa and colleagues determined that cyanamide (H₂NCN), an alcohol deterrent used in Europe, Canada, and Japan for clinical treatment of chronic alcoholism, is bioactivated by mitochondrial catalase to produce HNO.⁵⁹⁻⁶¹

(9)

Cyanamide is a potent inhibitor of aldehyde dehydrogenase (ALDH), which catalyzes the conversion of the acetaldehyde generated in the oxidative metabolism of ethanol to acetate. Inhibition of ALDH results in acetaldehydemia, provoking an unpleasant physiological response and ostensibly leading to alcohol avoidance. The inhibition mechanism involves association of HNO with an active site thiol.⁶²

These results provided the first clinical application of an HNO donor and demonstrated that HNO donors could be administered safely and with effect to humans. Consequently, a large series of prodrugs of HNO were developed to elicit this response *in vivo* (see for instance 63[–]68). Additionally, thiols were shown to be major targets of HNO. Subsequently, the interaction of HNO with thiols was shown to lead to reversible (Eq. 11) and potentially irreversible (Eq. 12) modifications, depending on the availability of a second thiol to bind to the *N*-hydroxysulfenamide (RSNHOH) intermediate (Eq. 10).^{69–73}

That protein thiols are able to be modified by HNO donors despite the presence of high concentrations of low molecular weight thiols such as glutathione (GSH) has now been shown in a number of systems.^{74–78} The mechanism by which HNO escapes scavenging by GSH is not entirely understood, but may relate to its hydrophobicity or to unique properties of protein thiols.

In a somewhat later study, Wink and coworkers demonstrated that an HNO donor (Angeli's salt) elicited significant cytotoxicity toward lung fibroblasts compared to NO donors.⁷⁹ This cytotoxicity is dependent upon an aerobic environment, is exacerbated by chemical depletion of cellular GSH and is induced in part by double strand DNA breaks and base oxidation.^{79–83} Moreover, this comparative analysis provided the first indication that HNO could affect cellular functions by altering the redox status of the cell in a manner unique from NO. Although HNO is capable of inducing oxidative stress, it can also act as an antioxidant via facile hydrogen atom donation to oxidizing radical species (akin to tocopherol) and subsequent generation of NO, which is an established antioxidant.⁸⁴ Significantly, the studies demonstrating the pro-oxidant effects were performed at high levels of HNO whereas the antioxidant properties were observed at much lower concentrations.

Later collaborative comparisons demonstrated that the *in vitro* toxicity of HNO could be replicated *in vivo* in a model of ischemia-reperfusion injury in rabbits in contrast to NO, which proved to be protective in the same model.⁸⁵ Importantly, a subsequent study showed that HNO could be protective toward reperfusion injury if administered prior to the ischemic

event.⁸⁶ Similar O₂-dependent responses to HNO were observed in neuronal channel response.⁸⁷

Together, these studies led to examination of the reaction of HNO with O₂, but the product has yet to be identified;^{79-83, 88-90} for further discussion of this reaction, see reference 52. Significantly, the autoxidation of HNO is generally too slow ($10^3 \text{ M}^{-1} \text{ s}^{-1}$) to be of kinetic consequence in many biological systems, particularly at pharmacological levels of HNO.^{91, 92} Furthermore, a number of comparisons of HNO and NO donors have now appeared in the literature (reviewed in 52, 53, 91, 93-97) and nearly universally demonstrate that the physiological properties of HNO and NO are discrete.

Perhaps most importantly, the analyses by Wink and colleagues led to intensive investigation of the cardiovascular properties of HNO in dogs (reviewed in 53, 93). HNO was found to enhance myocardial contractility even in failing hearts.^{98, 99} As such, HNO donors may act as a novel class of vasodilators and treatments for heart failure.¹⁰⁰ This discovery substantially increased interest in HNO and led to an accelerated publication rate. Ensuing analyses demonstrated that HNO targets key regulators of normal myocyte contractile function and increases sensitivity of myofilaments to calcium in a thiol-sensitive manner.^{101, 102, 103}

During the cardiovascular studies, Paolucci and colleagues⁹⁸ determined that the vascular effects of HNO in dogs were limited to the venous side of the circulatory system unlike NO donors, which are systemic hypotensive agents. In vascular smooth muscle, NO leads to dilation by binding to the regulatory ferrous heme of soluble guanylyl cyclase (sGC), which increases the rate of conversion of GTP to cGMP. The strong trans effect of NO induces cleavage of the proximal histidine upon binding, leading to an activating conformational change (Scheme 5). The observation that infusion of HNO donors did not lead to elevated cGMP levels in plasma both indicated that HNO and NO do not interconvert in blood and renewed interest in the vasoactive mechanisms of HNO.⁹¹

Several HNO donors have been observed to induce vasorelaxation *in vivo* or in rodent arterial/aortic ring assays.^{63, 104-106} These results instigated further examination of the effects of HNO donors on sGC function, usually by measurement of cGMP levels or determination of vasorelaxative potency in the presence of sGC inhibitors.^{63, 104, 106-114} The question of direct activation of sGC was addressed by Dierks and Burstyn,¹¹⁵ who exposed partially purified bovine lung sGC to donors of NO, NO⁺ and HNO and observed enhanced cGMP formation only upon introduction of NO. This finding in addition to earlier examinations of the reaction of HNO with myoglobin and hemoglobin^{69, 116, 117} led to the assumption that HNO does not react with ferrous hemes. Kinetic analysis later suggested that the primary cellular targets for HNO are thiols and oxidized metals while NO is thought to principally interact with other free radicals and with reduced metals.^{91, 118}

To explain the vasoactivity of HNO, the suggestion was made that HNO is converted to NO particularly in the aortic ring assays, for instance by superoxide dismutase (SOD), methHb, flavins¹⁰⁴ or by release of normally sequestered species during tissue preparation. Furthermore, it may be that addition of millimolar DTT, which is a vital stabilizing agent for sGC due to the oxidative instability of the heme and protein thiols under aerobic conditions, scavenged HNO before it could bind to the heme. This possibility in conjunction with the demonstration by Farmer and coworkers^{16, 17} of the thermally stable adduct of HNO with deoxymyoglobin led the Fukuto and Miranda labs to further investigate whether HNO can directly enhance the activity of sGC using bovine lung sGC purified in the Burstyn lab.

Exposure of sGC to two structurally distinct HNO donors in thiol-free media led to a concentration-dependent increase in cGMP formation.¹¹⁹ The extent of activation was

lower than that from NO but was significantly elevated compared to basal levels. That sGC was not affected by a metal-chelator but was modified by DTT clearly indicated that HNO can directly interact with sGC.

Both the heme and the multiple cysteine thiols of sGC are reasonable targets for HNO (Scheme 6). Removal of the heme decreased both HNO and NO-mediated activity, supporting a direct interaction with the heme for both nitrogen oxides. Surprisingly, HNO did not activate ferric sGC, which was expected to undergo reductive nitrosylation to form the ferrous heme complex (Eq. 13).

Ferric sGC has been previously noted to be substitutionally inert to cyanide.¹²⁰ The impact of the reactivity of HNO with the protein thiols was investigated by substituting the heme with the metal-free porphyrin, which activates the enzyme to a similar extent to the ferrous nitrosyl complex.¹²¹ In this case, HNO decreased activity, suggesting that cysteine thiols can function in a negative allosteric fashion when oxidized.

A second recent investigation¹²² appeared to confirm¹⁰⁴ that HNO does not affect sGC activity unless SOD is present. We suggest that as with other studies that suggest the requirement for oxidation of HNO to NO, buffer components may be lead to unexpected scavenging of HNO. In addition to thiols, HEPES buffer has been shown previously to scavenge HNO,⁸³ and related agents such as TEA, which was used in the recent activity studies, may have a similar effect.

Conclusion

Exposure to HNO is known to affect a variety of thiol containing proteins. That thermally stable complexes of HNO with heme proteins can now be readily produced suggests the possibility that metalloproteins may also be significant pharmacological targets for HNO. Since investigation of the chemical origin for the differing cardiovascular effects observed for HNO and NO donors in the studies by Paolucci *et al.* demonstrated that infusion of HNO did not result in a measurable increase in plasma levels of cGMP,⁹¹ questions remain about the role of HNO in whole organisms compared to *in vitro* assays or studies using excised tissues, which may have artifactual responses to HNO donors. Furthermore, whether HNO can activate sGC or coordinate to hemoglobin or myoglobin under physiological/cellular conditions remains to be determined. The coordination chemistry of HNO should be a fruitful area for both metalloprotein and small molecule chemistry for the foreseeable future.

MATERIALS AND METHODS

Horse skeletal muscle myoglobin (95-100%) adult human hemoglobin, sodium nitrite, sodium borohydride, sodium trimethoxyborohydride and zinc dust were purchased from Sigma-Aldrich and used as received. Piloty's acid was purchased from Cayman Chemicals. 4,4'-dimethyl-2,2'-dipyridyl (DTDP), tetracyanato nickelate,¹²³ and N-methyl-D-glucaminedithiocarbamate²⁶ were prepared and purified following literature procedures. Stock solutions of Piloty's acid were freshly prepared in deionized water before each experiment. Manipulation of various hemes and their adducts of NO and HNO were performed inside an anaerobic glove box. Purification of heme-adducts was carried out on a pre-equilibrated Sephadex G25 column in 50 mM phosphate buffer either at pH 7 or 9.4. All absorption spectra were recorded on a Hewlett-Packard 8453A spectrophotometer. Proton NMR experiments were recorded on Bruker Avance 600 or Varian 800 MHz spectrometers. The spectra were acquired by direct saturation of the residual water peak during the

relaxation delay. Chemical shifts were referenced to the residual water peak at 4.8 ppm. X-Band EPR spectra were recorded with a Bruker EMX spectrometer equipped with a standard TE₁₀₂ (ER 4102ST) or a high-sensitivity ER 4119HS resonator.

UV-Vis experiments

A sample of HNO-Fe^{II}Mb was concentrated on Centricon YM10. Several microliters were added to 2 mL of appropriate buffer (100 mM phosphate buffer for pH 7 and 8; 100 mM carbonate buffer for pH 10; appropriate concentration of NaOH with 50 mM NaCl for pH 11, 12 and 13). Spectra were collected in a glove box on a USB2000 spectrophotometer.

H/D exchange experiments

For the NMR experiments, HNO-Fe^{II}Mb was prepared using Piloty's acid, as described above. Aliquots were removed and concentrated on Centricon YM10 using 100 mM carbonate buffers at pH 10.0, 9.5, 9.0 and 100 mM phosphate buffer at pH 8.0. Two dilution concentration cycles were performed, until the pH of the solution in the waste reservoir was at the appropriate pH. The protein solution was concentrated to ~200 μ L. To the bottom a J-Young tube was added 360 μ L (60%) of D₂O, and to the top compartment was added total of 240 μ L (40 %) HNO-Fe^{II}Mb solution diluted with buffer. The solution was mixed and time course ¹H NMR spectra were collected. The HNO peak was integrated and plotted versus time. For the 95+% D₂O sample, the HNO-Fe^{II}Mb solution was fully exchanged with D₂O in a Centricon column.

Preparation of nitrosyl heme proteins

To a micromolar solution of heme (200 μ L) in 50 mM phosphate buffer at pH 7 was added sodium nitrite. After standing for 5 min, and sodium dithionite was added at a ratio of sodium nitrite to sodium dithionite of 1:3. The nitrosyl hemes were purified on size exclusion Sephadex G25 column and then concentrated on a Amicon YM10 membrane filters.

Nitrite/borohydride preparation of HNO-Fe^{II}Mb

To a 60 mg of Mb in 2 mL of carbonate buffer pH 9.4 was added 10-20 eq of sodium nitrite followed by careful addition of either solid sodium borohydride¹ (10 eq) or 200 μ L of 1 M solution of sodium borohydride in 1 M sodium hydroxide. Upon addition of sodium borohydride, a visible color change from brown to red was observed, which indicated formation of HNO-Fe^{II}Mb. The reaction solution was then added to a pre-equilibrated G-25 Sephadex column in 50 mM phosphate buffer at pH 7. The fast moving dark red band was collected and concentrated on an Amicon YM10 filter.

Reaction of tetracyanonickelate with nitrite/dithionite and nitrite/borohydride mixtures

Tetracyanonickelate (5 mg) was dissolved in 1 mL of 50 mM carbonate buffer pH 10 in a 1 cm cuvette. After addition of sodium nitrite (1 mg) followed by sodium borohydride (2 mg), the solution immediately turned to dark purple color. The absorbance spectrum was collected with no attempt to isolate the reaction products.

¹Sodium borohydride is highly reactive white solid powder that violently reacts with water and aid combustion, so proper protection and care should be taken while handling it.

Reaction of Fe(II) N-methyl-D-glucaminedithiocarbamate with nitrite-dithionite and nitrite/borohydride mixtures

Two samples of Fe^{II}MGD (5 mg) were dissolved in separate 1 ml of 50 mM carbonate buffer pH 10 in 1 cm cuvettes; to one was added sodium nitrite (1 mg) followed by sodium borohydride (2 mg); to the other sodium nitrite (1 mg) followed by sodium dithionite (2 mg). Both reaction mixtures immediately turned dark green, and aliquots were transferred to EPR tubes and frozen. No attempt was made to purify the reaction products.

Reduction of sodium nitroprusside by sodium borohydride

Sodium nitroprusside (10 mg) was dissolved in 1 mL of 50 mM carbonate buffer. Sodium nitrite and sodium borohydride were then added, and the brick red colored solution was then used for studies without further purification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.
Species involved in the nitrogen cycle

Scheme 2.
Reduction of coordinated NO^+ to NO and NO^-

Figure 1.

¹H-NMR spectra of the HNO adduct of myoglobin (0.5-1 mM) in 50 mM phosphate buffer at pH 7.0 either (A) prepared by the NaNO₂/NaBH₄ method or (B) generated by oxidation of A, reduction to Fe^{II}Mb and subsequent reaction with the HNO donor Piloty's acid at pH 10. H¹⁵NO-Fe^{II}Mb was prepared either by (C) DTDP reduction of NO-Fe^{II}Mb or (D) by reduction with Na¹⁵NO₂/NaBH₄. (E) shows the HNO adduct of iron 2,4-dimethyl deuterioporphyrin (0.1 mM) reconstituted myoglobin in 50 mM phosphate buffer at pH 7.

Scheme 3.
2,4-dimethyl deuterioporphyrin

Figure 2.

A). Electronic spectra of the reaction of $\text{K}_2[\text{Ni}(\text{CN})_4]$ (solid line) with $\text{Na}_2\text{S}_2\text{O}_4$ (dash dot) and $\text{NaNO}_2\text{-NaBH}_4$ (dashed) in carbonate buffer at pH 9.4. **B)** X-band EPR spectra of the reaction of $\text{Fe}^{\text{II}}\text{MGD}$ with $\text{NaNO}_2/\text{Na}_2\text{S}_2\text{O}_4$ (dashed line) and $\text{NaNO}_2/\text{NaBH}_4$ (solid line) in 50 mM phosphate buffer at pH 7.0 at 77 K.

Figure 3. Absorbance spectrum of $[\text{Fe}(\text{CN})_5\text{HNO}]^{3-}$ prepared by NaBH_4 reduction of $\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]$; inset shows the ^1H -NMR spectrum of $[\text{Fe}(\text{CN})_5\text{HNO}]^{3-}$ (ca. 1 mM) in 50 mM phosphate buffer.

Figure 4. Normalized electronic absorbance spectra of the Q-band region of HNO-Fe^{II}Mb (ca. 10 μM) at pH 7, 11 or 13.

Figure 5.

¹H-NMR assignment is shown for HNO-Fe^{II}Mb (top) and DNO-Fe^{II}Mb (bottom) samples in the HNO (left), the meso (center) and the Val68 methyl (right) regions.

Scheme 4.

Figure 6. NMR spectrum of the HNO adduct of human hemoglobin in pH 7 phosphate buffer. At bottom is the ^1H NMR spectrum **A**) in the HNO and **B**) valine regions. At top is the corresponding **C**) ^1H - ^{15}N HSQC spectrum of H^{15}NO adduct and **D**) the 2D ^1H - ^1H NOESY spectrum showing the interaction between HNO and the valine methyl protons.

Scheme 5.
Activation of sGC upon binding of NO to the regulatory heme.

Scheme 6. Routes of activation of sGC by HNO in purified, O_2 -free systems