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Patrick J. Farmer[a]; Mugaresson R. Kumar[a]; Elky Almaraz[a]
[a] Department of Chemistry and Biochemistry, Baylor University, Waco, TX, USA

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THE COORDINATION CHEMISTRY OF HNO: FROM WARREN ROPER TO HEMOGLOBIN

PATRICK J. FARMER, with MUGARESSON R. KUMAR, and ELKY ALMARAZ

Department of Chemistry and Biochemistry, Baylor University, Waco, TX, USA

The coordination chemistry of the one-electron reduced form of nitric oxide, termed a nitroxyl or nitrosyl hydride (NO- or HNO), is described anecdotally through the author's discovery of a stable HNO adduct of myoglobin, HNO-Mb, as an illustration of how different areas of chemistry, such as organometallic and bioinorganic, overlap and influence one another in unexpected ways. HNO is short-lived in solution, and many fundamental characteristics of HNO, such as the widely cited values of its oxidation potential and pKa, have been significantly revised through collaborations of theoreticians, biologists, and physical chemists. The very first HNO metal complex was discovered in Warren Ropers lab in 1970, with some 27 years before the next fully characterized example was reported by Hillhouse. Hillhouse in turn helped guide our characterization of HNO-Mb. As with oxymyoglobin, there are several possible descriptions of bonding for HNO-adducts, we present an analogy to the π-bonding interactions of a Fischer carbene. Roper's initial work demonstrated the importance of redox and protonation equilibria in these species, a current focus of ongoing work.

***Keywords*: carbine, electrochemistry, hemoglobin, HNO, hydrogen bonding, myoglobin, nitrosyl hydride, nitroxyl, NMR**

Address correspondence to Patrick J. Farmer, Department of Chemistry and Biochemistry, Baylor University, Sciences Building, One Bear Place, Waco, TX 97348, USA. E-mail: patrick_farmer@baylor.edu

F. Gordon Stone was instrumental in my recruitment, and ultimately the move of my lab from UC Irvine to Baylor University in the summer of 2009. I had first met FGAS two years before, and he impressed me with his dedication to the sciences at Baylor and by his strong support of younger colleagues. After 20 years at Baylor, the legacy of the man and his work at Baylor will be difficult to replace. For example, Gordon has been a determined voice against specialization within the department, saying that we must strive for excellence across the board. His point being that all disciplines contribute to the educational training of our students and the advancement of our research efforts. My own group's work on the coordination chemistry of HNO is illustrative of how different areas of chemistry, such as organometallic and bioinorganic, overlap in unexpected ways and also of how advances in one area may influence another.

HNO is a very simple molecule, often termed nitroxyl with its conjugate anion NO^-, but recent reports have shown it to have unique physiological effects that have led to much new interest. Of course, its redox congener NO also has a rich coordination chemistry that was well developed before its importance in biology and physiology was known. The recent discoveries on HNO complexation finds a similar interplay between the wet protein biochemists and the more austere inert-atmosphere organometallickers, somewhat complicated by the redox ambiguities of NO_x species, represented here in an anecdotal account.

REDOX CHEMISTRY

Our interest in HNO chemistry resulted from a project to investigate the reductive activation of nitrite and sulfite by heme proteins electrochemically, to evaluate redox mechanisms and sequential steps involved. We found that electrodes modified with myoglobin/surfactant films electrocatalytically reduced nitrite to a mixture of products, predominantly NH_3, N_2O and N_2,[1] and subsequently that a thermophilic cytochrome P450 produced NH_3 almost exclusively.[2,3] In both cases voltammograms of these electrodes in the presence of nitrite demonstrated sequential catalytic waves, which correlated to the production of N_2O and NH_3, related to the two enzymatic pathways for nitrite's conversion in plants and bacteria, the assimilatory that produces NH_3 and the dissimilatory that produces NO and ultimately N_2O.

We first identified the nitroxyl state in native myoglobin by fast scan voltammetry ($>1\,V/s$) under NO gas, which showed the kinetic

fingerprint of a reversible reduction of NO-Mb.[4] The same fingerprint was seen after several reductive scans in nitrite solution. The reversibility decreased with the concentration of H^+ and NO, implying that these species reacted with the Fe-bound nitroxyl.

To examine a *single-turnover* reduction of NO-Mb in the absence of exogenous NO, we fabricated electrodes directly from pre-formed NO-Mb in an anaerobic glovebox. Our expectations were that each fabricated electrode would yield only one or two scans before the NO_x ligand was lost, and therefore many electrodes were prepared for the initial experiments. Surprisingly, a reversible and reproducible single-electron reduction was obtained (Figure 1). The singly reduced product was long-lived at pH 10, as demonstrated by the reversibility at slow scan rate in Figure 1, but the lifetime of the product diminished at lower pH. At pH 7 the lifetime was determined by digital simulation to be on the order of a tenth of a second. In 1997, I presented these results at a national ACS meeting in Las Vegas, and David Stanbury, a physical inorganic chemist from Auburn, approached me afterward: "Why is the potential of nitric oxide so much lower when it is bound to an Fe^{2+} than when it is free in solution?"

At the time, there was much confusion and misinformation about the oxidation potential and pKa of NO^-. Experimental reports of the reduction potential of NO gave values scattered over a range of ca. $+0.4$ to -0.9 V NHE,[5] which led to much speculation in the medical literature concerning possible generation and reactivity of nitroxyl in vivo. Stanbury, who had previously reviewed the thermochemical data related to NO,[6] could not understand why the reduction potential should decrease so dramatically in complex with ferrous deoxyMb.

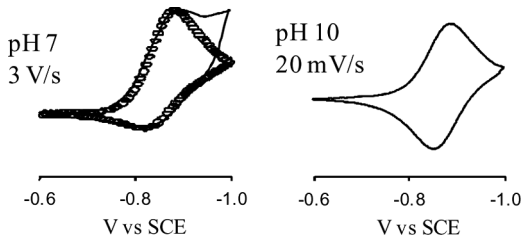


Figure 1. Voltammograms of NO-Mb/DDAB films in different pH solutions; circles represent simulation used to derive lifetime of reduced species. Figure adapted.[4]

In collaboration with Jon Fukuto and Ken Houk at UCLA, we reinvestigated the reduction of NO both experimentally and theoretically.[7] We demonstrated that under equivalent conditions, the reduction of NO occurred at significantly lower potential than O_2 (−330 mV) at pH 7, and using a series of low potential viologens as redox indicators, we boxed in the reducing ability of nitroxyl releasing compounds methylsulfohydroxamic acid (CH_3SO_2NHOH, MSHA) and disodium trioxodinitrate ($Na_2N_2O_3$, Angeli's salt). Interpretation of such behavior is complicated by the rapid dimerization of nitroxyl, which limits the concentration and lifetime of nitroxyl in solution, but gave qualitative proof of the reducing potential of the short lived species.

$$HN_2O_3^- \rightleftarrows HNO + NO_2^- \quad pH\ 4\ to\ 8 \tag{1}$$

$$MeSO_2NHO^- \rightleftarrows MeSO_2^- + HNO \quad pH\ 8\ to\ 13 \tag{2}$$

$$2\ HNO \rightarrow [H_2N_2O_2] \rightarrow (N_2O_{(g)} + H_2O \tag{3}$$

Ultimately, the theoretical and experimental data pointed to a potential ca. −0.8 V (at 1 M vs. NHE) for the reduction of NO to nitroxyl anion ($^3NO^-$). Likewise, the pK_a of $^3NO^-$ was revised from a previously quoted value of 4.7[8] to above 11.5.[9] This results from the change in ground-state spin from the neutral nitrosyl hydride, ^1HNO, to the nitroxyl anion, $^3NO^-$. Therefore, upon deprotonation of HNO it must undergo a spin flip to avoid a high energy intermediate.[10] Thus nitric oxide is much harder to reduce than molecular oxygen, and consequently nitroxyl generation in much less likely in a physiological setting.

HNO-Mb

Concurrently, we had been working with the late Barbara Burgess on characterizing an unusual all-ferrous oxidation state of a four-Fe ferrodoxin termed "the Fe protein,"[11] which is the electron donor to the famous MoFe nitrogenase from Azotobacter vinelandii.[12] The question driving our collaboration was whether this all-ferrous state was biologically feasible; our redox titration analysis put the reduction potential at ca. −650 mV NHE, on the edge of what may be considered biologically attainable. In order to generate bulk samples of this species, we resorted to a variety of powerful inorganic reductants such as Ti(II) and Cr(II) salts as well as the low potential viologen radicals. By happenstance, the reduction potential of the all-ferrous Fe protein was close to that

of our "nitroxyl" myoglobin, and we used these same reagents to generate nitroxyl myoglobin in bulk solution (Equation (4)).[13] This species was best generated at high pH but once formed it seemed quite stable, with a half-life greater than weeks.

$$\text{NO-Mb} + \text{X} + \text{H}^+ \rightarrow \text{HNO-Mb} + \text{X}^+ \quad (4)$$

At this time I attended a Gordon conference in which Greg Hillhouse from U. Chicago described a new Re nitroxyl complex synthesized in his lab, $Re(Cl)(CO)_2(HNO)(PPh_3)_2$, only the second such species crystallographically characterized.[14] These species, formally HNO adducts, were quite air sensitive and reactive, disproportionating in solution over time. After his talk, I raised my hand and noted that we had generated the equivalent "nitroxyl" adduct of Mb, which was stable in aqueous solution for weeks. Hillhouse told me emphatically: "Take an NMR!" He said that the handful of reported HNO complexes were formally low-spin d^6 diamagnetic metal ions, and all displayed a characteristic ^{1}H NMR nitrosyl hydride signal at ca. 20 ppm.[15] It took a few months to convince both students and our NMR facility, but we did indeed find a unique proton peak at 14.8 ppm in the ^{1}H NMR spectra of "nitroxyl-myoglobin" samples, which splits into a doublet ($J_{NH} = 72$ Hz) in samples made from ^{15}NO-Mb, consistent with protonation at the nitrogen.

The nitrosyl hydride peak is in a relatively clean region of the ^{1}H NMR spectra of proteins, and has subsequently proven to be a very valuable method in following the generation of HNO-Mb,[16] and in characterizing such adducts in species including leghemoglobin, human and clam hemoglobins, and the heme oxygenases.[17] With Gerd La Mar at UC Davis, we used NOESY and COSY experiments on HNO-Mb (Figure 2) to determine that the HNO had a fixed orientation, perpendicular to the trans proximal histidine, likely facilitated by hydrogen bonding between the nitroxyl oxygen and the distal histidine.[18]

Our first presentation of synthesis and NMR spectra of HNO-Mb was at a local ACS meeting in Ontario, CA. After my talk, Chris Reed from USC stood and made a cryptic comment: "I'd like to point out that the first HNO metal complex was made by a New Zealander!" Chris was a friend and neighbor in Irvine, the husband of my collaborator Barbara Burgess, and not typically so laconic. It was the following week, in preparation for our communication on this work, that I noticed his name, C.A. Reed, as third author on the first report of an HNO complex from Warren Roper's lab in 1970![19]

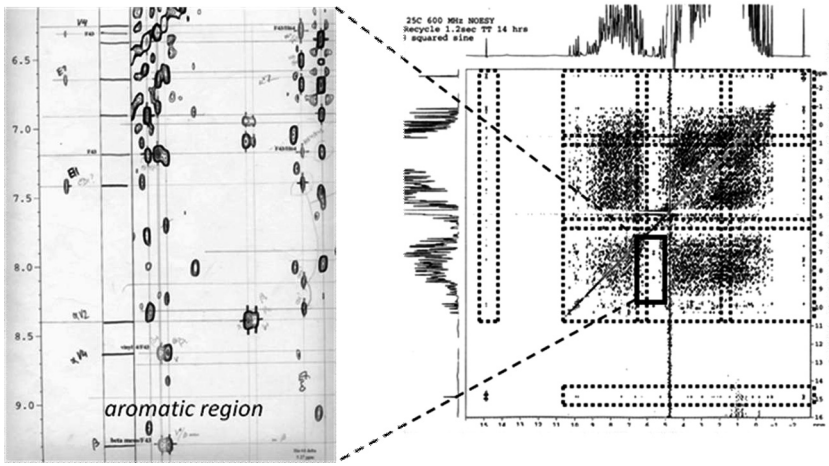


Figure 2. 2D ^{1}H NOESY NMR spectra of HNO-Mb with blowup of aromatic region used in assignment of the mesoprotons on the heme edge.

HNO COMPLEXES

As Tony Hill pointed out during the Stone Symposium, Roper's work was ahead of its time in many ways. His 1970 communication suggested an HNO adduct as an intermediate in the conversion of $Ir(NO)(PPh_3)_3$ to $IrCl_3(NH_2OH)(PPh_3)_2$ in reaction with excess HCl[19]; stoichiometric addition of HCl to the analogous $OsCl(CO)(NO)(PPh_3)_2$ generated $OsCl_2(CO)(HNO)(PPh_3)_2$, characterized by a ν_{NO} stretch at 1410 cm^{-1}. Ibers confirmed this formulation crystallographically in 1980,[20] and first identified the nitrosyl hydride by a ^{1}H NMR absorbance at 21.1 ppm, split into a doublet when containing ^{15}N with an ^{15}N-^{1}H ($^{1}J_{NH}$) coupling of 75 Hz. The crystal structure shows a long N–O bond, at 1.193 Å, as well as a short N–H bond at 0.94 Å as compared to that in free HNO at 1.026 Å, all indicative of strong backbonding interactions.

Subsequently, many different routes to HNO complexes have been found (Figure 3). In the 1990s, Hillhouse investigated complexes first identified by Roper, $IrHCl_2(HNO)(PPh_3)_2$, and La Monica, $Re(Cl)(CO)_2(HNO)(PPh_3)_2$.[21,14] His group generated the cationic $[Re(CO)_3(HNO)(PPh_3)_2]^+$ by two routes: first by oxidizing a hydroxylamine adduct $[Re(CO)_3(NH_2OH)(PPh_3)_2]^+$ with $Pb(OAc)_4$;[14] then by of NO^+ insertion into metal-hydride bond in $ReH(CO)_3(PPh_3)_2$.[22] Sellman reported the first ruthenium nitroxyl complex, [Ru(HNO)-('py^{bu}S$_4$')],[23] prepared by

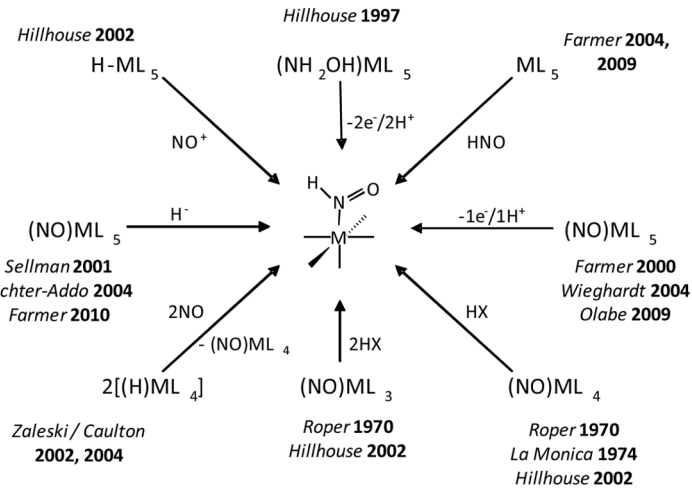


Figure 3. Synthetic routes to HNO-metal ion complexes. Figure adapted.[16]

addition of $NaBH_4$ to a cationic octahedral nitrosyl complex, [Ru(NO)-('$py^{bu}S_4$'). Caulton and Zaleski identified similar adducts $MHCl(HNO)(CO)(P^iPr_3)_2$(M = Ru, Os), during reaction of NO with a five coordinate metal hydride, $MHCl(CO)(P^iPr_3)_2$.[24] Richter-Addo's group synthesized the first metalloporphyrin HNO adduct, in Ru(HNO)-TPPy by hydride addition to a cationic Ru(II) nitrosyl;[25] the observed ^1H NMR nitrosyl hydride resonance of this complex at 13.64 ppm is comparable to that seen for HNO-Mb. Olabe demonstrated that reduction of nitroprusside produces a stable anionic $Fe^{II}(CN)_5(HNO)^{3-}$,[26] showing that all three redox states of nitrosyl (NO^+, NO, and NO-/HNO) can be obtained reversibly for on the nitroprusside platform. Doctorovich has generated an isolable anionic nitroxyl adduct $Fe^{II}(TFPPBr_8)NO^-$ by reduction of the ferrous porphyrin nitrosyl; perbromination of the porphyrin effectively stabilizes the reduced form, which is suggested as a low spin Fe^I antiferromagnetically coupled to the coordinated NO.[27]

$$Fe^{II}\text{-globin} + HNO \rightarrow HNO\text{-globin} \quad (5)$$

Only Fe^{II}Mb and ferrous states of other oxygen-binding proteins, such as hemoglobin (Hb), leghemoglobin (legHb), and an H_2S-binding hemoglobin from the clam *L. pectinata*, have been shown to trap free HNO in solution to form HNO-adducts (Equation (5)).[15] The trapping of HNO by tetrameric human hemoglobin is particularly interesting. In

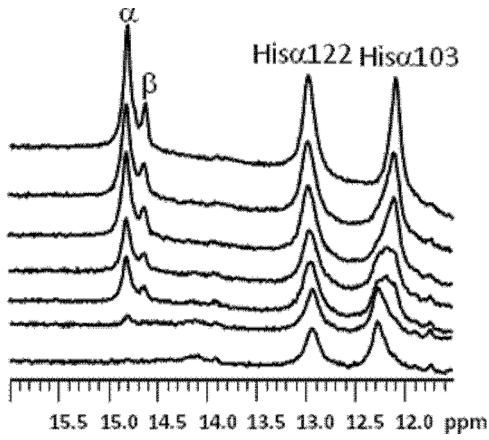


Figure 4. HNO-Hb generated by reaction of MSHA with deixtHb (23:1) at pH 9 and 25°C, spectra taken at hour intervals over 16 hours. Figure adapted.[15]

our recent report on HNO trapping by globins, we reported two broad resonances between 12 and 13 ppm are observed in the ^{1}HNMR of HNO-Hb due to protons at the α/β interface (His122 and His103) that form strong H-bonds between subunits (Figure 4). Shifts of these ^{1}H NMR signals have been linked to transitions between tensed and relaxed allosteric states.[28] Such shifts are seen in time course spectra obtained during HNO trapping by deoxyHb, using the donor MSHA which generates HNO at a slow rate (Figure 4). The two nitrosyl hydride peaks have a persistent 3:1 ratio during the trapping reaction, suggesting a kinetic binding difference between these sites.

BONDING IN Mb-HNO

Insight into bonding parameters and structure of HNO-Mb was obtained by collaborations with David Bocian at UC Riverside on resonance Raman vibrational spectra, and with Peter Lay at the University of Sidney on X-ray absorption (Fe K-edge).[29] On first glance, the data obtained data was as anticipated (Table 1): a decrease in N−O stretching frequency from 1617 cm^{-1} in Mb-NO to 1385 cm^{-1} in Mb-HNO is consistent with the formally-ligand based reduction, and the corresponding decrease as compared to free HNO at 1510 cm^{-1} demonstrates that the HNO ligand acts as a strong π-acceptor in complex with Fe^{II}. The EXAFS structure, which orients the HNO perpendicular to the distal

Table 1. Bonding parameters for Mb-HNO obtained from X-ray absorbance and resonance Raman spectroscopies

XAFS/Raman	$Mb^{II}HNO^a$	$Mb^{II}NO^b$	$Mb^{III}NO^b$
Fe-N (por), Å	2.00	1.99	2.00
Fe-N (His), Å	2.09	2.05	2.04
Fe-N (xNO), Å	1.82	1.76	1.68
Edge Energy, eV	7122.5	7124.7	7125.4
N−O, Å	1.24	1.12	1.13
Fe-N-O, deg	131	150	180
$\nu_{N\text{-}O}$ cm^{-1}	1385	1613	1927
$\nu_{Fe\text{-}N}$ cm^{-1}	651	551	595

[a]Data from[27].
[b]XAFS data from,[30] IR data from[19].

His trans to it, was fully consistent with the ^1H NMR structure. Less expected was the marked increase in Fe−N stretching frequency from 554 cm^{-1} to 651 cm^{-1}, suggesting an increase in the Fe−N bond strength that was at odds with the EXAFS best-fit model showing a lengthened Fe−N bond. Even more unexpected was the difference of over 2 eV in ionization edge energy from XANES measurements of NO-Mb and HNO-Mb, implying a significant increase in electron density at the Fe in the nitroxyl adduct. This led to the suggestion of an Fe^I character to HNO-Mb, which caused quite a debate within our collaboration.

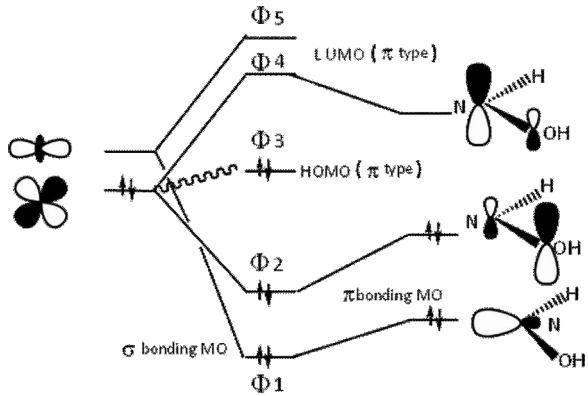


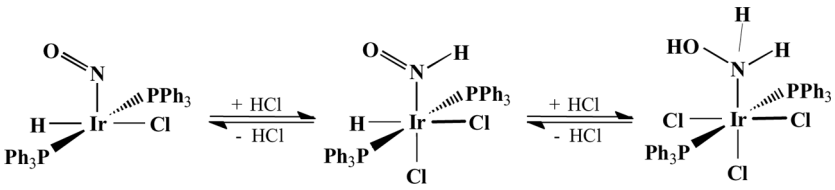
Figure 5. Molecular orbital diagram suggesting a 3 center, 4 electron π-interactions in M−HNO bonding.

We preferred the analogy of HNO complexes to the electrophilic Fischer carbenes, derived from the effect of a beta-oxygen on the metal-ligand bond (Figure 5).[31] As with HNO complexes, the stable Fischer carbenes are formed with d^6 metal ions. Like the Fischer carbenes, protonation at the terminal oxygen of a metal-bound HNO should lead to a formal reorganization of the bonding: weakening the N–O bond, as observed in νNO of [Ru(HNO)-('py^{bu}S$_4$')],[23] and a corresponding strengthening of the M–N bond. Such an effect may explain the anomalously high Fe–N bond stretch of Mb–HNO and the comparatively low XANES oxidation energy; strong hydrogen-bonding to the distal histidine might induce such an internal reorganization, and like the alkoxycarbenes, increase a charge buildup on the metal.

PROTONATION EQUILIBRIA

The dramatic effect of protonation on metal nitrosyls was demonstrated the Roper's initial reports of the reversible conversion of $Ir(NO)(PPh_3)_3$ to $IrCl_3(NH_2OH)(PPh_3)_2$ with excess HCl: reversible conversions between the NO, HNO, and NH_2OH adducts were induced by treatment with acid and base (Scheme 1). In a similar way, Hillhouse attempted to deprotonate $Re(Cl)(CO)_2(HNO)(PPh_3)_2$ by reactions with strong Brønsted bases, but this resulted in dehydrohalogenation (loss of HX), not deprotonation. We have also been unable to characterize the deprotonated form of HNO-Mb; while changes in the electronic spectra suggestive of deprotonation are seen in samples of HNO-Fe^{II}Mb at pH above 10, the protein is not stable under these conditions.[32]

Spiro has recently suggested that hydrogen-bonding to the nitrogen or oxygen atoms of a coordinated nitrosyl changes the effective electronic states of such adducts of heme proteins.[25] Hydrogen bonding at the oxygen strengthens back bonding with the metal, as in the Fischer carbenes, but hydrogen-bonding to the nitrogen weakens



Scheme 1. Protonation/redox equilibria.

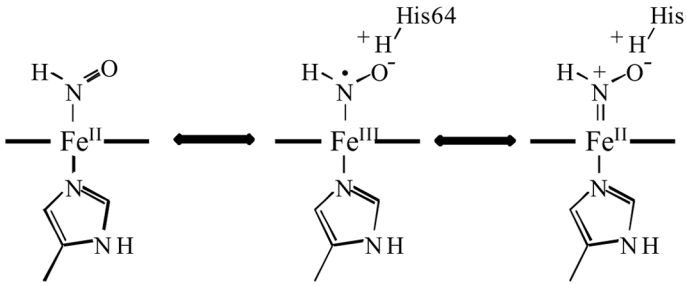


Figure 6. Possible resonance states associated with H-bonding within distal pocket of HNO-Mb.

both the Fe–N and N–O bonds, thus may facilitate reduction to the HNO-Fe^{II} state.

H-bonding interactions with the distal His in HNO-Mb may likewise promote charge buildup on the Fe moiety (Figure 6). Protonation at the O would stabilize the resonance form with full charge delocalized onto the ligand, i.e., an Fe^{III}-HNO^{-}; alternatively, true covalency within the Fe–N bond would suggest a cationic buildup on the N, as in the Fischer carbenes. We do have unexpected evidence of such resonance states: photolysis of HNO-Mb generates transient Fe^{III}-Mb and presumably HNO^{-}, which then recombine on the microsecond time scale (Figure 7).[33] The transient generation of Fe^{III}-Mb was quite unexpected, confirming experiments were done in the photochemical labs of Harry Gray at Caltech and Doug Magde at UCSD. The photogenerated byproduct H_2NO is another rare NO_x species, the aminoxyl radical; we have further evidence of its generation in other reactions of HNO-globins.[17]

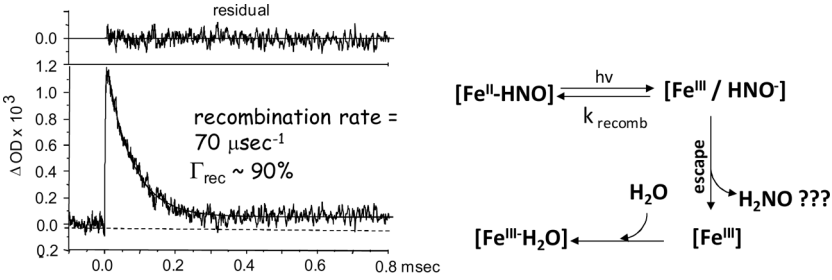


Figure 7. Left: a fit of transient absorbance traces at 395 nm of 4 uM MbHNO after excitation at 532 nm; right: proposed photolysis reaction sequence.

CONCLUSION

As my account here illustrates, the interactions between the various chemical disciplines and between the scientists themselves play an important role in the progress of science. As Gordon likes to say, "a strong tide lifts all boats"; we need strength in all areas to truly have strength in any. F. Gordon Stone was certainly a strong tide, as seen by his influence on several generations of chemists; his influence will live on in the body of work he has produced, as well as in the department and all his colleagues here at Baylor.

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