The HNO Adduct of Myoglobin: Synthesis and Characterization

Rong Lin and Patrick J. Farmer*

Department of Chemistry, University of California, Irvine
Irvine, California 92697-2025

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The nitroxyl, a one-electron reduced form of nitric oxide, has been suggested to play an important role in many biological systems which involve heme proteins.[1,2] Like nitric oxide, the nitroxyl has been linked to processes such as vasodilation[3] and cytotoxicity.[4] It has been proposed to be the released product of arginine oxidation by inducible $P450_{NOS}$.[5] Nitroxyl−heme adducts are postulated as intermediates in mechanisms of several types of nitric oxide reductases.[6,7] In a previous paper, we described the reversible electrochemical reduction of nitrosyl myoglobin (NO−Mb) to a long-lived nitroxyl adduct.[8] In this work, we describe the characterization and unusual stability of nitroxyl myoglobin in aqueous solution, and clearly identify it as an HNO-adduct.

Voltammetry of NO−Mb adduct at high pH and in the absence of exogenous NO gave evidence of a reversible formation of a one-electron reduced product at −0.63 V vs NHE, eq 1. The potential of this reduction is at the edge of those known in biological systems, but in the range of certain highly reduced ferredoxin and siroheme proteins which are accessible in aqueous medium.[9,10] The product was postulated as a nitroxyl anion, NO^-, adduct in analogy to several such adducts formed from reductions of Fe porphyrin nitrosyl in organic solvents.[11,12] The lifetime of the nitroxyl adduct, as indicated by voltammetric reversibility, was dramatically increased at high pH in these measurements.

$$Fe^{II}-NO \underset{-\,e^-}{\overset{+\,e^-}{\rightleftharpoons}} Fe^{II}-NO^- \qquad (1)$$

The chemical reduction of solution-based NO−Mb, **1**, to HNO−Mb, **2**, was achieved using an excess of Cr^{II} reagents (as the tacn or edta complexes), and could be followed by the shift in the Soret absorbance from 421 to 423 nm, Figure 1.[13]

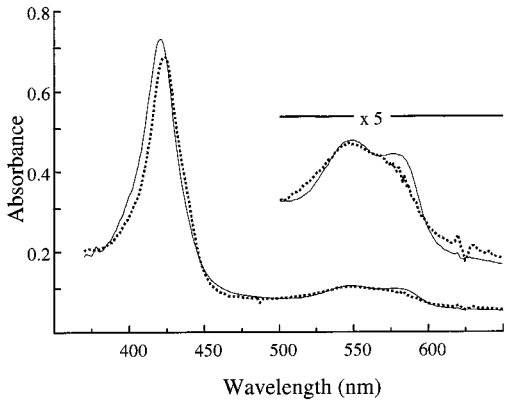

Figure 1. Absorbance spectra of NO−Mb, **1**, (solid line) and HNO−Mb, **2**, (dotted line) at ∼0.3 mM concentration in 50 mM borate buffer, pH 10.

Electrochemical reduction of **1,** using *N*-methyl-4,4′-bipyridine iodide as a mediator ($E_{1/2}^{NHE} = -800$ mV), also produces the spectral changes we attribute to **2**.[14] The reduced protein thus formed can be separated by size-exclusion chromatography to yield purified samples for analysis.

$$\underset{\mathbf{1}}{Mb-NO} + Cr^{II} \rightarrow \underset{\mathbf{2}}{Mb-HNO} \qquad (2)$$

The formation of **2** by chemical reduction is pH dependent, and only at pH > 9.5 are high yields obtained. At lower pH, the reaction is incomplete and susceptible to side reactions resulting in deoxy−Mb from ligand loss. Once formed, the nitroxyl adduct **2** is very long-lived, with a half-life greater than weeks. Purified samples of **2** may be dialyzed and the pH adjusted to 6 without a change in the apparent stability of the adduct. The reduced state of **2** is demonstrated by its titration with methyl viologen, (MV^{2+} $E_{1/2}^{NHE} = -440$ mV); reaction of **2** with a stoichiometric amount of MV^{2+} regenerates NO−Mb over several minutes (spectra are given in the Supporting Information).[15] There is no reaction between **2** and metMb over several hours. More extensive reactivity studies of **2** are underway in our labs.

Protonation of the nitroxyl was confirmed by NMR. As nitroxyl is iso-electronic with O_2, its Mb adduct may be expected to be diamagnetic; indeed, the ^1H NMR of **2** is similar to that of oxy−

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(13) (a) Nitrosyl myoglobin was generated by reducing metmyoglobin with sodium dithionite in the presence of sodium nitrite.[13b] The resulting deep red solution was purified by passing through the Sphedax G-25 size-exclusion column. The reductant Cr(II)(EDTA) solution was made by adding 25 mM EDTA solution at pH 7.0 to $CrCl_2$ anhydrous powder (Aldrich).[13c] The solution turned light blue immediately and gradually changed to purple. An excess of fresh Cr(EDTA) was added to the nitrosyl myoglobin solution at pH 10.0 to give essentially quantitative production of **2**, by UV−vis. The reduced protein was repurified through a G-25 column, and the resulting solution was concentrated down by centrifugation before spectral measurements. To generate solutions of **2** at different pH, the buffer was replaced by dilution and centrifugation with desired buffer solution. (b) Arnold, E. V.; Bohle, D. S. in *Methods in Enzymology*, Packer, L., Ed.; Academic Press: San Diego, 1996; Vol. 268, Part B, pp 41−55. (c) Crane, B. R.; Siegel, L. M.; Getzoff, E. D. *Biochemistry* **1997**, *36*, 12101.

(14) (a) Monomethyl 4,4′-bipyridine was synthesized following published procedures.[14b] For the electrochemical reduction of nitrosyl myoglobin, monomethyl 4,4′-bipyridine was used as the mediator. A three-neck UV−vis cuvette was employed with a gold working electrode, an SCE reference electrode, and a Pt counter electrode. The solution was degassed with nitrogen, and both nitrosyl myoglobin and monomethyl 4,4′-bipyridine were added. As a −750 mV vs NHE potential was imposed, the spectra change was followed by UV−vis. (b) Yonemoto, E. H.; Riley, R. L.; Kim, Y. I.; Atherton, S. J.; Schmehl, R. H.; Mallouk, T. E. *J. Am. Chem. Soc.* **1992**, *114*, 8081.

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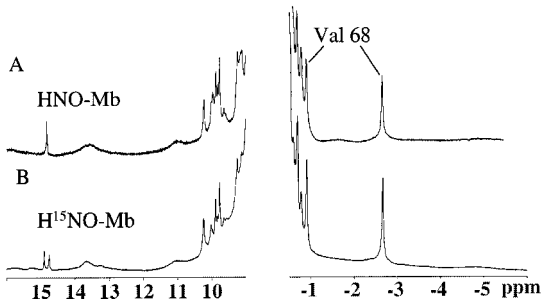
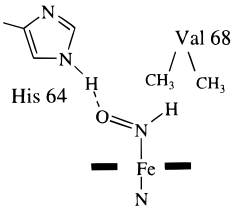


Figure 2. Water-decoupled ^{1}H NMR spectra of (A) natural abundance **2**, (B) ^{15}N-labeled **2**. Broad peaks are due to paramagnetic Mb impurities.

Scheme 1



Mb at room temperature.[16] A unique feature of the ^{1}H NMR of **2** is proton peak at 14.8 ppm, Figure 2, well-separated from the protein peaks. This peak is split into a doublet (72 Hz) in **2** formed by reduction of ^{15}N-labeled NO−Mb, consistent with protonation at the nitrogen.[17] The chemical shift of labeled **2** by ^{15}N NMR, at +788 ppm vs $^{15}NH_4^+$, is similar to RS−NO adducts as well as several $Co^{II}NO$ complexes.[18]

Although HNO has been well-studied in the gas phase,[19] very few examples of transition metal adducts have been reported.[20−22] These HNO complexes have been synthesized by oxidative addition of HCl to a metal nitrosyl,[20] or by the oxidation of a metal-bound hydroxylamine.[21,22] Characteristic of these HNO complexes is an ^{1}H NMR peak assignable to the H−(NO) at ∼20 ppm, with ^{15}N coupling ∼70 Hz in ^{15}N-labeled samples, Table 1.[22]

The stability of **2** suggests an unusual protection of the HNO adduct within the distal Mb pocket. By comparison, the half-life of an analogous one-electron reduced product generated by flash photolysis of NO−Fe(tpps) in aqueous pH 6 solution was only 2 s.[23] A possible source of the stability of **2** is direct H-bonding between the bound ligand and the distal pocket histidine, His64, analogous to that known to stabilize the dioxygen adduct.[24] To test this hypothesis, NOESY experiments were conducted on **2**, which show two cross-peaks at −0.93 and −2.67 ppm due to dipolar relaxation with the nitroxyl H (data given in the Supporting Information). In NMR spectra of oxy−Mb, these peaks have been assigned to the methyl groups of Val68, located at one side of the distal pocket. The relative peak integrals of the methyls are ∼3:1 compared to the H−(NO) peak, Figure 2.

Table 1. Characterized HNO−Metal Complexes

complex	^{1}H NMR (ppm)	J_{NH} (Hz)	ref
$Os(HNO)(CO)(PPh_3)_2Cl_2$	21.2	75	20
$Re(HNO)(CO)_3(PPh_3)_2^+$	21.7	72.5	22

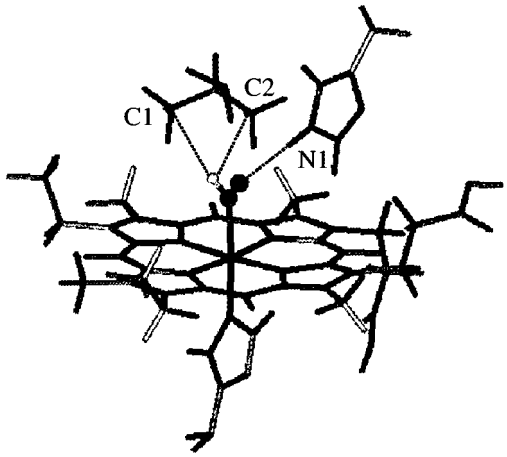


Figure 3. Modeled active-site structure of HNO−Mb, as described in the text. The large dark circle is the nitroxyl oxygen, the small white circle the nitroxyl H. The lines show nearest neighbor interactions between the HNO ligand and the protein Val62 and His64 residues. H−(NO) to the methyl carbons of Val62 distances are 2.85 A (C1) and 2.52 A (C2); (HN)−O to N1 of His64 distance is 2.88 A in this model.

Molecular modeling of **2** yielded an active-site structure that is consistent with the NOESY results, Figure 3.[25] In this model, the sp^2-hybridized N is bound to the Fe, with the nitroxyl H within 3 Å of the two Val methyl rotors. This orientation points the nitroxyl O atom toward the distal His64, and suggests a H-bonding interaction between this residue and the nitroxyl, Scheme 1. The HNO plane is at ∼85° relative to the proximal His93 plane, Figure 3, a swing of over 60° from the relative orientation in the published structures of NO−Mb.[26] The implied reorientation of the NO moiety upon reduction is perhaps driven by the π back-bonding competition between the two ligands, in conjunction with H-bonding and steric interactions.

In conclusion, we have described a very rare example of a stable HNO metalloprotein adduct, and as such a potential source of free nitroxyl for chemical and biochemical studies. We also believe it to be of possible physiological importance in relevance to the action of the various nitric oxide synthase enzymes. Further experiments are underway to elucidate the active-site structure of **2**, as well as the source of its stability.

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Supporting Information Available: Absorbance spectra illustrating the titration of HNO-Mb with methyl viologen, and the 2D NOESY spectra of HNO-Mb (PDF). This material is available free of charge via the Internet at http:/pubs.acs.org.

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(15) A methyl viologen dichloride (Aldrich) solution was made by dissolving 3 mg of methyl viologen in pH 10.0 buffer, and then it was added by aliquots to the solution of **2** in a gastight UV−vis cell. Resulting spectra are given in the Supporting Information.

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