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Hemoglobin as a Nitrite Anhydrase: Modeling Methemoglobin-Mediated N₂O₃ Formation

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Abstract

Nitrite has recently been recognized as a storage form of NO in blood and as playing a key role in hypoxic vasodilation. The ion is readily reduced to NO by hemoglobin in red blood cells, which, however, also presents a conundrum. Given NO's enormous affinity of ferrous heme, a key question concerns how it escapes capture by hemoglobin as it diffuses out of the red cells and to the endothelium, where vasodilation takes place. Dinitrogen trioxide (N₂O₃) has been proposed as a vehicle that transports NO to the endothelium, where it dissociates to NO and NO₂. Although N₂O₃ formation might be readily explained via the reaction

the exact manner in which methemoglobin (Hb-Fe³⁺), nitrite and NO interact with one another is unclear. Both an 'Hb-Fe³⁺-NO₂⁻ + NO' pathway and an 'Hb-Fe³⁺-NO + NO₂⁻' pathway have been proposed. Neither pathway has been established experimentally. Nor has there been any attempt until now to theoretically model N₂O₃ formation, the so-called nitrite anhydrase reaction. Both pathways have been examined here in a detailed density functional theory (DFT, B3LYP/TZP) study and both have been found to be feasible based on energetics criteria. Modeling the 'Hb-Fe³⁺-NO₂⁻ + NO' pathway proved complex. Not only are multiple linkage-isomeric (*N*- and *O*- coordinated) structures conceivable for methemoglobin-nitrite, multiple isomeric forms are also possible for N₂O₃ (the lowest-energy state has an N-N-bonded nitro-nitrosyl structure, O₂N-NO). We considered multiple spin states of methemoglobin-nitrite as well as ferromagnetic and antiferromagnetic coupling of the Fe(III) and NO spins. Together, the isomerism and spin variables result in a diabolically complex combinatorial space of reaction pathways. Fortunately, transition states could be successfully calculated for the vast majority of these reaction channels, both *M_S* = 0 and *M_S* = 1. For a six-coordinate Fe³⁺-*O*-nitrito starting geometry, which is plausible for methemoglobin-nitrite, we found that N₂O₃ formation entails barriers of about 17–20 kcal/mol, which is reasonable for a physiologically relevant reaction. For the 'Hb-Fe³⁺-NO + NO₂⁻' pathway, which was also found to be energetically reasonable, our calculations indicate a two-step mechanism. The first step involves transfer of an electron from NO₂⁻ to the Fe³⁺-heme-NO

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Supporting Information. Optimized geometries, spin densities and relative energies for alternative reaction pathways, as discussed in the text.

($\{\text{FeNO}\}^6$) center, resulting in formation of nitrogen dioxide and an Fe^{2+} -heme-NO ($\{\text{FeNO}\}^7$) center. Subsequent formation of N_2O_3 entails a barrier of only $8.1 \text{ kcal mol}^{-1}$. From an energetics point of view, the nitrite anhydrase reaction thus is a reasonable proposition. Although it is tempting to interpret our results as favoring the ' $\{\text{FeNO}\}^6 + \text{NO}_2^-$ ' pathway over the ' $\text{FeIII-nitrite} + \text{NO}$ ' pathway, both pathways should be considered energetically reasonable for a biological reaction and it seems inadvisable to favor a unique reaction channel based solely on quantum chemical modeling.

Introduction

The ubiquitous role of nitric oxide (NO) as a biological signaling molecule, in processes such as vasodilation, neurotransmission, immune responses, and mitochondrial respiration, has been recognized for some time.^[1,2,3,4] For vasodilation in mammalian systems, eNOS (endothelial NO synthase) was long thought to be the exclusive source of NO. Recently, blood nitrite has been shown to play a key role in hypoxic vasodilation and has been recognized as an important storage form of NO.^[5,6] A variety of heme proteins are able to reduce nitrite to NO,^[7,8,9] of which globins such as hemoglobin, cytoglobin and neuroglobin appear to be particularly important in mammalian systems. In blood, this reduction takes place in the red blood cells:

As a basis for vasodilation, however, equation (1) produces a conundrum: To elicit vasodilation, the NO produced has to diffuse out of the red cells and into the endothelium; this, however, is an impossibly unlikely process given NO's enormous affinity for ferrous heme (i.e. deoxyhemoglobin).^[10,11] One attractive hypothesis is that nitric oxide is converted to a less reactive transport molecule, which can be transformed back into NO at the target site.^[10] Dinitrogen trioxide (N_2O_3) has been proposed as a likely candidate for such a transport molecule.^[12,13,14,15] The uncharged N_2O_3 molecule is thought to be able to diffuse through the cell membrane, and can also easily be converted back to NO (and NO_2^-) via homolytic cleavage.^[16]

Verification of the role of N_2O_3 in nitric oxide signaling requires elucidation of the pathways leading to conversion of nitrite and/or NO to N_2O_3 . Several recent studies indicate that N_2O_3 formation might be mediated by methemoglobin.^[12,13,17,18,19] Basu *et al.* have suggested that N_2O_3 is formed by the reaction of methemoglobin-nitrite with NO.^[12] The overall reaction implies conversion of two molecules of nitrite to one molecule of N_2O_3 and is thought to happen simply by recombination of the products of equation (1), as depicted below:^[12]

Indirect evidence suggests that such a 'nitrite anhydrase' reaction is chemically viable. Although NO should possess a 10-to 100-times higher affinity for ferric heme than NO_2^- , [12] thermal reduction studies in glass matrix indicate that NO molecules entering the distal heme-pocket of the nitrite-coordinated methemoglobin would not displace the nitrite ligand to form Fe^{3+} -NO.^[20,21] Instead a ferrous intermediate is observed, which is consistent with formation of Fe^{2+} - N_2O_3 .^[21,22]

An alternative reaction mechanism for methemoglobin-mediated formation of N_2O_3 has been postulated by Ford and coworkers,^[19] involving direct nitrite attack on Hb-Fe^{3+} -NO,

as shown in equation 3. Two variants of this mechanism have been proposed; one involves straightforward nucleophilic attack by NO_2^- on $\text{Hb-Fe}^{3+}\text{-NO}$; the other entails initial electron transfer from NO_2^- to create an $\text{Hb-Fe}^{2+}\text{-NO}$ or $\{\text{FeNO}\}^7$ center, followed by reaction of the NO_2 (nitrogen dioxide) so produced with free or heme-bound NO to give N_2O_3 :

Quantum chemical studies of Fe^{3+} -heme- NO_2^- complexes indicate that the reactivity of methemoglobin-bound nitrite is likely to depend on the exact coordination mode of nitrite.^[12,23] Three main linkage isomers are known, involving coordination through the nitrogen atom (*N*-nitrito) or through the oxygen atom (*O*-nitrito α and *O*-nitrito β , Figure 1). The X-ray crystal structure of wild-type human methemoglobin exhibits both the *O*-nitrito α and β forms.^[24] The *N*-nitrito form is well-known from bacterial nitrite reductases.^[25,26,27] Theoretical studies show that the nitrite linkage isomers display different relative energies and spin density profiles, which are likely to influence available reaction pathways.^[12,23,28,29] Experimental and theoretical results on heme proteins and different $\text{Fe}^{\text{III}}\text{-NO}_2^-$ complexes also indicate that the preferred binding mode might depend on the presence and orientation of distal hydrogen-bond donors.^[30,31,32]

Here we present an extensive quantum chemical study of different reaction pathways for N_2O_3 formation from five- and six-coordinate Fe^{3+} -porphyrin (*met*-heme) complexes. We have examined in detail the effect of nitrite linkage isomerism linkage, overall coordination number, N_2O_3 isomerism, and overall spin multiplicity on the activation barriers and reaction free energies for N_2O_3 formation. We have also explored the alternative reaction pathway, involving N_2O_3 formation from $\text{Hb-Fe}^{3+}\text{-NO}$ and free nitrite. The calculations show that Fe^{3+} -mediated N_2O_3 formation is indeed energetically feasible, consistent with the proposed role of N_2O_3 in nitric oxide signaling.

Computational Details

Models— N_2O_3 formation was studied with three different quantum chemical models shown in Figure 2: Model A, comprising five-coordinate $\text{Fe}^{3+}\text{-Por-NO}_2^-$ (Por = porphin) reacting with free NO; Model B, comprising six-coordinate $\text{Fe}^{3+}\text{-Por-Im-NO}_2^-$ (Im = imidazole) reacting with free NO; and Model C, comprising six-coordinate $\text{Fe}^{2+}\text{-Por-Im-NO}$ ($\{\text{FeNO}\}^7$) reacting with free NO_2 radical. Different Fe-NO_2^- linkage isomers (Figure 1) were studied for Models A and B. The overall charge of all three models is 0.

For computations of electron affinities (EA) and ionization potentials (IP), calculations were performed on a 6-coordinated $\{\text{FeNO}\}^6 \text{Fe}^{3+}\text{-Por-Im-NO}$ complex ($S = 0$, charge +1) and the corresponding reduced $\{\text{FeNO}\}^7 \text{Fe}^{2+}\text{-Por-Im-NO}$ complex ($S = 1/2$, charge 0), as well as on the free NO_2^- and $\text{NO}_2\cdot$ molecules.

Methods—All calculations were performed with the DFT functional B3LYP,^[33,34] as implemented in the Gaussian 03 package.^[35] Optimizations of Model A were carried out with the triple- ζ basis set 6-311+G(d,p). For Models B and C and for IP calculations, geometry optimizations were performed with the double- ζ basis set 6-31G(d,p), followed by single point calculations with 6-311+G(d,p) to obtain improved energies for evaluation of reaction barriers. The effect of the surrounding solvent was modeled with single point calculations with the IEFPCM model, employing water as solvent and a dielectric constant of $\epsilon = 4$ (resembling the protein environment).

All reaction pathways were analyzed on different spin surfaces, corresponding to an overall $M_S = 0$ state (referred to as S1) or an overall $M_S = 1$ state (T1 or T2, Figure 3). For open-shell $M_S = 0$ calculations, broken-symmetry wavefunctions were obtained with the stable = opt utility and were then employed as a starting guess for geometry optimizations. Frequency calculations were performed on all optimized geometries to ensure only positive eigenvalues for minima and a single imaginary frequency for saddle points (TS). Thermochemical data and temperature corrections were computed at 298.15 K for all optimized geometries. Reported energies are solvent-corrected Gibbs Free energies (ΔG^{sol}) unless otherwise indicated. Relative energies are given referenced to the lowest-energy conformer of each model. Geometries and spin density plots were visualized with Chemcraft.^[36]

Results

(a) Some general remarks

The various isomerism and spin state possibilities lead to a formidable combinatorial space of reaction channels, which we have nonetheless explored quite thoroughly. Before presenting a systematic account of the different pathways considered, some additional remarks on these variables might be helpful. As mentioned above, we have taken into account the three linkage isomers of $\text{Fe}^{3+}\text{-NO}_2^-$ and different isomers of N_2O_3 (Figure 1). The asymmetric form of N_2O_3 (*asym*- N_2O_3) has a primarily ionic bond between the nitrogen centers and is the form observed in gas phase.^[37] The symmetric (*sym*- N_2O_3) and *trans-cis* forms have been observed in low temperature matrix.^[38] Theoretical studies show that the *sym*- and *trans-cis*- N_2O_3 forms are respectively ~ 1 and ~ 6 kcal mol⁻¹ less stable than *asym*- N_2O_3 .^[39] While concentrating on the formation of the two more stable N_2O_3 isomers, *asym*- and *sym*- N_2O_3 , we have not neglected to examine the formation of *trans-cis*- N_2O_3 .

We have investigated the ' $\text{Fe}^{3+}\text{-heme-NO}_2^- + \text{NO}$ ' pathway [equation (2)] suggested by Basu *et al.*^[12] with both five- and six-coordinate heme models (Models A and B, Figure 2). Although consideration of five-coordinate models might not seem absolutely required, the proximal histidine has been shown to be susceptible to dissociation in a number of heme proteins.^[40,41,42] We therefore examined both types of models. The coordination number is likely to affect the spin state of the iron center, which in turn should lead to different reaction channels. For low-spin Fe(III) ($S = 1/2$), reaction with free NO can occur on two different surfaces, depending on the relative alignment of the spins of the NO and of the iron center. Antiparallel alignment results in an open-shell $M_S = 0$ surface (denoted hereafter as S1, Figure 3, I), whereas parallel alignment produces an $M_S = 1$ surface (denoted T1, Figure 3, II). If the Fe(III) center is intermediate spin state ($S = 3/2$), reaction with NO would occur on a different $M_S = 1$ surface (hereafter referred to as T2, Figure 3, III). Studies of the S1 and T2 surfaces entailed rather challenging broken-symmetry transition state optimizations; to our considerable satisfaction, the vast majority of these were successful.

To keep track of the extensive combinatorial space of isomerism and spin variables, we will refer to each optimized geometry with a four-part subscript, indicating the overall coordination number, nitrite linkage isomerism, the spin surface, and the N_2O_3 isomer produced as shown below in Scheme 1:

(b) N_2O_3 formation from $\text{Fe}^{3+}\text{-Por-NO}_2^-$

Briefly stated, B3LYP/6-311G(d,p) calculations indicate an intermediate-spin state ($S = 3/2$) iron center for the five-coordinate $\text{Fe}^{3+}\text{-Por-O-nitrito}$ complex, which implies that N_2O_3 formation occurs on the T2 surface. Formation of *asym*- N_2O_3 on this surface is significantly

lower in energy (by $\sim 9 \text{ kcal mol}^{-1}$) than formation of *sym*-N₂O₃. Some additional details are as follows.

The reactant structures of the five-coordinate ($S = 3/2$) *O*-nitrito linkage isomers and free NO have similar relative energies (Table 1). The *O*-nitrito β isomer (Reac_{A-O β -T2}, Figure 4) was employed as the energy reference for all calculations on the five-coordinated system in this study. The *O*-nitrito a reactant is only $0.4 \text{ kcal mol}^{-1}$ higher in energy (Table 1 and Supporting Information Figure S1).^[43]

The preferred reaction pathway for formation of N₂O₃ from five-coordinate ($S = 3/2$) Fe³⁺-Por-NO₂⁻ involves generation of *asym*-N₂O₃ from the *O*-nitrito β isomer (Reac_{A-O β -T2}, Figure 4). At the transition state (TS_{A-O β -T2-*asym*}), the incipient N_{NO}-N_{NO2} bond is about $1.90 \text{ \AA}$. The displacement vector of the imaginary frequency ($176i \text{ cm}^{-1}$) shows that bond formation occurs simultaneously with Fe-O dissociation, resulting in N₂O₃ release. The overall spin population on NO at the TS is -0.53 , compared to -1.01 for the reactant, indicating significant bond formation between NO and NO₂⁻. Simultaneously with NO attack, an electron is transferred from the NO₂⁻ ligand to the iron center, resulting in formation of intermediate-spin ($S = 1$) Fe²⁺. The overall barrier for this reaction pathway is $11.6 \text{ kcal mol}^{-1}$ (Figure 4, top), indicating a very feasible pathway. The reaction energy is positive ($12.0 \text{ kcal mol}^{-1}$),^[44] making product formation endothermic. It is likely, however, that the displaced N₂O₃ molecule would be replaced by another ligand (such as NO or H₂O), which would reduce the reaction energy.

Formation of *asym*-N₂O₃ from the *O*-nitrito a isomer of intermediate-spin Fe³⁺-Por-NO₂⁻ occurs analogously to the *O*-nitrito β isomer and displays similar reactivity (TS_{A-O α -T2-*asym*}, $\Delta G^{\text{sol}} = 12.4 \text{ kcal mol}^{-1}$, $\Delta H^{\text{sol}} = 7.5 \text{ kcal mol}^{-1}$, $\Delta E = 7.3 \text{ kcal mol}^{-1}$, Supporting Information Figure S2A). For the *N*-nitrito isomer, *asym*-N₂O₃ formation is not possible, as it would require dissociation of the nitrite ligand from the iron ion to allow for N_{NO}-N_{NO2} bond formation. Instead of an N_{NO}-N_{NO2} interaction, N_{NO}-O_{NO2} bond formation can occur, leading to generation of *sym*-N₂O₃. The optimized transition states for formation of *sym*-N₂O₃ from the three nitrite linkage isomers of intermediate-spin Fe³⁺-Por-NO₂⁻ display barriers of 20.0 to $20.8 \text{ kcal mol}^{-1}$, which is $\sim 9 \text{ kcal mol}^{-1}$ higher than for formation of *asym*-N₂O₃ (Figure 4, top). At the optimized geometry for the *O*-nitrito β TS, the incipient N_{NO}-O_{NO2} bond is $1.75 \text{ \AA}$ (TS_{A-O β -T2-*sym*}, imaginary frequency = $306i \text{ cm}^{-1}$, Figure 4 bottom, right). The NO radical has an overall spin of only -0.23 , and the iron center is partially reduced with a spin population of 2.35 . The optimized product (Prod_{A-*sym*}) indicates an endothermic reaction energy ($14.5 \text{ kcal mol}^{-1}$), which is slightly higher than that for *asym*-N₂O₃. The *O*-nitrito a isomer shows a similar spin density profile and reactivity (TS_{A-O α -T2-*sym*}, $\Delta G^{\text{sol}} = 20.0 \text{ kcal mol}^{-1}$, $\Delta H^{\text{sol}} = 16.0 \text{ kcal mol}^{-1}$, $\Delta E = 14.0 \text{ kcal mol}^{-1}$, Supporting Information Figure S2B). Formation of *sym*-N₂O₃ from the *N*-nitrito isomer entails slightly different bond distances (TS_{A-N-T2-*sym*}, Supporting Information Figure S3), but a similar barrier of $20.3 \text{ kcal mol}^{-1}$ ($\Delta H^{\text{sol}} = 15.3 \text{ kcal mol}^{-1}$, $\Delta E = 14.0 \text{ kcal mol}^{-1}$).

The results for the five-coordinate complexes show facile formation of *asym*-N₂O₃ from the intermediate-spin *O*-nitrito linkage isomers. If the five-coordinate Fe(III) center instead adopts a low-spin state ($S = 1/2$, S1 and T1 surface, Figure 3), the reaction barriers are increased by several kcal mol^{-1} . The low-spin reactant states of three nitrite-linkage isomers display energies of ~ 5 to 11 kcal mol^{-1} relative to the intermediate-spin reactant complexes (Table S1, Supporting information), underlining the preference for an intermediate-spin state at the five-coordinated iron-center (for optimized geometries, see Supporting Information Figure S4–S6). Formation of *asym*-N₂O₃ from the low-spin *O*-nitrito- β isomer has a barrier of $14.7 \text{ kcal mol}^{-1}$ (T1 surface, Figure 5, top), compared with $11.6 \text{ kcal mol}^{-1}$ for the

intermediate-spin complex. At the TS (TS_{A-Oβ-T1-*asym*}, Figure 5), the incipient N_{NO}-N_{NO2} bond distance is 2.49 Å, which is much longer than that on the T2 surface (1.90 Å, TS_{A-Oβ-T2-*asym*}, Figure 4). The iron center is essentially reduced to Fe²⁺ (spin population of 1.97), which results in significant radical character of the NO₂ ligand (spin population of -0.58, Figure 5). This allows N_{NO}-N_{NO2} bond formation to occur earlier on the T1 surface than on the T2 surface. Formation of *sym*-N₂O₃ from the low-spin *N*-nitrito isomer has a barrier of 23.0 kcal mol⁻¹ ($\Delta H^{\text{sol}} = 17.4$ kcal mol⁻¹, $\Delta E = 16.6$ kcal mol⁻¹, Supporting Information Figure S6C), which is also a few kcal mol⁻¹ higher than that on the T1 surface (20.0 kcal mol⁻¹).

In summary, the computational results suggest that five-coordinate Fe³⁺-Por-NO₂⁻ prefers an intermediate-spin ($S = 3/2$) iron center (T2 surface) over a low-spin one ($S = 1/2$). Attack of nitric oxide on the coordinated nitrite ion preferably leads to formation of *asym*-N₂O₃ (with a computed barrier of 11.6 kcal mol⁻¹ on the T2 surface).

(c) N₂O₃ formation from Fe³⁺-Por-Im-NO₂⁻

Our studies of N₂O₃ formation from six-coordinate Fe³⁺-Por-Im-NO₂⁻, which are very possibly more faithful mimics of methemoglobin, may be summarized as follows. Low-spin Fe(III) (S1 and T1) surfaces are most relevant for this model. Formation of a T2 species with intermediate-spin Fe(III) center requires partial loss of the imidazole ligand so the complex would no longer be truly 6-coordinate. *N*-nitrito coordination of NO₂⁻ is slightly favored over *O*-nitrito coordination. For the *O*-nitrito models, *asym*-N₂O₃ formation is ~9 kcal mol⁻¹ lower in activation-barrier than *sym*-N₂O₃ formation. Both *sym*- and *asym*-N₂O₃ initially remain coordinated to the iron complex, displaying a noninnocent electronic structure. For the *N*-nitrito isomer, the results indicate a preference for formation of *trans-cis*-N₂O₃ over *sym*-N₂O₃. Some details of our findings are discussed below.

Six-coordinate Fe³⁺-Por-Im-NO₂⁻ has an unambiguous low-spin Fe(III) center ($S = 1/2$), so the S1 and T1 surfaces (Figure 3) are expected to be most relevant (however, the T2 surface has been considered as well). On the low-spin surface, *N*-nitrito coordination is favored by 1.3 kcal mol⁻¹ over *O*-nitrito coordination. Parallel (T1) or antiparallel (S1), alignment of the spin on NO results in similar relative energies (Table 2 and Supporting Information Figure S7 and S8). Our results are in line with previous theoretical calculations on Fe³⁺-Por-L-NO₂⁻ complexes (L = DMS,^[28] His,^[12] NO,^[29] Im^[23]), where the *N*-nitrito form was favored over the *O*-nitrito isomers by 1.9 to ~10 kcal mol⁻¹ (electronic energies only). Experimental results by Kurtikyan *et al.* have shown that whereas five-coordinate Fe³⁺-Por-NO₂⁻ complexes prefer *O*-nitrito coordination, the stable form of the six-coordinate complexes is the *N*-nitrito bound form, in agreement with our results.^[45]

N_{NO}-N_{NO2} bond formation and generation of *asym*-N₂O₃ from the low-spin *O*-nitrito α -isomer of Fe³⁺-Por-Im-NO₂⁻ on the T1 surface has a barrier of 19.4 kcal mol⁻¹ (TS_{B-O α -T1-*asym*}, Figure 6, top). For the S1 surface, a similar barrier is obtained ($\Delta G^{\text{sol}} = 20.2$ kcal mol⁻¹, $\Delta H^{\text{sol}} = 14.6$ kcal mol⁻¹, $\Delta E = 14.2$ kcal mol⁻¹, Supporting Information Figure S9). The spin population on NO at the TS is reduced to 0.6 (TS_{B-O α -T1-*asym*}, Figure 6) showing significant electron pairing with NO₂⁻. In contrast to five-coordinate Fe³⁺-Por-NO₂⁻, the unpaired electron generated on NO₂⁻ at the TS is not transferred to the iron, which therefore remains low-spin Fe(III). The displacement vector of the imaginary frequency of the optimized TS (258i cm⁻¹) reveals that bond formation between NO and NO₂⁻ is not accompanied with Fe-O bond breakage, i.e. the *asym*-N₂O₃ product remains coordinated to iron (Figure 6). The bound-product states display a noninnocent electronic structure, with a spin-population of 0.94 on the *asym*-N₂O₃ ligand. The N-N bond length is rather short, 1.49 Å (compared to 1.89 Å in free N₂O₃), which is a consequence of radical character of the non-innocent ligand.

Formation of *asym*-N₂O₃ from the *O*-nitrito β -isomer of low-spin Fe³⁺-Por-Im-NO₂[−] occurs as from the *O*-nitrito α -isomer, but involves a slightly lower barrier of 17.7 kcal mol^{−1} (TS_{B-O β -T1-*asym*}, T1 surface, $\Delta H^{\text{sol}} = 14.3$ kcal mol^{−1}, $\Delta E = 15.7$ kcal mol^{−1}, Supporting information Figure S10). The geometry parameters and spin profiles are similar for *O*-nitrito α and β , supporting a similar reactivity towards N₂O₃ formation for the two six-coordinate low-spin *O*-nitrito isomers.

Analysis of the alternative N_{NO}-O_{NO2} bond formation pathways show that *sym*-N₂O₃ formation is less feasible than *asym*-N₂O₃ formation, as also observed for the five-coordinate complex. Similar results were obtained for both low-spin surfaces, S1 and T1, with barriers of 25.8 kcal mol^{−1} (TS_{B-O α -S1-*sym*}, Supporting Information Figure S11) and 30.1 kcal mol^{−1} (TS_{B-O α -T1-*sym*}, Figure 7, top), respectively. The *sym*-N₂O₃ product (Prod_{B-O α -T1-*sym*}) displays noninnocent character (spin population of 0.54, Figure 7, bottom), while remaining coordinated to the iron center. Release of the product is accompanied by reduction of the iron to intermediate-spin (*S* = 1) Fe(II) (Figure 7).

The reaction pathway for formation of *sym*-N₂O₃ from low-spin *N*-nitrito Fe³⁺-Por-Im-NO₂[−] proceeds as for the *O*-nitrito complexes and has a barrier of 24.5 kcal mol^{−1} (TS_{B-N-S1-*sym*}, S1 surface, $\Delta H^{\text{sol}} = 18.5$ kcal mol^{−1}, $\Delta E = 17.2$ kcal mol^{−1}, Supporting Information Figure S12). An alternative and more feasible route to N₂O₃ formation was obtained for the *N*-nitrito linkage isomer when N-O bond formation leads to *trans-cis*-N₂O₃; this process has a barrier of 19.8 kcal mol^{−1} (Figure 8, top). The *trans-cis* product also exhibits noninnocent character (Figure 8, bottom). The spin population on iron decreases from 1.11 at the reactant (Reac_{B-N-S1}) to 0.39 at the bound product geometry (Prod_{B-N-S1-*trans-cis*}), indicating that the iron center is partially reduced. The spin density on the *trans-cis*-N₂O₃ ligand is −0.45, which is centered on the iron-bound oxygen and the adjacent nitrogen. The electronic structure of the product is thus intermediate between Fe³⁺-N₂O₃[−] and Fe²⁺-N₂O₃. The results indicate that formation of *trans-cis*-N₂O₃ and *asym*-N₂O₃ is approximately equally feasible, despite the significant energy difference between the free forms of the two N₂O₃ isomers. Following release of *trans-cis*-N₂O₃, it is likely to isomerize to *sym*- or *asym*-N₂O₃, which are calculated to be 5 to 6 kcal mol^{−1} more stable.^[39] The computed transition state for isomerization of free *trans-cis*-N₂O₃ to *sym*-N₂O₃ has a barrier of only 4.1 kcal mol^{−1} (Supporting Information Figure S13). The computational results show that N₂O₃ formation from the six-coordinate low-spin (*S* = ½) complex occurs in two steps: in the first step, N-N or N-O bond formation occurs, resulting in formation of a coordinated one-electron-reduced N₂O₃ form, followed by electron transfer to the iron center in the second step and release of free N₂O₃. Product dissociation involves formation of an intermediate-spin electronic structure (*S* = 1) at the iron center. This observation led us to also examine N₂O₃ formation from an intermediate-spin Fe³⁺-Por-Im-NO₂[−] reactant structure (T2 surface), from which N₂O₃ formation and release can occur in a single step (Figure 9). Formation of an intermediate-spin reactant complex of Fe³⁺-Por-Im-NO₂[−] is accompanied by a lengthening of the Fe-N_{Im} bond to 2.44 Å (Supporting Information S14A), highlighting the importance of the proximal histidine in modulating the spin state of the iron center. The resulting complex has a relative energy of 0.8 kcal mol^{−1}, making it energetically accessible. The optimized transition state for *sym*-N₂O₃ formation on the T2 surface exhibits an elongated Fe-O_{NO2} bond, implying that product release occurs concertedly with N_{NO}-O_{NO2} bond formation (TS_{B-O α -T2-*sym*}, Supporting Information S14B). The incipient N_{NO}-O_{NO2} bond is very short, 1.57 Å. The barrier for this transition state is 22.1 kcal mol^{−1} (Figure 9). The products consist of a dissociated closed-shell N₂O₃ molecule and an intermediate-spin Fe(III)-Im complex (Figure 9). Similar results were also obtained for the *N*-nitrito isomer, for which the formation of *sym*-N₂O₃ on the T2 surface has a barrier of 21.2 kcal mol^{−1} ($\Delta H^{\text{sol}} = 20.0$ kcal mol^{−1}, $\Delta E = 19.1$ kcal mol^{−1}, TS_{B-N-T2-*sym*}, Supporting Information Figure S15).

The results for intermediate-spin $\text{Fe}^{3+}\text{-Por-Im-NO}_2^-$ are thus analogous to the five-coordinate complex, with product formation and release occurring in the same step. It therefore appears that N_2O_3 formation from six-coordinate heme-nitrite complexes would be more facile if the *trans* ligand would be weakened, for example, via hydrogen-bond interactions with other residues.

(d) N_2O_3 formation from $\text{Fe}^{3+}\text{-NO}$

Ford and coworkers have suggested that N_2O_3 formation from methemoglobin might occur via an alternative pathway in which an NO-coordinated heme reacts with free NO_2^- (eq. 3).^[19] Two variants of this pathway have been proposed, a direct attack of the nitrite anion on $\text{Fe}^{3+}\text{-heme-NO}$ ($\{\text{FeNO}\}^6$),^[19] or a two-step pathway, involving electron transfer from NO_2^- to the heme complex in the first step (yielding $\text{Fe}^{2+}\text{-heme-NO}$ or $\{\text{FeNO}\}^7$), followed by attack of the NO_2 radical so produced on NO.^[19] We have analyzed the proposed pathways computationally and the results may be summarized as follows: Electron transfer from free NO_2^- to the porphyrin complex is exothermic, indicating that the initial step involves formation of an NO_2 radical. Reaction of the NO_2 radical with $\text{Fe}^{2+}\text{-Por-Im-NO}$ ($\{\text{FeNO}\}^7$) to give N_2O_3 has a low barrier, indicating that this could provide a very facile way for N_2O_3 formation. Details of our findings are given below.

We have evaluated whether a diradical reactant state (originating from electron transfer from NO_2^- to the $\text{Fe}^{3+}\text{-Por-Im-NO}$ complex, Figure 10) is uphill or downhill from the charge-separated reactant by computing the ionization potential (IP) of NO_2^- and comparing it to the electron affinity (EA) of $\text{Fe}^{2+}\text{-Por-Im-NO}^+$. The computed EA of $\text{Fe}^{2+}\text{-Por-Im-NO}^+$ is $-118.1 \text{ kcal mol}^{-1}$, compared to a computed IP for NO_2^- of $103.9 \text{ kcal mol}^{-1}$ (ΔG^{sol}). Transfer of an electron from NO_2^- to the iron-NO complex is thus favorable by $14.2 \text{ kcal mol}^{-1}$. The results indicate that N_2O_3 formation is more likely to occur via a diradical reactant complex ($\text{Fe}^{2+}\text{-Por-Im-NO}$ with $\text{NO}_2^\cdot$) rather than directly from a charge-separated complex ($\text{Fe}^{2+}\text{-Por-Im-NO}^+$ and NO_2^-).

The NO_2 radical can react with the $\text{Fe}^{2+}\text{-Por-Im-NO}$ ($\{\text{FeNO}\}^7$) center to yield N_2O_3 . The reaction has to occur on an open-shell $M_S = 1$ surface (S1) to allow bond formation between the radicals. At the optimized reactant state, the spin population on NO_2 is exactly 1, aligned antiparallel to the unpaired electron on $\text{Fe}^{2+}\text{-Por-Im-NO}$ ($\text{Reac}_{\text{C-S1}}$, Figure 11, bottom). The spin density of the $\{\text{FeNO}\}^7$ complex resides primarily on the NO ligand (0.79), with only a small amount on the iron. Although formation of different isomers of N_2O_3 might be anticipated, only the transition state for formation of *trans-cis*- N_2O_3 was successfully located ($\text{TS}_{\text{C-S1-trans-cis}}$, imaginary frequency $214i \text{ cm}^{-1}$, Figure 11). At the TS, the incipient $\text{N}_{\text{NO}}\text{-O}_{\text{NO}_2}$ bond is $2.17 \text{ \AA}$. The spin density on the NO_2 radical is reduced to -0.33 , indicating strong electron pairing with the coordinated NO. The barrier for this transition state is $8.1 \text{ kcal mol}^{-1}$ (solvent corrections not included^[46]) relative to the reactant complex, implying a very facile pathway for N_2O_3 formation. The closed-shell N_2O_3 generated remains coordinated to the iron center and has a relative energy of $7.3 \text{ kcal mol}^{-1}$ (Figure 11).

Conclusions

An unresolved aspect of nitric oxide physiology involves processes that prevent capture of NO by heme species. A possible scenario involves transformation of NO into an intermediate that is less likely to be captured, allowing it to diffuse to relevant target sites. A key candidate for such an intermediate is the N_2O_3 molecule.^[12] N_2O_3 is also a potent nitrosating agent so signaling could also take place via formation of nitrosothiols.^[14,47,48] Different pathways for methemoglobin-mediated formation of N_2O_3 have been put forward, involving initial formation of an $\text{Fe}^{3+}\text{-nitrite}$ ^[12] or $\text{Fe}^{3+}\text{-NO}$ ^[19] complex. We have

presented an extensive DFT study of the various mechanistic alternatives, including careful consideration of the effect of coordination number, nitrite linkage conformation, electronic state, and N_2O_3 isomerism. For a six-coordinate *O*-nitrito starting geometry, which is plausible for methemoglobin-nitrite, N_2O_3 formation entails barriers of about 17–20 kcal/mol. For N_2O_3 formation via nitrite attack on a six-coordinate Fe^{3+} -heme-NO ($\{\text{FeNO}\}^6$) center, our calculations indicate such a two-step mechanism; the first step involves transfer of an electron from NO_2^- to the Fe^{3+} -heme-NO complex, resulting in formation of nitrogen dioxide and an Fe^{2+} -heme-NO ($\{\text{FeNO}\}^7$) center. Subsequent formation of *trans-cis*- N_2O_3 entails a barrier of only 8.1 kcal mol⁻¹ (Figure 11). It is tempting to interpret these energetics results as favoring the ' $\{\text{FeNO}\}^6 + \text{NO}_2^-$ ' pathway over the ' Fe^{III} -nitrite + NO' pathway; both pathways, however, should be considered energetically feasible for a biological reaction.

The above results clearly add to the evidence in favor of hemoglobin's role as a nitrite anhydrase and of N_2O_3 's viability as a NO vehicle. Experimental support for N_2O_3 formation consists of the formation of *S*-nitrosothiols *in vivo* and *in vitro*.^[6,12,13,49] In addition, as mentioned above, Friedman and coworkers have observed reactions of nitrite-bound methemoglobin with NO in a glass matrix that strongly support the formation of N_2O_3 .^[20,21] Clearly, much additional work is necessary to determine the importance of N_2O_3 in nitrite-mediated NO and *S*-nitrosothiol signaling. Also, the possible relevance of the reaction pathways studied here for heme proteins other than hemoglobin remains an intriguing question for the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Nitrite-linkage isomers and free N₂O₃ isomers.

Figure 2.
Computational models investigated in this study.

Figure 3.

Spin surfaces on which the reaction of nitric oxide and $\text{Fe}^{3+}\text{-NO}_2^-$ can occur.

Computed reaction pathways:

Optimized geometries:

Figure 4.

Top: Computed reaction pathways and energies (kcal mol^{-1}) for formation of *asym*- and *sym*- N_2O_3 from intermediate-spin Fe^{3+} -Por- NO_2^- (Model A, O-nitrito β , surface T2). Bottom: Optimized geometries and spin densities for this pathway (distances are in Å, major and minor spin densities are in blue and magenta, respectively, contour value 0.03 eÅ^{-3} , spin populations are in italic).

Computed reaction pathway: T1 surface

Optimized geometries:

Figure 5.

Top: Reaction pathway and energies (in kcal mol⁻¹) for formation of *asym*-N₂O₃ from low-spin Fe³⁺-Por-NO₂⁻ (Model A, O-nitrito β, surface T1). Bottom: TS geometry (TS_{A-Oβ-T1-asym}).

Computed reaction pathway:

T1 surface

Optimized geometries:

Figure 6.

Top: Computed reaction pathway and energies (kcal mol $^{-1}$, relative to $\text{Reac}_{\text{B-N-T1}}$) for formation of $\text{asym-N}_2\text{O}_3$ from low-spin (T1) Fe^{3+} -Por-Im- NO_2^- (Model B, O-nitrito a). Bottom: Optimized geometries and spin densities for the TS ($\text{TS}_{\text{B-O}\alpha\text{-T1-asym}}$) and product ($\text{Prod}_{\text{B-O}\alpha\text{-T1-asym}}$).

Computed reaction pathway:

T1 surface

Optimized geometries:

Figure 7.

Top: Computed reaction pathway and energies (kcal mol⁻¹, relative to $\text{Reac}_{\text{B-N-T1}}$) for formation of $\text{sym-N}_2\text{O}_3$ from low-spin (T1) Fe^{3+} -Por-Im- NO_2^- (Model B, *O*-nitrito a). Bottom: Optimized geometries and spin densities for the transition state ($\text{TS}_{\text{B-O}\alpha\text{-T1-sym}}$), the bound product ($\text{Prod}_{\text{B-O}\alpha\text{-T1-sym}}$) and the free product ($\text{Prod}_{\text{B-sym}}$).

Computed reaction pathway:

Optimized geometries:

Figure 8.

Top: Computed reaction pathway and energies (kcal mol⁻¹, relative to Reac_{B-N-T1}) for formation of *trans-cis*-N₂O₃ from low-spin *N*-nitrito Fe³⁺-Por-Im-NO₂⁻ (Model B). Bottom: Optimized geometries and spin densities for the TS (TS_{B-N-S1-trans-cis}) and the bound product (Prod_{B-N-S1-trans-cis}).

T2 surface

Figure 9.

Computed reaction pathways and energies (kcal mol⁻¹, relative to Reac_{B-N-T1}) for formation of *sym*-N₂O₃ from intermediate-spin (T2) Fe³⁺-Por-Im-NO₂⁻ (Model B, O-nitrito a).

S1 surface

Figure 10.

Computed energies for transfer of an electron from NO_2^- to Fe^{2+} -Por-Im- NO^+ .

Computed reaction pathway:

S1 surface

Optimized geometries:

Figure 11.

Top: Computed reaction pathway and energies (in kcal mol⁻¹, relative to Reac_{C-S1}) for formation of *trans-cis*-N₂O₃ from Fe²⁺-Por-Im-NO and NO₂ (Model C, surface S1).

Bottom: Optimized geometries and spin densities.

Scheme 1.
 Illustration of employed subscripts.

Table 1Computed energies (kcal mol⁻¹) for linkage isomers of (S = 3/2) Fe³⁺-Por-NO²⁻ with free NO (T2 surface).^a

	ΔE^b	ΔG^b	$\Delta H^{\text{sol}} b,c$	$\Delta G^{\text{sol}} b,c$
Reac _{A-Oβ-T2}	0.0	0.0	0.0	0.0
Reac _{A-Oα-T2}	0.7	0.8	0.4	0.4

^aEnergies are given relative to Reac_{A-O β -T2},^bCorrected for basis set effects,^cIncludes solvent effects ($\epsilon = 4$).

Table 2

Computed energies (kcal mol⁻¹) for reactant geometries of low-spin Fe³⁺-Por-Im-NO₂⁻ with free NO (Model B, S1 and T1 surface).^a

	ΔE^b	ΔG^b	$\Delta H^{sol\ b,c}$	$\Delta G^{sol\ b,c}$
Reac _{B-N-S1}	0.0	0.5	0.0	0.5
Reac _{B-N-T1}	0.0	0.0	0.0	0.0
Reac _{B-Oα-S1}	1.7	1.4	1.2	1.4
Reac _{B-Oα-T1}	1.7	1.2	1.3	1.3
Reac _{B-Oβ-S1}	2.6	2.5	3.1	3.6
Reac _{B-Oβ-T1}	2.6	0.3	3.1	1.4

^aEnergies are given relative to Reac_{B-N-T1}

^bCorrected for basis set effects

^cIncludes solvent effects ($\epsilon = 4$).