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Heme-Nitrosyls: Electronic Structure Implications for Function in Biology

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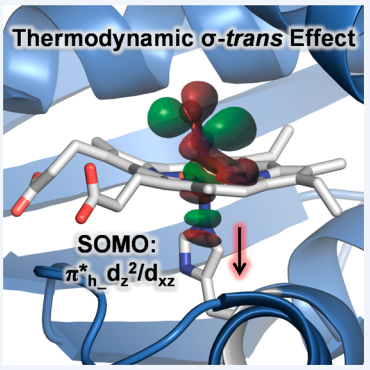
CONSPECTUS: The question of why mammalian systems use nitric oxide (NO), a potentially hazardous and toxic diatomic, as a signaling molecule to mediate important functions such as vasodilation (blood pressure control) and nerve signal transduction initially perplexed researchers when this discovery was made in the 1980s. Through extensive research over the past two decades, it is now well rationalized why NO is used in vivo for these signaling functions, and that heme proteins play a dominant role in NO signaling in mammals. Key insight into the properties of heme-nitrosyl complexes that make heme proteins so well poised to take full advantage of the unique properties of NO has come from in-depth structural, spectroscopic, and theoretical studies on ferrous and ferric heme-nitrosyls. This Account highlights recent findings that have led to greater understanding of the electronic structures of heme-nitrosyls, and the contributions that model complex studies have made to elucidate Fe−NO bonding are highlighted. These results are then discussed in the context of the biological functions of heme-nitrosyls, in particular in soluble guanylate cyclase (sGC; NO signaling), nitrophorins (NO transport), and NO-producing enzymes.



Central to this Account is the thermodynamic *σ-trans* effect of NO, and how this relates to the activation of the universal mammalian NO sensor sGC, which uses a ferrous heme as the high affinity "NO detection unit". It is shown via detailed spectroscopic and computational studies that the strong and very covalent Fe(II)−NO *σ*-bond is at the heart of the strong thermodynamic *σ-trans* effect of NO, which greatly weakens the proximal Fe−N_{His} (or Fe−S_{Cys}) bond in six-coordinate ferrous heme-nitrosyls. In sGC, this causes the dissociation of the proximally bound histidine ligand upon NO binding to the ferrous heme, inducing a significant conformational change that activates the sGC catalytic domain for the production of cGMP. This, in turn, leads to vasodilation and nerve signal transduction. Studies on ferrous heme-nitrosyl model complexes have allowed for a quantification of this thermodynamic *σ-trans* effect of NO, through the use of high-resolution crystal structures, binding constant studies, single-crystal vibrational spectroscopy and density functional theory (DFT) calculations. These studies have further identified the singly occupied molecular orbital (SOMO) of the NO complexes as the key MO that mediates the thermodynamic *σ-trans* effect of NO.

In comparison to ferrous heme-nitrosyls, ferric heme-nitrosyls display thermodynamically much weaker Fe−NO bonds (from NO binding constants), but *at the same time* much stronger Fe−NO bonds in their ground states (from vibrational spectroscopy). Using spectroscopic investigations coupled to DFT calculations, this apparent contradiction has been rationalized with the involvement of at least three different electronic states in the binding/dissociation of NO to/from ferric hemes. This is of key significance for the release of NO from NO-producing enzymes like NOS, and further forms the basis for ferric hemes to serve as NO transporters in biological systems.

1. INTRODUCTION

For the longest time, nitric oxide (NO) had a rather bad reputation due to its contributions to smog and its highly toxic nature. This, however, changed dramatically in the 1980s when it was realized that NO is an endogenously produced molecule in mammals that is involved in signaling in the cardiovascular system and in the brain.[1] In addition, mammals make use of the fact that NO is toxic to microbes at micromolar concentrations and use it for immune defense. Other important functions of NO include the inhibition of platelet aggregation in blood. Hence, NO has morphed its reputation from being solely a noxious molecule and became a subject of primary interest for biomedical research and bioinorganic chemistry.

Importantly, the production, detection, transport, and detoxification of NO in biological systems are all strongly dependent on metalloproteins, especially those containing iron.[2−6] Other metal centers that interact with NO are copper (especially in copper-containing nitrite reductase[7]) and cobalt (in cobalamines). Ruthenium and manganese complexes have been developed as NO-releasing molecules for applications as antimicrobials and in cancer therapy.[8] For signaling and immune defense in mammals, NO is generated by the two-step oxidation of L-arginine to NO and citrulline, which is

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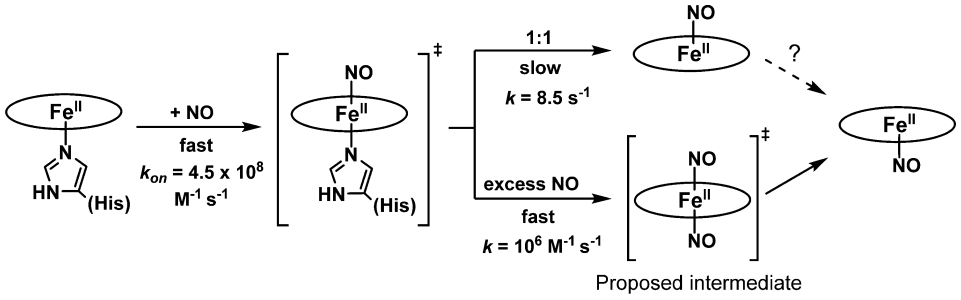
Scheme 1. Proposed Mechanism for sGC Activation by NO



mediated by the nitric oxide synthase (NOS) isozymes.[3] These enzymes are relatives of the cytochrome P450 superfamily, both containing heme-thiolate active sites. Another proposed pathway for NO generation in mammals uses nitrite as a biological pool for NO.[9] Once NO is generated in the cardiovascular system or the brain, it is detected by the universal mammalian NO sensor, soluble guanylate cyclase (sGC), which uses a ferrous heme as a high affinity NO sensor.[10] Interestingly, gene sequences homologous to those of sGC's sensor domain are also widespread among prokaryotes. These "H-NOX" (heme-nitric oxide/oxygen binding) domains usually sense NO or O_2.[11] Other NO sensor proteins in bacteria contain either non-heme iron centers[12] or iron−sulfur clusters.[13]

The transport of NO in the saliva of blood-sucking insects is mediated by another class of heme proteins, called nitrophorins (Np).[3,14] These proteins transport heme-bound NO in their ferric oxidation state, to allow for facile NO release once the Np is injected into the victim's blood.

The detoxification of NO in biological systems is again catalyzed by iron-containing enzymes. Under aerobic conditions, mammals degrade NO by reaction with oxy-Hb/Mb,[15] generating nitrate. Under anaerobic conditions, prokaryotes reduce NO to less toxic nitrous oxide (N_2O) by NO reductases that use heme and nonheme iron active sites.[16]

Due to this paramount significance of transition metal-NO interactions for the biologically relevant chemistry of NO, much work has been devoted to better understand the electronic structures of transition-metal−NO complexes, and the implications thereof for the functions of NO in biology. This Account focuses on the electronic structure of heme-nitrosyls, and particularly the contributions that model complex studies have made to elucidate Fe−NO bonding in these systems. Central to this paper are the thermodynamic *σ-trans* effect of NO, and how this relates to the activation of the mammalian NO sensor sGC, and the electronic structure of ferric heme-nitrosyls, which is of key importance for the ability of ferric hemes to transport and release NO.

2. ACTIVATION OF SOLUBLE GUANYLATE CYCLASE (sGC): THE THERMODYNAMIC *σ-trans* EFFECT OF NO

sGC is the primary NO sensor protein in mammals and a key component in the NO-signaling pathway.[3,10] For example, this enzyme is involved in vasodilation (blood pressure control) and nerve signal transduction in the brain. sGC is a heterodimer,[17] with $\alpha1\beta1$ being most common. Different domains of sGC have been structurally characterized, but the crystal structure of the complete protein is lacking. Insight into the flexibility of sGC's heterodimeric structure has been obtained recently from cryo-electron microscopy.[18] The β-subunit of sGC contains an H-NOX domain with a five-coordinate (5C) ferrous heme with proximal histidine coordination as the primary NO sensor.[19] The catalytic domain of sGC takes GTP to 3′,5′-cyclic GMP (cGMP) and pyrophosphate when NO is bound at the sensor unit. cGMP then serves as a secondary messenger that, for example, induces the relaxation of the arterial smooth muscle tissue, and in this way, induces vasodilation.

Kinetic experiments have shown that the interaction of NO with sGC involves a two-state activation process. NO binding to the 5C ferrous sensory heme of sGC is fast ($k_{on} = 4.5 \times 10^8$ M^{-1} s^{-1}),[20,21] leading to the formation of a transient six-coordinate (6C) NO complex (Scheme 1). The thermodynamic *trans* effect of NO then induces breaking of the Fe−N_{His} bond, generating a corresponding 5C ferrous heme-nitrosyl complex. Interestingly, this step is dependent on NO concentration, and proceeds slowly with stoichiometric NO (1:1 ratio of NO to sGC; $k = 8.5$ s^{-1}) and very rapidly in the presence of excess NO ($k = 10^6$ M^{-1} s^{-1}),[21] potentially via a dinitrosyl intermediate, generating a 5C complex with NO in the proximal site (Scheme 1). NO binding to the heme is communicated to the cyclase domain of sGC via a significant conformational change that is induced by the breaking of the Fe−N_{His} bond and that is accompanied by heme flattening.[22] This leads to activation of the cyclase catalytic domain and production of cGMP.

sGC shows two different levels of activation, tonic and acute, which depends not only on the concentration of NO, but also on the presence of GTP and ATP, which complicates our detailed mechanistic understanding of sGC activation by NO in vivo.[10,23] One activation state is low, but stable, and clearly linked to NO binding to the heme at low NO concentrations (10−20% activity for cGMP production). At higher levels of NO, a more transient state is observed where the cyclase is fully activated (100%). Correspondingly, Marletta and co-workers concluded that NO binding to heme is insufficient for full sGC activation, and that a second nonmetallo binding site must exist, which is presumed to be cysteine-based.[10]

The Thermodynamic *σ-trans* Effect of NO

Based on these results, a central signal of NO binding in sGC results from the breaking of the proximal Fe−N_{His} bond.[10] It was early recognized that this is due to a *trans* effect of NO,[24] but the exact nature of the *trans* effect and how it relates to the electronic structure of ferrous heme-nitrosyls was not known. Detailed structural and spectroscopic studies on corresponding model systems, coupled to quantum-chemical calculations, were of key importance in understanding the nature of this phenomenon.[25] Model systems allow for the study of high-resolution crystal structures, binding constants, and single-crystal spectroscopy in a straightforward way. Synthetic models

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can also be designed to directly probe different aspects of the Fe−NO bond in ways that would be difficult to accomplish with proteins alone.

The most straightforward way to probe the *trans* effect of NO is to measure binding constants of N-donor ligands, especially imidazole (Im) or pyridine (Py) derivatives, to 5C ferrous heme-nitrosyl model complexes:

$$[\mathrm{Fe(Porph)(NO)}] + \text{N-donor} \rightleftarrows [\mathrm{Fe(Porph)(N\text{-}donor)(NO)}] \quad (1)$$

With tetraphenylporphyrin ($Porph^{2-}$ = TPP^{2-}) and 1-methylimidazole (MI) as the N-donor, the binding constant K_{eq} of MI to [Fe(TPP)(MI)] is 7.8×10^4 M^{-1},[26] whereas for [Fe(TPP)(NO)] the MI binding constant is only 26 M^{-1}.[27] N-donor binding constants of 1−100 M^{-1}, corresponding to free binding energies ΔG° of −1 to −2 kcal/mol, are typical for ferrous heme-nitrosyls and demonstrate that the *trans* effect of NO actually corresponds to a ground state property and, hence, should be called a thermodynamic *trans* effect, or a *trans* interaction between NO and the N-donor ligand. High-resolution crystal structures directly support this notion, showing greatly elongated Fe−(N-donor) bond lengths trans to NO.[4] For example, in [Fe(TPP)(MI)(NO)], the Fe−N_{MI} distance is 2.17−2.19 Å,[4] whereas in [Fe(TPP)$(MI)_2$] the Fe−N_{MI} distance is only 2.01 Å.[28]

Accordingly, the binding of NO to a ferrous heme active site in a protein leads to a great weakening of the axial Fe−N_{His} or Fe−S_{Cys} (in Cytochrome P450s and NOS) bonds, and in many cases 5C ferrous heme nitrosyls are formed. This is not only the case in sGC, but also deoxy α-Hb, cytochrome c', the CO sensor CooA, and certain nNOS mutants.[29] These observations highlight the difficulty of keeping an axial donor ligand bound to a ferrous heme-nitrosyl. Correspondingly, it is difficult to design a model complex with an imidazole or pyridine "tail" that remains 6C upon NO binding. Figure 1 shows a selection of tailed porphyrins that systematically probe the effect of chain length and rigidity on the binding affinity of the linker to the corresponding Fe(II)−NO heme complexes.[30] The binding of the tethered nitrogen ligand can be monitored with different spectroscopic methods, as 5C and 6C ferrous heme-nitrosyls clearly differ in their spectroscopic properties.[3,4,25] 5C ferrous heme-nitrosyls with TPP^{2-}-type coligands show Soret bands around 400−405 nm, rhombic electron paramagnetic resonance (EPR) spectra with clearly resolved three-line hyperfine splittings on the minimum g-value, and N−O stretching frequencies around 1680−1700 cm^{-1}. In contrast, the analogous 6C complexes show Soret bands around 420−425 nm, rhombic EPR spectra with a nine-line hyperfine splitting on the medium g-value, and N−O stretching frequencies around 1620−1630 cm^{-1}. Both the N−O stretching frequencies and the imidazole ^{14}N-hyperfine coupling constants (from EPR) show a good correlation with the Fe−(N-donor) bond strengths in these systems.[30] The complexes with covalently attached pyridyl and alkyl-imidazole linkers only exhibit weak interactions of the nitrogen ligands with the iron(II)−NO unit. However, the stable six-coordinate complex [Fe(T*o*-F_2PP-BzIM)(NO)] is obtained when a rigid benzyl-linker is applied, which fixes the proximal imidazole ligand in trans position to NO. This is evident from the absorption and EPR data of this complex (Figure 2). Figure 3 shows a crystal structure of the

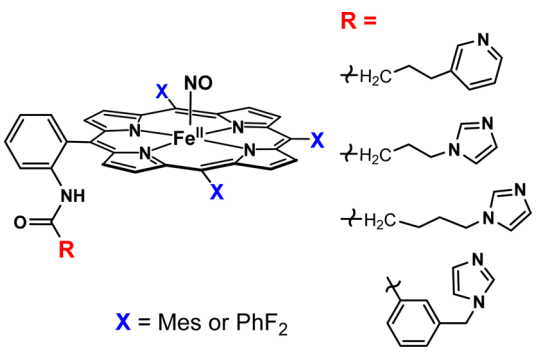

Figure 1. Iron(II)−porphyrin NO complex with covalently attached N-donor ligands.

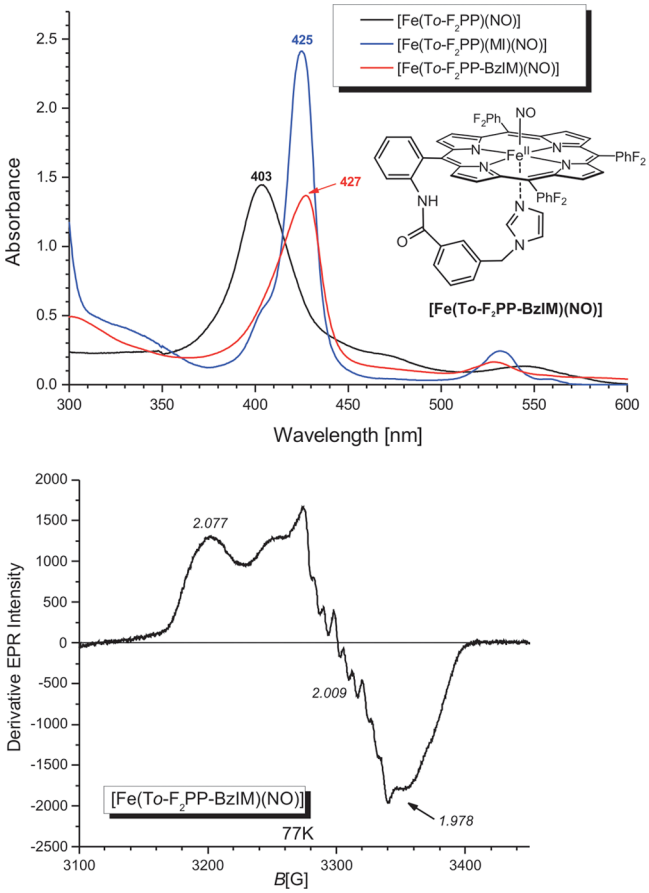

Figure 2. Top: UV−vis spectra of the "tailed" iron(II)−porphyrin NO complex [Fe(T*o*-F_2PP-BzIM)(NO)]. Bottom: EPR spectrum of [Fe(T*o*-F_2PP-BzIM)(NO)] in frozen DMSO at 77 K. Reprinted with permission from ref 30. Copyright 2009 American Chemical Society.

corresponding Zn^{2+} complex to illustrate this point. [Fe(T*o*-F2PP-BzIM)(NO)] is the first 6C ferrous heme-nitrosyl complex that is stable in solution at room temperature in the absence of excess axial N-donor ligand.

Quantification of the *trans* Effect Using Vibrational Spectroscopy

Since both NO and the proximal Im ligand influence each other via their *trans* interaction, the presence of the axial Im ligand in 6C ferrous heme-nitrosyls should also lead to a weakening of the Fe−NO bond in comparison to the analogous 5C complexes. The best way to probe such changes in metal−

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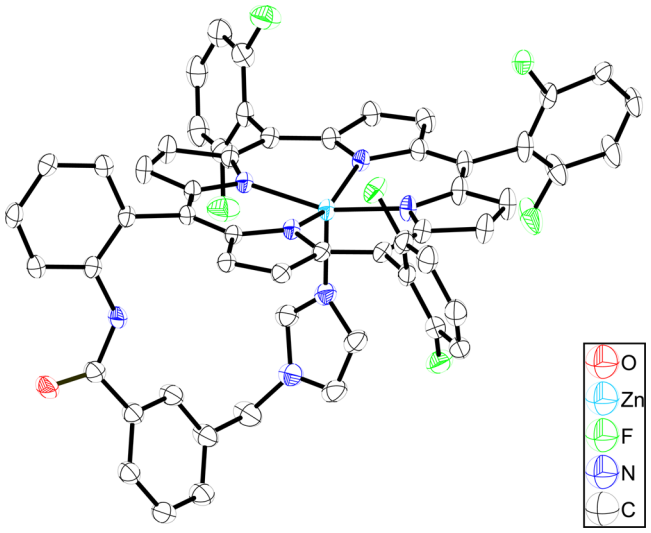


Figure 3. Molecular structure of [Zn(To-F_2PP-BzIM)]. Two CH_2Cl_2 solvent molecules and hydrogen atoms are omitted for clarity.[30]

ligand bond strengths is via measurement of the corresponding metal−ligand stretching frequencies, followed by normal coordinate analysis (NCA) to determine the corresponding force constants (since vibrational frequencies are not directly proportional to the corresponding bond strengths in the presence of vibrational mixing, whereas force constants are independent of this effect).[31] For 5C ferrous heme-nitrosyls, the Fe−NO stretching frequency can be determined in a straightforward way via resonance Raman or nuclear resonance vibrational spectroscopy (NRVS), and is generally observed around 520−540 cm^{-1} (see Figure 4 for assignments).[32,33] On

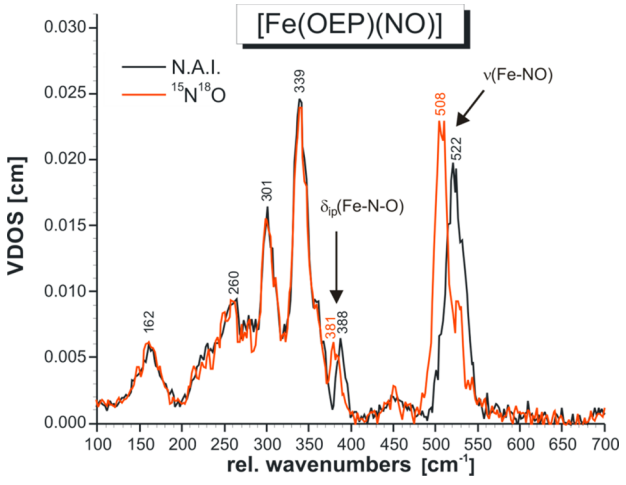


Figure 4. Vibrational density of states (VDOS) for [^{57}Fe(OEP)(NO)]. Reprinted in part with permission from ref 33. Copyright 2010 American Chemical Society.

the other hand, the vibrational assignments of the corresponding 6C complexes are more complicated.[25,34] From resonance Raman spectroscopy, only one isotope sensitive feature is observed around 550−575 cm^{-1},[25] the assignment of which to either the Fe−NO stretch or the Fe−N−O bend remained controversial.[35] To resolve this issue, we applied single-crystal NRVS to unambiguously assign the Fe−NO stretch in 6C ferrous heme-nitrosyls.[34] Here, the directionality of iron motion can be determined based on the orientation of the heme in the excitation beam. As shown in Figure 5, top, there are two $^{15}N^{18}O$-isotope sensitive features observed at 563 and 437 cm^{-1} for [Fe(TPP)(MI)(NO)].[36] The feature at 437 cm^{-1} is strongly out-of-plane polarized (i.e., the iron moves orthogonal to the heme plane), which is the expected behavior for the Fe−NO stretch (Figure 5, bottom). Further QCC-NCA simulations then allowed us to unequivocally assign the Fe−NO stretch in [Fe(TPP)(MI)(NO)] to the feature at 437 cm^{-1},[34] and a similar assignment is reported for Mb(II)-NO.[37]

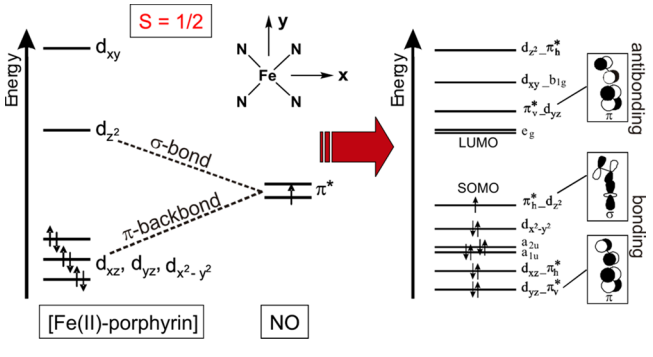
Analysis of the vibrational data of [Fe(TPP)(NO)] and [Fe(TPP)(MI)(NO)] by NCA delivers force constants of the Fe−N−O units in these exactly analogous complexes. In the 5C compound, the Fe−NO and N−O force constants are 2.98 and 12.53 mdyn/Å, which drop to 2.57 and 11.55 mdyn/Å upon coordination of MI to the ferrous iron center. *These results are in exact agreement with the idea of a thermodynamic trans interaction between the coordinated NO and Im ligands, where the presence of NO greatly weakens the Fe−N_{Im} bond, and vice versa the presence of Im weakens the Fe−NO bond.* These results have important implications for the electronic structures of these complexes. Because the binding of MI leads to a simultaneous weakening of both the Fe−NO and N−O bonds in 6C ferrous heme-nitrosyls, this cannot originate from a change in the Fe−NO π-backbond, which would lead to an inverse correlation between the Fe−NO and N−O bond strengths and vibrational frequencies (as observed in ferrous heme−CO complexes). *The direct correlation between the Fe−NO and N−O bond strengths in 5C and 6C ferrous heme-nitrosyls clearly shows that the binding of MI leads to a weakening of the Fe−NO σ-bond.*[27,34]

The Fe−N_{MI} stretch in [Fe(TPP)(MI)(NO)] is observed at 149 cm^{-1}, which is distinctively lower than the corresponding vibration in deoxy Hb/Mb, observed at 210−220 cm^{-1}.[38] The particularly low Fe−N_{MI} stretching frequency in [Fe(TPP)(MI)(NO)] again reflects the weak Fe−N_{MI} bond in the ground state of 6C ferrous heme-nitrosyls.

Electronic Structure of Ferrous Heme-Nitrosyls

These experimental findings are used to *calibrate* density functional theory (DFT)-calculated electronic structure descriptions.[25,27,33,34,39] This is particularly important here since ferrous heme-nitrosyls constitute significant challenges for DFT.[3,25] Based on the combination of experimental and theoretical results, the electronic structures of 5C and 6C ferrous heme-nitrosyls can be understood. These complexes are classified as low-spin {FeNO}^{7} in the Enemark−Feltham notation (the exponent '7' reflects the number of valence electrons: six d-electrons of iron(II), plus one unpaired electron of NO).[40] As indicated in Scheme 2, left, the singly occupied π^*

Scheme 2. Electronic Structure of Ferrous Heme-Nitrosyls



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orbital of NO forms a strong σ-bond with the empty d_{z^2} orbital of low-spin iron(II). The corresponding bonding combination, $\pi^*_h\_d_{z^2}$ (h = horizontal, the π^* orbital in the Fe−N−O plane), constitutes the singly occupied molecular orbital (SOMO) of the complexes (Scheme 2, right). In the 5C case, this leads to a very covalent σ-bond and results in an even distribution of the unpaired electron density of NO over the Fe−N−O unit. As shown in Figure 6, left, the calculated spin densities are about

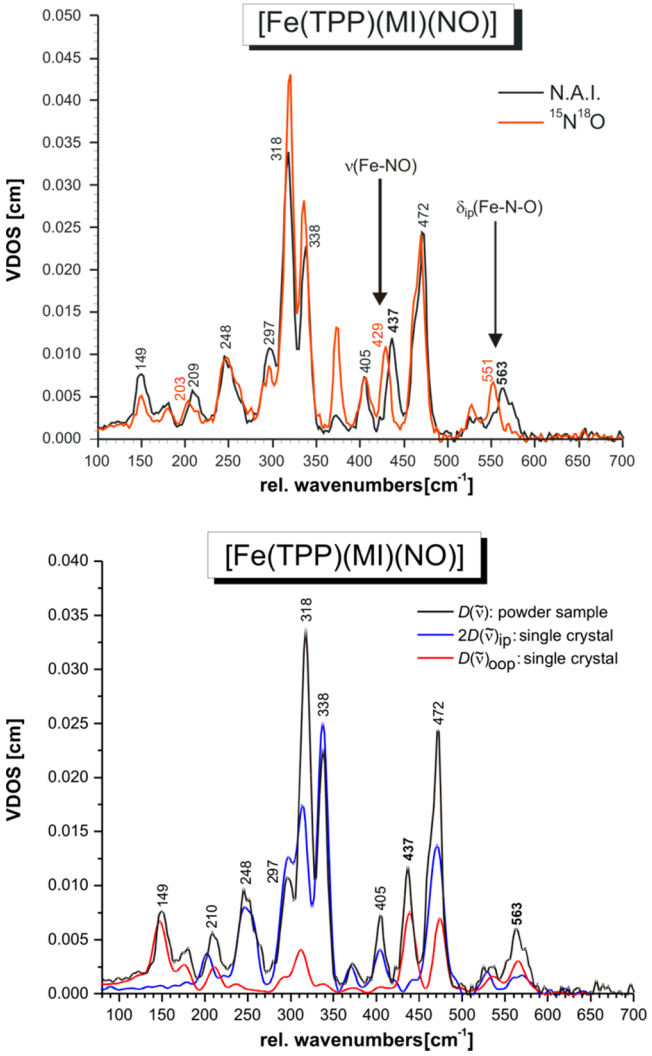

Figure 5. Top: Vibrational density of states (VDOS) for powder samples of [^{57}Fe(TPP)(MI)(NO)]. Bottom: Powder versus oriented single-crystal VDOS data of [^{57}Fe(TPP)(MI)(NO)]. Bottom panel reprinted with permission from ref 34. Copyright 2010 American Chemical Society.

50% on iron and 50% on NO, in agreement with this description. The empty π^* orbital of NO, π^*_v (v = vertical, the π^* orbital orthogonal to the Fe−N−O plane), forms a strong π-backbond with one of the t_{2g}-type orbitals of iron(II), d_{yz} in the coordinate system used in Scheme 2. NO therefore corresponds to a "classic" σ-donor/π-acceptor ligand in ferrous heme-nitrosyls. Based on the significant donation of electron density from π^*_h of NO into d_{z^2} of iron, the 5C complexes have a certain degree of Fe(I)−NO^+ character, reflected by their high N−O stretching frequencies (1680−1700 cm^{-1}) and strong Fe−NO bonds with force constants around 3 mdyn/Å.

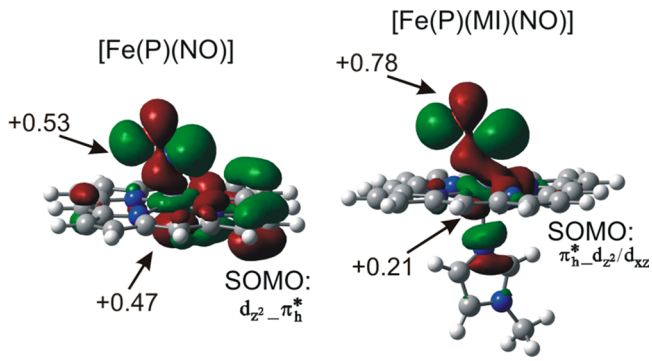

Figure 6. Contour plots of the SOMOs of [Fe(P)(NO)] and [Fe(P)(MI)(NO)] calculated with B3LYP/LanL2DZ*. Spin densities are indicated. Reprinted with permission from ref 27. Copyright 2006 American Chemical Society.

Binding of imidazole in trans position to NO weakens the Fe−NO σ-bond (Figure 6, right). This is due to (a) a loss of covalency in the orbital interaction between π^*_h and d_{z^2}, and (b) mixing of d_{xz} character into d_{z^2}, resulting in a rotation of d_{z^2} off the Fe−N(O) axis. The *trans* interaction between NO and the proximal Im ligand is best rationalized as a "competition" between the σ-donor orbitals π^*_h of NO and Im(σ) (the sp^2 donor orbital of the Im-nitrogen) for d_{z^2} of iron, where π^*_h makes the stronger bond and ends up with the bonding combination, whereas the interaction between Im(σ) and d_{z^2} in the SOMO is actually *antibonding* (Figure 6, right). This defines the electronic-structural reason for the thermodynamic *trans*-effect of NO. Consequently, the d_{z^2} orbital of iron then rotates off the Fe−N(O) axis to lower this antibonding interaction. The reduction in σ-donation from π^*_h of NO into d_{z^2} of iron(II) in the 6C complex causes a weakening of the Fe−NO bond, reflected by reduced Fe−NO force constants of 2.5−2.6 mdyn/Å. The resulting, increased electron density in π^*_h causes the simultaneous weakening of the N−O bond in 6C ferrous heme-nitrosyls, reflected by low N−O stretching frequencies (1620−1630 cm^{-1}). This further causes a shift in spin density distribution (see Figure 6, right), where spin densities increase to about 80% on NO and decrease to about 20% on iron in the 6C case, again reflecting the reduced donation of the unpaired electron of NO to the iron center. MCD and EPR spectroscopy further confirm this shift in spin density distribution.[25,27,39] 6C ferrous heme-nitrosyl complexes therefore represent the paradigm for Fe(II)−NO(radical) type electronic structures.

In summary, the *trans interaction between NO and proximal N-donor ligands in ferrous heme-nitrosyls corresponds to a thermodynamic σ-trans effect that is mediated by the SOMO of the complexes*. The Fe-Im antibonding interaction in the SOMO is directly reflected by the low binding constants of N-donor ligands trans to NO, the low Fe−N_{Im} stretching frequencies and long Fe−N_{Im} bonds in corresponding 6C complexes, and is the underlying key interaction that induces a breaking of the Fe−N_{His} bond in sGC.

Interrogating the SOMO of Ferrous Heme-Nitrosyls

Since the SOMO is responsible for the thermodynamic *σ-trans* effect in ferrous heme-nitrosyls, this could be further probed by one-electron reduction of the complexes, leading to a double-occupation of the SOMO (which becomes the HOMO in the reduced complex) and formation of ferrous nitroxyl, Fe(II)-NO(−), complexes (classified as low-spin {FeNO}^{8}; see Scheme 3 and reviewed in detail in ref 16). However, this is

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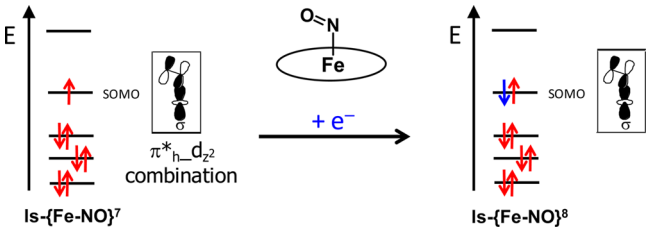
Scheme 3



only meaningful if the reduction leads to minimal electronic relaxation, that is, the composition of the key Fe−N−O bonding MOs, especially the SOMO, does not change in the reduced state. Surprisingly, this is in fact the case: as shown in Figure 7, there is a strong correlation between the N−O

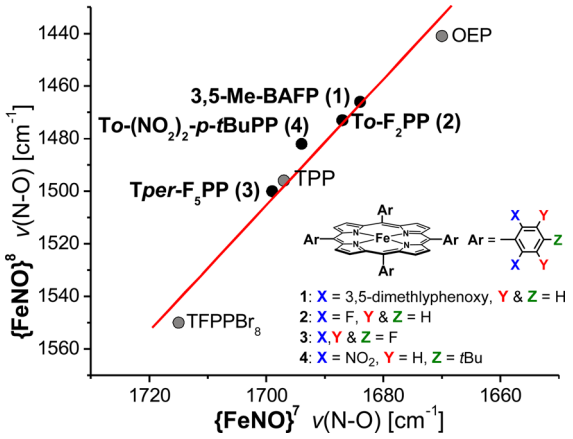


Figure 7. Comparison of N−O stretching frequencies in analogous {FeNO}^{7} and {FeNO}^{8} porphyrin complexes. Reprinted in part with permission from ref 41. Copyright 2013 American Chemical Society.

stretching frequencies along a diverse series of {FeNO}^{7}/{FeNO}^{8} couples.[41] This implies that electronic differences between different {FeNO}^{7} complexes are "carried over" into the corresponding {FeNO}^{8} systems, which requires minimal electronic relaxation of Fe−NO bonding MOs upon reduction. DFT calculations support this finding and show identical S/HOMOs for [Fe(Porph)(NO)] and [Fe(Porph)(NO)]$^-$ (Figure 8).[41] The thermodynamic *σ-trans* effect requires that the SOMO has distinct Fe−NO *σ*-bonding character. Double occupation of this orbital should therefore amplify these properties, and lead to a strengthening of the Fe−NO bond, and further increase of the *trans* effect in the {FeNO}^{8} complexes. Experimental data verify that this is indeed the case: resonance Raman studies by Ryan and co-workers show that the Fe−NO stretching frequency increases from 525 cm^{-1} in [Fe(TPP)(NO)] (in THF) to 549 cm^{-1} in the reduced {FeNO}^{8} complex (despite the fact that reduction increases the Coulomb repulsion between the now double occupied π^*_h orbital of NO and d_{xz} of iron, which counteracts this effect),[42] directly demonstrating that the SOMO is Fe−NO bonding in nature. Furthermore, the binding constant of MI in trans position to NO drops by several orders of magnitude in the reduced {FeNO}^{8} complexes, directly confirming that the SOMO is in fact responsible for the thermodynamic *σ-trans* effect of NO.[41] In this way, studies on the reduced iron(II)-nitroxyl model complexes confirm the previous findings about the properties of the SOMO in ferrous heme-nitrosyls. These results could only be obtained with model complexes, as {FeNO}^{8} complexes are protonated in an aqueous environment.[41]

Why NO?

This is the question that Traylor and Sharma posed in their pioneering 1992 *Biochemistry* paper.[24] Their answer is that NO is a stable, diffusible radical that has an extraordinary affinity to ferrous heme and, in this way, can be detected at nanomolar concentrations, and, via its *trans* effect, can transduce a signal in an enzyme or transcription factor. At the same time, NO is reactive enough to be detoxified straightforwardly by reaction with oxy-Hb/Mb. We would like to add that 5C and 6C ferrous heme-nitrosyls (the latter with axial His ligation) generally display a low reactivity toward O_2, CO, and so forth (at physiologically relevant concentrations) and are not easily reduced or oxidized. The bound NO ligand in ferrous heme-nitrosyls does not show any reactivity toward excess NO, and these complexes do not show any tendency to dimerize, forming reactive hyponitrite complexes:[43]

$$2[Fe^{II}(Porph)(NO)(L)] \not\rightarrow \{[Fe^{III}(Porph)(L)]_2(\mu\text{-}N_2O_2)\} \quad (2)$$

In fact, experimentally, ferric hyponitrite-bridged dimers decompose back into ferrous heme-nitrosyl monomers due to the great thermodynamic stability of the latter species. This reaction is slow, due to significant kinetic barriers.[43] The lack of

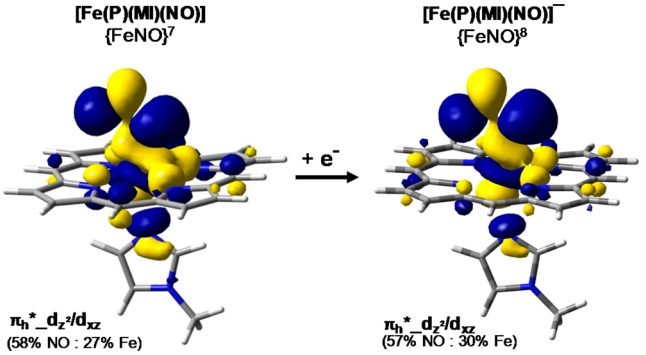


Figure 8. Key π^*_h_d_{z^2}/d_{xz} molecular orbitals of [Fe(P)(MI)(NO)] and [Fe(P)(MI)(NO)]$^-$. Calculated with B3LYP/TZVP on BP86/TZVP optimized structures. Reprinted with permission from ref 41. Copyright 2013 American Chemical Society.

reactivity of ferrous heme-nitrosyls formed in the sensor units of proteins is therefore another attractive feature of NO as a signaling molecule. Since ferrous heme-nitrosyls have been proposed as reactive intermediates in respiratory bacterial NO reductases, this poses the interesting question of how these generally very stable complexes could be activated for catalysis in these enzymes.[16]

3. COMPARISON TO FERRIC HEME-NITROSYLS

At this point it is insightful to make a comparison between analogous ferrous and ferric heme-nitrosyls. Thermodynamically, the Fe−NO bond is dramatically weaker in ferric heme-nitrosyls, as reflected by the corresponding NO binding constants, K_{eq},[3,25] which are usually in the 10^3−10^5 M^{-1} range. This corresponds to free NO binding energies, ΔG° = −4 to −7 kcal/mol for ferric hemes. In contrast, K_{eq} = 10^{11}−10^{12} M^{-1} for NO binding to ferrous hemes, which translates into free binding energies of ΔG° = −15 to −16 kcal/mol. *Clearly, NO is bound more strongly to ferrous hemes, based on these*

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Acc. Chem. Res. 2015, 48, 2117−2125

thermodynamic considerations. However, spectroscopic methods that probe the ground states of these complexes paint a different picture. In ferric heme-nitrosyls, the ground state has Fe(II)−NO^+ character with a strong Fe−NO bond, evidenced by the very short Fe−NO distances in these complexes (~1.65 Å) and the large Fe−NO stretching frequencies, ν(Fe−NO), of about 590 cm^{-1}. For example, in ferric [Fe(TPP)(MI)(NO)]-(BF_4), the Fe−NO stretching frequency and Fe−NO force constant are 580 cm^{-1} and 3.92 mdyn/Å, which drop to 437 cm^{-1} and 2.57 mdyn/Å, respectively, in the one-electron reduced complex [Fe(TPP)(MI)(NO)]. *Clearly, the Fe−NO bond is stronger in the ground state of ferric compared to ferrous hemes.* This contradiction between the overall thermodynamic stability and the ground state properties of ferric versus ferrous heme-nitrosyls is intriguing. Using spectroscopic investigations coupled to DFT calculations, we demonstrated that the unusual properties of ferric heme-nitrosyls relate to the presence of multiple low-lying excited states that are involved in NO bonding/dissociation (Figure 9).[44] The calculations show that in the Fe(II)−NO^+ ground state of ferric heme-nitrosyls (blue curve in Figure 9) the Fe−NO bond is indeed very strong, with an Fe−NO dissociation energy of ~30 kcal/mol, in agreement with the spectroscopic findings. Upon movement of the NO away from the Fe center, there is a change in the electronic state at an Fe−NO distance of ~1.7 Å: here, one electron is transferred back from the Fe(II) to the NO^+ ligand, and the complex transitions into the low-spin Fe(III)-NO(radical) electronic state (red curve in Figure 9), where the unpaired electrons of low-spin Fe(III) and NO are antiferromagnetically coupled (giving rise to a diamagnetic state). In this state, the NO dissociation energy has dropped to ~10 kcal/mol, which is comparable to the dissociation energies of ferrous heme-nitrosyls. Upon further elongation of the Fe−NO bond, the iron(III) undergoes a spin-crossover at an Fe−NO distance of ~1.9 Å, and the complex enters the high-spin Fe(III)-NO(radical) state (black curve in Figure 9). Most importantly, this excited state is *dissociative* with respect to the Fe−NO bond, which not only lowers the thermodynamic NO dissociation free energy to about 5 kcal/mol, but also explains the large kinetic k_{off} rates for NO when bound to ferric hemes: once the complex enters the high-spin Fe(III)-NO(radical) state, the NO is effectively "pushed away" from the iron center, and dissociates easily.[44] In this sense, *NO can form a strong Fe−NO bond to a ferric heme and be a weak ligand at the same time* (since this depends on the properties of different electronic states)! The biological significance of this finding relates to the fact that ferric hemes, in contrast to ferrous hemes, can release NO easily, and are therefore well suited for NO transport in nitrophorins. Ferric heme-nitrosyls are also ideal enzyme−product complexes in NO-producing enzymes (e.g., NOS), as these can release NO quite readily.[44]

In ferric heme-nitrosyls with axial thiolate coordination, an interesting change in the geometric and electronic structure is observed.[45,46] In particular, the Fe−N−O unit becomes bent in the presence of the axial thiolate ligand, and both the Fe−NO and the N−O bonds become weaker.[25] Theoretical work shows that this is due to a σ-backbond into a fully antibonding Fe−N−O σ^* orbital, and that the strength of this backbond is proportional to the donor strength of the anionic axial ligand trans to NO.[45,46]

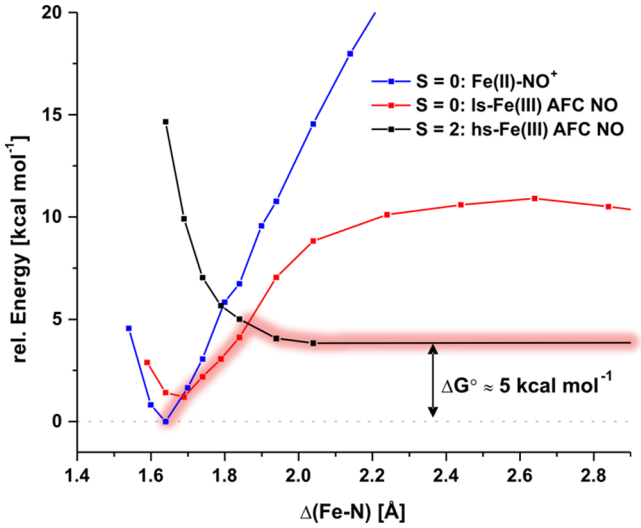

Figure 9. Calculated potential energy surfaces for 6C ferric heme-nitrosyls using the model system $[Fe(P)(MI)(NO)]^+$ (P = $porphine^{2-}$; MI = 1-methylimidazole). The NO binding/dissociation pathway is highlighted. AFC = antiferromagnetically coupled. Reprinted in part with permission from ref 44. Copyright 2008 American Chemical Society.

4. CONCLUSIONS

In conclusion, in-depth structural, spectroscopic, and theoretical studies on model systems for ferrous- and ferric heme-nitrosyls have provided key insights into their electronic structures, and this information relates directly to their function in biology. Due to the strong Fe(II)−NO σ-bond, NO exerts a strong thermodynamic *σ-trans* effect on the proximal ligand in ferrous heme-nitrosyls, which is of key significance for NO to act as a signaling molecule in mammals. Studies on analogous model systems have allowed one to quantify this effect, and to identify the SOMO of the complexes as the key MO involved in this interaction. On the other hand, ferric heme-nitrosyls are labile, which is in contradiction to their ground state properties. This is rationalized with the involvement of at least three different electronic states in the binding/dissociation of NO to/from ferric hemes. This is of key significance for ferric hemes to serve as a platform for NO transport in biology.

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Notes

The authors declare no competing financial interest.

Biographies

Andrew P. Hunt received his B.S. in Chemistry from Kent State University in 2013, where he worked with Prof. Nicola E. Brasch, and is currently a Ph.D. student at the University of Michigan in Prof. Nicolai Lehnert's group. His research focuses on the synthesis and spectroscopic characterization of ferric heme-thiolate complexes, and their reactivity with NO as models for Cytochrome P450s.

Nicolai Lehnert obtained a Diploma in Chemistry in 1995 from the Heinrich-Heine-University Düsseldorf, Germany. In 1999, he received his Ph.D. from the Johannes Gutenberg-University Mainz, Germany, working on model systems for nitrogenase under supervision of Priv.-Doz. Dr. F. Tuczek and Prof. P. Gütlich. He carried out postdoctoral

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Acc. Chem. Res. 2015, 48, 2117−2125

research, from 1999 to 2001, under Prof. E. I. Solomon at Stanford University. He received his Habilitation in 2006 from the Institute of Inorganic Chemistry, Christian-Albrechts-University Kiel, Germany, working with Prof. F. Tuczek. In 2006, he accepted a faculty position at the University of Michigan. In 2012, he was promoted to Associate Professor with tenure.

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