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Mechanisms of HNO Reactions with Ferric Heme Proteins

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Abstract

Many HNO scavenging pathways exist to regulate its in vivo and in vitro biological and pharmacological activities, including the involvement of numerous ferric heme proteins. Such reactions also build an important basis for HNO probe development. However, mechanisms of HNO reactions with ferric heme proteins are largely unknown. A computational investigation was performed to provide the first detailed pathways, using metmyoglobin and catalase as representative ferric heme proteins with neutral and negatively charged axial ligands. Results well reproduced experimental barriers with an average error of 0.11 kcal/mol. The rate-limiting step was found to be the dissociation of the resting ligand or HNO coordination where there is no resting ligand. Unlike the non-heme case, the reductive nitrosylation step for both heme proteins was found to be barrierless proton-coupled electron transfer, providing the major thermodynamic driving force for the overall reaction. Origin of experimental reactivity difference between metmyoglobin and catalase was revealed. Results will facilitate studies of other heme-based HNO scavenging systems and probe development.

Graphical Abstract

HNO binding process is rate-limiting and reductive nitrosylation is barrierless proton-coupled electron transfer to provide the major thermodynamic driving force. Protein axial ligand is a dominant factor to influence reactivity.

Keywords

nitrogen oxides; bioinorganic chemistry; heme proteins; reaction mechanism; computational chemistry

HNO is involved in numerous biological activities, such as vascular relaxation, enzyme activity regulation, and neurological function regulation.^[1] Its favorable biological effects have enabled HNO donors to be a promising new class of pharmacological agents in heart failure treatment,^[2] as a potential treatment for reduction of neuronal damage during stroke,^[1a] and in anti-cancer research.^[3] But it can also lead to some deleterious effects, such as enhancement of oxidative stress and neutrophil infiltration,^[4] and blood-brain barrier disruption.^[5] To regulate its *in vivo* and *in vitro* activities, there are a number of biological HNO scavenging pathways,^[1a] and the use of various heme proteins^[1a] such as metmyoglobin (metMb),^[6] methemoglobin,^[6a, 7] catalase (CAT),^[6b] ferricytochrome *c*,^[6a, 6c, 8] oxymyoglobin,^[6c] myoglobin,^[9] hemoglobin,^[10] leghemoglobin,^[10] cytochrome P450,^[11] and horseradish peroxidase (HRP),^[6b, 12] have been experimentally investigated. Such work builds an important basis for the development of heme-based HNO probes.^[1f, 13]

Despite decades long research in this area,^[1a, 1f, 6a, 6c, 14] many mechanistic questions remain unanswered. For instance, how does HNO bind to the active site and how does HNO get reduced? Which step is rate-limiting? How does the protein environment affect biological HNO consumption rate?^[1a, 6b] In fact, the protein axial ligand that is *trans* to HNO in the binding site was found to influence the HNO scavenging process.^[1a, 15] For example, metMb and HRP with a neutral axial His ligand have larger HNO consumption rate constants ($8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ respectively) than CAT with a negatively charged axial ligand Tyr ($3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$).^[6b] The mechanistic details may also help rational design of heme-based HNO probes. In particular, fewer heme-based HNO probes have been developed,^[1f, 13] in contrast with many non-heme based HNO probes.^[16]

Therefore, a computational investigation was performed to provide the first detailed HNO scavenging pathways, using metMb and CAT as representative ferric heme proteins with neutral and negatively charged axial ligands. Results not only quantitatively well reproduced experimental barriers, but also revealed key mechanisms to understand the origin of different experimental reactivities important for HNO biological and pharmacological activities.

Active site models along both the HNO binding and conversion pathways for metMb (called Mb in model names) and CAT containing proximal and nearby residues that could influence HNO/NO binding were investigated, using Ca-truncated residues and non-substituted porphyrins based on the previous work.^[17] All models were subject to geometry optimization and frequency analysis, in a medium to simulate the bulk protein environment using the PCM approach,^[18] with a DFT method that yields excellent predictions of experimental HNO reactivities,^[16h, 17a, 19] see Supporting Information (SI) for computational details and results of all conformations and electronic states studied, with results of the most favorable species shown in Figure 1 and Table 1.

Since metMb is the mostly used heme protein to scavenge HNO,^{1, 6, 11, 13, 25–27} its reaction pathway was first studied. In metMb resting state, there is a water ligand *trans* to proximal His ligand, see **Mb-I-1** in Figure 1. Its experimental ground state has $S=5/2$,^[20] as evidenced by the calculated Mulliken spin density of Fe ($\rho^{\alpha\beta}_{\text{Fe}}$), ~ 4.5 e. Although distal His64 could have either N δ or N ϵ protonated, our calculations show that the N δ form is more favorable by 7.61 kcal/mol in Gibbs free energy (ΔG), and the same trend holds for electronic energies (E 's), zero-point energy corrected electronic energies (E_{ZPE} 's), and enthalpies (H 's), see Table S2. This favorable structure is associated with a shorter water...His hydrogen bond (H-bond) length ($R_{\text{H}\cdots\text{His}}$) by 0.433 Å. In the case of HNO binding with this ferric center (the ground state was found to be low spin^[15]), **Mb-I-5**, again the N δ -protonated form is more stable by 11.89 kcal/mol in ΔG and a shorter H-bond distance by 1.004 Å. These trends agree well with the previous experimental and computational work on ferric Mb with H₂O and NO as axial ligands.^[21] Therefore, the N δ -protonated His64 was used in the following reaction pathway study.

In principle, ligand substitution of water by HNO could be either associative or dissociative. However, all associative trials (i.e. adding HNO to Fe before water leaves) were failed, which is not unexpected since **Mb-I-1** is already six-coordinate with basically no room for additional coordination. Then, the dissociative pathway (see Figure 1) was studied. In this case, when HNO comes, water still binds and an H-bond complex (**Mb-I-2**) was formed. This is enthalpically favorable due to H-bond formation but entropically unfavorable due to the association, see Table 1. Then, water dissociates to form **Mb-I-3** with ~ 7 kcal/mol enthalpy loss. To minimize the energy penalty due to water dissociation, both H-bond's with His64 and HNO were maintained. With the favorable H-bond mode between water and His64, there are still three possible ways to form H-bond between water and HNO: 1) $\text{HN}=\text{O}\cdots\text{HOH}$, 2) $\text{O}=\text{N}(\text{H})\cdots\text{HOH}$, and 3) $\text{O}=\text{NH}\cdots\text{OH}_2$, see Figure S3 for optimized structures. Their ΔG 's are not of significant differences (<1 kcal/mol, Table S4). Results in Tables 1 and S5 show that the H-bond distances between water and His64 are significantly shorter by $\sim 0.2\text{--}0.6$ Å than those between water and HNO ($R_{\text{H}_2\text{O}\cdots\text{HNO}}$), suggesting a more important role of water and His64 interaction. The first two H-bond modes have similar water...His H-bond distances in **Mb-I-2** and **Mb-I-3**, which are ~ 0.05 Å longer (and weaker) than that for the third mode having the lowest energies for all energetic terms (Table S4) for **Mb-I-3**. Therefore, the third H-bond mode was chosen for **Mb-I-2** and **Mb-I-3**.

Breaking the HNO and water H-bond in **Mb-I-3** is favorable since its formation in **Mb-I-2** has positive ΔG . The subsequent rearrangement of HNO and water to allow HNO binding with Fe in **Mb-I-4** is also favorable with ΔG reduction of 1.28 kcal/mol. The initial setup to have HNO with an H-bond with His64 while maintaining the original H-bond between water and His64 was optimized to have only HNO's H-bond with His64, see Figure 1. This step is largely enthalpy driven, with ΔH of -6.30 kcal/mol. The ~ 2.3 Å distance between water and HNO in ferric **Mb-I-4** suggests that this interaction is unfavorable, unlike in the ferrous system.^[17a] So the loss of water to form **Mb-I-5** is driven by both enthalpy (-4.53 kcal/mol) and entropy (-7.27 kcal/mol).

After HNO binds to the ferric center, the reductive nitrosylation was found to occur with a proton-coupled electron transfer (PCET) transition state, **Mb-TS**, to form the final ferrous

NO heme product as indicated by 0.142 e spin density for Fe and 0.858 e spin density for NO, and the doubly protonated distal His64 (see Figure 1) in **Mb-I-6**. Unlike the PCET mechanism of HNO to NO conversion mediated by the non-heme metal system^[16b] which has a reasonably high barrier, the PCET process via metMb is basically barrierless, with a large thermodynamic tendency (ΔG : -13.69 kcal/mol), see Table 1. To confirm this feature, four additional sets of calculations were performed. First, the intrinsic reaction coordinate calculations were done to confirm that **Mb-TS** is indeed connected with **Mb-I-5** and **Mb-I-6** and the potential energy surface between **Mb-I-5** and **Mb-TS** is flat (barrierless), see Figure S4. Second, since **Mb-TS** has little spin density change compared to **Mb-I-5**, to examine if a separate proton transfer mechanism followed by an electron transfer process could operate, the proton-transfer-only product was studied using three broken-symmetry setups of **a**) Fe^{III} (S=1/2) and NO⁻ (S=0); **b**) Fe^{III} (S=1/2) and NO⁻ (S=1); **c**) Fe^{III} (S=-1/2) and NO⁻ (S=1), covering both singlet and triplet NO⁻, and both ferromagnetic and anti-ferromagnetic couplings. For comparison, the broken-symmetry setup of **d**) Fe^{II} (S=0) and NO (S=1/2) was also examined. As shown in Table S6, after geometry optimizations, all trials with total S=1/2 (**a**, **c**, **d**) converged to basically the same structure as reported in Table 1, while the trial with S=3/2 (**b**) maintains the basic feature in the initial setup, but of much higher energy. These results suggest that there is no favorable separated proton transfer and further support results in Table 1. Third, a different type of DFT method with dispersion correction was used, which yielded the same mechanism discussed above (see SI). Fourth, this pathway was re-investigated using the whole protein system via the quantum mechanics and molecular mechanics (QM/MM) approach, which again supports the barrierless PCET feature (see SI).

As seen from Figure 2A, the overall rate-limiting step is water dissociation in the HNO binding process (**Mb-I-3**), with the calculated Gibbs free energy of activation ($\Delta G^\ddagger$) of 9.46 kcal/mol in good agreement with experiment: 9.39 kcal/mol.^[6b] The major driving force for this step is the stronger binding of HNO compared to water as indicated by ΔH of -5.63 kcal/mol, despite a small entropy penalty of 2.01 kcal/mol. But the dominant thermodynamic driving force for HNO scavenging is the PCET from **Mb-I-5** to **Mb-I-6**, yielding ΔG of -10.07 kcal/mol.

The varied experimental reaction rates of different ferric heme proteins^[1a, 6b, 13a, b, 13d] indicate that the protein environment may modulate the HNO scavenging details. To understand such an effect, we next investigated CAT, which is the only ferric heme protein having a negatively charged ligand with experimental reaction rate constant reported.^[6b] Compared to metMb with a neutral axial ligand, its reactivity is almost half.

The N δ -protonated distal His was also used for CAT based on the above results of metMb. However, in the resting state, a difference from metMb is that many studies show that CAT has no axial water bound,^[22] see **CAT-I-1** in Figure 1, which is supported by the calculated unfavorable binding ΔG of +4.54 kcal/mol in Table S11. For **CAT-I-1**, the negatively charged Tyr ligand has a stronger interaction with positively charged Fe center than the neutral His ligand in **Mb-I-1**, as evidenced by ~0.25 Å shorter Fe-axial-ligand (R_{FeL}) distance in Table 1, which results in stronger *trans* effect to destabilize the binding of axial water.

The cavity in **CAT-I-1** is ready for HNO binding to form **CAT-I-2**, which results in ΔH of -5.18 kcal/mol. However, the entropic effect makes a ΔG of 10.12 kcal/mol. This is different to metMb with ΔG of -3.62 kcal/mol, but is consistent with the previous report that HNO binding with a neutral ligand is significantly more stable than with a negatively charged ligand, in ferric porphyrins,^[15] due to stronger *trans* effect of the latter, as demonstrated by the longer Fe-NO bond length (R_{FeNO}) in corresponding species of CAT vs. metMb in reaction pathways (Table 1). The stronger interaction between the negatively charged Tyr ligand and the positively charged Fe in CAT compared to that in metMb also makes Fe displacement below the heme plane (0.422 Å) significantly larger than that in metMb (0.372 Å), in corresponding five-coordinate structures **CAT-I-1** and **Mb-I-3**. This renders Fe in CAT farther away and thus more difficult for HNO binding.

The subsequent reductive nitrosylation from **CAT-I-2** to **CAT-I-3** via **CAT-TS** is also a barrierless PCET process, the same with metMb. **CAT-I-3** has basically ferrous NO feature as indicated by $\rho^{\alpha\beta}_{\text{Fe}}$ of 0.106 e and $\rho^{\alpha\beta}_{\text{NO}}$ of 0.884 e, and also the doubly protonated distal His64 (see Figure 1) as in **Mb-I-6**. As shown in Table S12, other possible spin coupling patterns were also investigated as for metMb, which further supports the results in Table 1 as the most favorable ones and are consistent with the $S=1/2$ ground state found experimentally.^[23]

As seen from Figure 2B, the rate-determining step for CAT is HNO binding. The calculated $\Delta G^\ddagger$ of 10.12 kcal/mol is in good agreement with the experimental value of 9.97 kcal/mol.^[6b] The calculated trend of higher reactivity with metMb vs. CAT is also consistent with experiment. In addition, the reductive nitrosylation process here also provides ΔG of -11.13 kcal/mol from the HNO bound system to NO bound species, which makes the net reaction thermodynamically favorable as observed experimentally.^[6b]

Overall, the above results provide the first detailed mechanisms of HNO reactions with ferric heme proteins. The HNO binding process was found to be rate-limiting, with excellent predictions of experimental $\Delta G^\ddagger$'s for both metMb and CAT. The experimentally found slower HNO reactivity with CAT vs. metMb was also reproduced and found to be associated with the stronger *trans* effect of the negatively charged Tyr ligand in CAT than that of the neutral His ligand in metMb. Unlike the non-heme case, the reductive nitrosylation step for both heme proteins is a barrierless PCET to provide the major thermodynamic driving force for the overall reaction. These results will facilitate studies of other heme-based HNO scavenging systems and probe development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Optimized heme-containing species along the reaction pathways for metMb and CAT. Atom color scheme: C- cyan, N- blue, O- red, H – grey, Fe - black. Residue numbers are from PDB files 1DWR and 4B7H for metMb and CAT respectively.

Figure 2.
Energy profiles for metMb (A) and CAT (B).

Table 1.

Key Energies, Geometric Parameters, and Spin Densities

Species	E (kcal/mol)	E _{ZPE} (kcal/mol)	H (kcal/mol)	G (kcal/mol)	R _{FeL} ^{a)} (Å)	R _{FeOH₂} (Å)	R _{FeNO} (Å)	R _{H...His} (Å)	R _{H₂O...HNO} (Å)	ρ ^{ab} _{Fe} (e)	ρ ^{ab} _{NO} (e)
Mb-I-1+HNO	0.00	0.00	0.00	0.00	2.150	2.153	/	1.614	/	4.494	0.000
Mb-I-2	-4.08	-2.38	-1.66	5.35	2.141	2.180	4.486	1.584	2.129	4.534	0.001
Mb-I-3	2.90	4.33	5.20	9.46	2.059	4.804	5.880	1.762	1.931	4.263	0.001
Mb-I-4	-5.15	-1.37	-1.10	8.18	2.017	5.124	1.869	1.631	2.289	1.251	-0.029
Mb-I-5+H₂O	-7.85	-5.80	-5.63	-3.62	2.004	/	1.884	1.584	/	1.224	-0.033
Mb-TS+H₂O	-7.60	-7.56	-7.57	-5.54	2.036	/	1.871	1.385	/	1.254	-0.059
Mb-I-6+H₂O	-18.74	-15.76	-15.46	-13.69	2.100	/	1.762	1.019	/	0.142	0.858
CAT-I-1+HNO	0.00	0.00	0.00	0.00	1.902	/	/	/	/	4.245	0.000
CAT-I-2	-7.72	-4.00	-5.18	10.12	1.911	/	1.895	1.658	/	1.174	-0.028
CAT-TS	-6.89	-5.94	-7.05	8.94	1.948	/	1.879	1.400	/	1.177	-0.028
CAT-I-3	-20.17	-16.28	-17.30	-1.01	2.082	/	1.745	1.014	/	0.106	0.884

^{a)} L = N(His) for Mb and O(Tyr) for CAT.^{b)} H is from the closest H-bond partner.