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Density Functional Theory Modeling of the Proposed Nitrite Anhydrase Function of Hemoglobin in Hypoxia Sensing

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Supporting Information

ABSTRACT: The role of NO and nitrite-bound methemoglobin ($Hb^{III}NO_2^-$) in hypoxic signaling is highly controversial. One provoking possibility is that hemoglobin (Hb) functions as a nitrite anhydrase, producing N_2O_3 (from nitrite) as an NO carrier. The ability of Hb to generate N_2O_3 would provide an intriguing means of NO release from red blood cells. We have investigated this proposed new reactivity of Hb using density functional theory (DFT) calculations. For this purpose, models of the Hb/myoglobin (Mb) active site have been constructed. Our results show that the O-bound (nitrito) form of $Hb/Mb^{III}NO_2^-$ is essential for the formation of N_2O_3. The formation and release of N_2O_3 is shown to be energetically favorable by 1−3 kcal/mol, indicating that the anhydrase function of Hb/Mb is biologically feasible.

The role of nitrite in mammalian hypoxic vasodilation is currently a highly contested topic within the field of biological chemistry.[1] One intriguing possibility, as investigated experimentally by Basu et al., is that an intermediate methemoglobin nitrite species ($Hb^{III}NO_2^-$) is able to activate nitrite to produce the metastable species N_2O_3 in the presence of free NO.[2a] Such a scenario would provide a convenient means of NO escape (via N_2O_3) from hemoglobin (Hb)-rich red blood cells (RBCs) under hypoxic conditions. Because N_2O_3 possesses a half-life of ~1 ms and a diffusion coefficient of 1000 $\mu m^2/s$, it is not unreasonable to expect that some fraction of N_2O_3 can diffuse out of the erythrocyte.[2] Once released from the RBCs, the NO (obtained via decomposition of N_2O_3 in blood) would then induce vasodilation and, in this way, would direct blood flow to hypoxic tissue.[1] The proposed physiological reactions are shown below in eqs 1−4. Deoxyhemoglobin, Hb^{II} in eq 1, is simply obtained by the release of O_2 from oxyhemoglobin in O_2-poor tissue.

$$Hb^{II} + NO_2^- + H^+ \rightarrow Hb^{III} + NO + OH^- \quad (1)$$

$$Hb^{III} + NO_2^- \rightarrow Hb^{III}NO_2^- \quad (2)$$

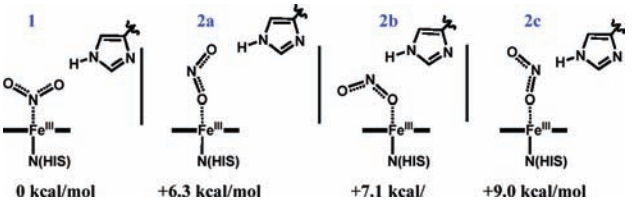
$$Hb^{III}NO_2^- + NO \rightarrow Hb^{III}N_2O_3(-) \quad (3)$$

$$Hb^{III}N_2O_3(-) \rightarrow Hb^{II} + N_2O_3 \quad (4)$$

It is known that Hb and myoglobin (Mb) can act as nitrite reductases[3] (eq 1). However, the feasibility of the nitrite anhydrase reaction (eq 3) is unknown. One important detail in this regard is the binding mode of nitrite. It has been shown through a variety of methods that nitrite can bind to ferric hemes in either an N-bound (nitro) or O-bound (nitrito) conformation.[4] Most commonly, nitrite shows a preference for the nitro binding mode. Interestingly, however, recent crystallographic results indicate that the unusual nitrito form is found in both Hb and Mb.[5] This paper seeks to clarify the geometric structure of the heme nitrite complex within ferric (met-)Hb/Mb, and to elucidate the feasibility of eqs 3 and 4 as a function of the nitrite binding mode, using DFT calculations. To model the interaction of NO with nitrite-bound met-Hb/Mb, a structural model for the Hb/Mb active site was generated based on a high-resolution crystal structure of oxymyoglobin (PDB code: 1A6M).[6] The model consists of heme b, stripped of its peripheral substituents, one axial (proximal) histidine ligand, and, fixed at the crystallographically determined distance from the iron center, the distal histidine residue. The distal histidine is only fixed at the terminal α-C atom, such that its orientation relative to bound substrates is free to optimize. The model system obtained in this way serves as a general model for both the Hb and Mb active sites (therefore referred to as Hb/Mb).

The first task in order to investigate eq 3 is to assess the preferred binding mode of nitrite. For this purpose, nitrite was positioned within the distal pocket of our model system in several different orientations, inspired by crystallographically observed binding modes of nitrite[4,5] and recent DFT calculations that explored nitrite binding geometries using a more restricted Mb active site model.[2a] This includes a nitro (N-bound) and three distinct nitrito (O-bound) coordination modes, which are shown in Scheme 1. All orientations were optimized at the BP86/TZVP theory level, employing only the constraints indicated above. Experimentally, structures **1**, **2a**, and **2b** have been observed, but **2b** is seen only in Mb mutants.[5a] The final structure, **2c**, has not been observed experimentally. Finally, a distorted Fe-ONO unit similar to **2b** is observed in the β-subunit of Hb, but this tilted structure is not predicted by our DFT results.

Scheme 1. Potential $Hb/Mb^{III}NO_2^-$ Structures and Their Relative Energies (BP86/TZVP)[a]



[a] All species show $S = {}^1/_2$ ground states.

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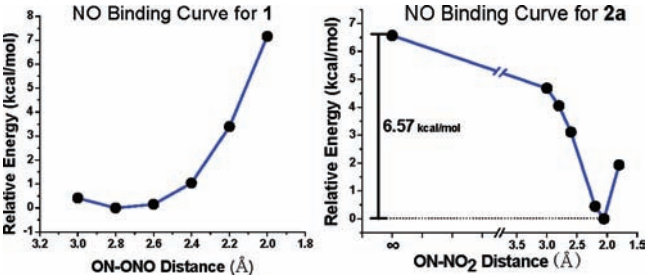

Figure 1. Binding curves for NO interacting at either the N atom (**2a**) or O atom (**1**) of ferric Hb/Mb-bound nitrite. The PES is associative (favorable) for the formation of N_2O_3 only in the case of **2a**. B3LYP/LanL2DZ and BP86/TZVP calculations were used to construct these energy surfaces.

Overall, the N-bound nitro configuration (**1**) is predicted to be the lowest-energy conformation, which is not in agreement with the crystal structures of ferric Hb/Mb-nitrite adducts. This indicates that, besides the hydrogen bond from the distal histidine, other interactions within the active sites of these proteins must contribute to their preference for O-bound nitrite. The lowest-energy nitrito structure (**2a**), in which the remaining O atom of nitrite forms a hydrogen bond with the distal histidine, is located only 6.3 kcal/mol higher in energy than **1**. The two other nitrito structures, **2b** and **2c**, are only slightly higher in energy than **2a**. Our calculations follow the experimentally observed results and predict **1** and **2a** to be energetically most favorable. In particular, **2a** corresponds closely to the experimentally observed structures of met-Hb/Mb nitrite adducts. Our computational models favor hydrogen bonding between the terminal (not bound to Fe) O atom of NO_2^- and the distal histidine residue, whereas, experimentally, hydrogen bonding to the internal O atom of nitrite is also observed. This finding is attributed to secondary protein effects not accounted for in our model.

The interaction of NO with the two lowest-energy nitrite-bound structures, **1** and **2a**, was then analyzed in order to assess the potential of each binding mode for N_2O_3 generation. To accomplish this, potential energy surfaces (PESs) were generated for NO binding to $Hb/Mb^{III}NO_2^-$ in both **1** and **2a**. Importantly, in the case of the N-bound nitrite complex **1**, the N atom of nitrite is involved in strong binding to the Fe^{III} center and, hence, cannot interact with NO. Any attempts to form an N−N bond led to the movement of NO toward nitrite's uncoordinated O atoms. This, however, does not allow for NO coupling to the coordinated NO_2^- ligand either: as seen in Figure 1, left, NO addition to **1** leads to a very shallow energy minimum around 2.8 Å, corresponding to the formation of a very weakly associated $[ONO\text{-}NO]^-$ adduct. Below ONO−NO distances of 2.8 Å, the nitrite−NO interaction is dissociative. These results demonstrate that *in the normally encountered nitro complex of ferric heme, nitrite is not reactive toward NO and, hence, eq* 3 *is not feasible.*

In the case of **2a**, which is the experimentally observed binding mode in met-Hb, the NO_2^- ligand is coordinated with one O-atom to ferric heme (cf. Scheme 1). Importantly, the N atom of nitrite is now accessible to react with free NO and, indeed, is able to form the necessary N−N bond to generate N_2O_3. Figure 1, right, shows the calculated PES for NO binding to $Hb/Mb^{III}NO_2^-$. The calculated $Fe^{III}N_2O_3(-)$ structure at the energy minimum predicts a long $ON-NO_2^-$ distance of 2.05 Å and is stabilized by ~7 kcal/mol relative to the NO-free precursor. This provides a significant driving force for NO addition

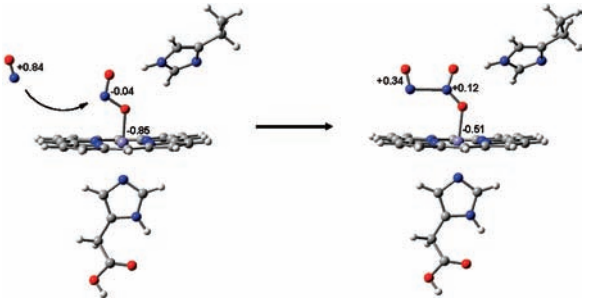

Figure 2. Intermediate N_2O_3-bound structure resulting from reaction of the nitrito complex **2a** with NO. Calculated spin-density values for the NO adduct of **2a** (on the right) indicate a delocalized radical throughout the $Fe^{III}N_2O_3(-)$ unit. Spin-density values are indicated for NO, NO_2^-, and Fe. Calculated with BP86/TZVP.

to **2a**. No energy barrier (intermediate) is observed for formation of the $N_2O_3(-)$ ligand. A further thermodynamic driving force will originate from the entropy gain upon formation of the N−N bond, which can, in general, be estimated to be about −10 kcal/mol at room temperature for small-molecule binding to transition-metal complexes.[7] On the basis of this estimate, *the free energy for NO addition to the nitrito complex* **2a** *is favorable by −15 to −20 kcal/mol at room temperature, and no energy barrier is observed for this process.*

Spin-density analysis of **2a** prior to NO association shows values of +0.84 on NO and −0.86 on Fe^{III} (see Figure 2, left). These values are consistent with the radical nature of NO and the low-spin Fe^{III} electronic structure of the $Hb/Mb^{III}NO_2^-$ complex. As NO approaches the bound nitrite, the spin density decreases in magnitude to +0.34 and −0.51 on NO and Fe, respectively. This observation is in agreement with the eventual reduction of iron from the ferric to the ferrous state upon dissociation of N_2O_3. The formally $Hb/Mb^{III}N_2O_3(-)$ complex, therefore, represents an intermediate state at which N_2O_3 is bound to the low-spin Fe center and the unpaired electron of NO is delocalized throughout the $Fe-O_2N-NO$ π system. The calculated structure of this N_2O_3-bound intermediate is shown in Figure 2, right. The calculated spin-density plot of the $Mb^{III}N_2O_3(-)$ intermediate is shown in Figure 3, left which further illustrates this observation.

Based on these results, NO attack on the bound nitrite of $Hb/Mb^{III}NO_2^-$ is not likely to occur through a radical−radical coupling mechanism between $NO_2^{\bullet}$ (as a resonance structure of the ferric nitrite complex) and the incoming $NO^{\bullet}$, as has been previously proposed.[2a] Calculated spin densities on the NO_2^- moiety consistently show values of <0.05 during the NO approach (see Figure 3, right). These data are consistent with a $Fe^{III}NO_2^-$ electronic structure. Additionally, the redox potential of the $NO_2^-/NO_2^{\bullet}$ pair is relatively high at approximately +1 V.[8] In comparison, the heme sites within Mb and Hb typically show redox potentials in the range of +50 to +200 mV and, therefore, are not likely capable of forming a $Hb/Mb^{II}NO_2^{\bullet}$ species.[9]

Upon formation of the $Hb/Mb^{III}N_2O_3(-)$ intermediate, N_2O_3 must be able to dissociate from the heme site in order to export NO out of the RBC. To model the dissociation process, N_2O_3 was stepwise dissociated from the low-spin Fe center of our Hb/Mb model to yield low-spin five-coordinate Hb/Mb^{II} and free N_2O_3, and the energy at each distance was calculated. The dissociation energy of N_2O_3 from the Fe center in the low-spin state is ~16 kcal/mol. Importantly, however, because five-coordinate ferrous Hb/Mb sites are high-spin, the spin crossover of the Fe

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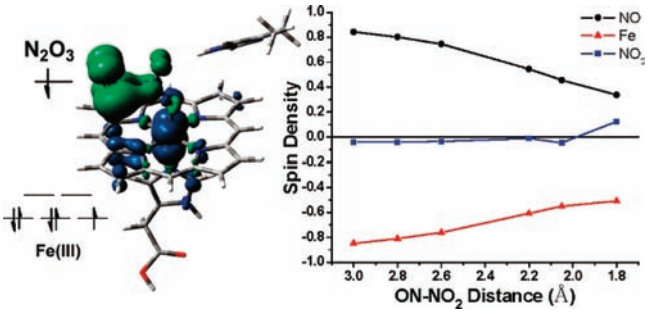

Figure 3. (left) Calculated (BP86/TZVP) spin-density plot of the Hb/$Mb^{III}N_2O_3(-)$ intermediate. The results show low-spin Fe^{III} bound to $N_2O_3(-)$ with a delocalized radical in the π system (green contour). (right) Spin-density plots for NO, NO_2^-, and Fe within **2a**. As NO approaches the $Fe^{III}ONO^-$ moiety, the spin density becomes delocalized across the whole Fe−ONO−NO(−) π system. Calculated with BP86/TZVP.

center upon dissociation of N_2O_3 also needs to be considered. Taking this into consideration, the N_2O_3 dissociation energy drops to 4−6.5 kcal/mol (B3LYP/TZVP, depending on the heme structure).[10] The predicted high-spin Hb/Mb^{II} product state, therefore, makes the N_2O_3 dissociation process energetically feasible. The spin crossover of the Fe center is expected very early on the PES because $N_2O_3(-)$ is only a weak ligand to a ferric heme. Because of this early spin crossover, the energy barrier for N_2O_3 dissociation should be very small. We employed B3LYP/TZVP calculations in order to estimate the Fe−$N_2O_3(-)$ distance at which the spin crossover can be expected. The calculations predict that the energy-minimized $Fe^{III}N_2O_3(-)$ structure shown in Figure 2, right, is, in fact, very close to the spin-crossover point, which can be estimated to occur at Fe−$N_2O_3(-)$ distances below 2.5 Å.

In summary, our calculations show that both the addition of NO to Hb/$Mb^{III}NO_2^-$ and the dissociation of N_2O_3 from the resulting Hb/$Mb^{III}N_2O_3(-)$ species are energetically feasible, resulting in a total energy for the complete process of −1 to −3 kcal/mol. The net entropy contribution is likely to be negligible because NO association followed by N_2O_3 dissociation can be expected to be close to entropically neutral. The free energy for N_2O_3 production by met-Hb can, therefore, conservatively be estimated to be slightly exothermic, which indicates that the reaction is biologically feasible. One important restriction for this process is that this reaction is only possible in the ferric nitrito (O-bound) binding mode of nitrite; otherwise, this reaction is energetically very unfavorable. Through generation of the metastable species N_2O_3, it would be possible for NO to escape inactivation (trapping) within RBCs as previously proposed.[3] N_2O_3 could then partition out of the RBC and release NO, effectively acting as an NO transporter or, alternatively, could induce RS-NO formation which could then act as the NO carrier.[12] The released NO would then induce arterial vasodilation in the area of hypoxic tissue. The calculations presented in this paper provide corroborative evidence that the underlying nitrite anhydrase reaction of met-Hb is indeed energetically feasible, which provides support for the idea of nitrite-dependent signaling in the cardiovascular system, as previously proposed by Basu et al.[2a] While this paper was under revision, Hopmann et al. published DFT results that also show the feasibility of the nitrite anhydrase reaction.[13] Importantly, the incorporation of the distal histidine residue in our Hb/Mb active site model (in contrast to ref 13) significantly lowers the predicted free energy of this process and further supports the possibility of N_2O_3-mediated hypoxic signaling.

■ ASSOCIATED CONTENT

Supporting Information. Computational details, structures, and tables of optimized Cartesian coordinates. This material is available free of charge via the Internet at http://pubs.acs.org.

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