Nitric oxide coupling to generate N₂O promoted by a single-heme system as examined by density functional theory

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Abstract: Bacteria utilize a heme/non-heme enzyme system to detoxify nitric oxide (NO) to N₂O. In order to probe the capacity of a single-heme system to mediate this NO-to-N₂O transformation, various scenarios for addition of electrons, protons, and a second NO molecule to a heme nitrosyl to generate N₂O were explored by density functional theory calculations. We describe, utilizing this single-heme system, several stepwise intermediates along pathways that enable the critical N–N bond formation step yielding the desired Fe–N₂O product. We also report a hitherto unreported directional second protonation that results in either productive N₂O formation with loss of water, or formation of a non-productive hyponitrous acid adduct Fe{HON=NOH}.

Keywords: nitric oxide coupling, hyponitrite, density functional theory, iron porphine, nitrous oxide

1. Introduction

The diatomic molecule nitric oxide (NO) is toxic to bacteria. It is thus not too surprising that bacteria have evolved sophisticated enzymes to detoxify NO into the less toxic nitrous oxide (N₂O) molecule. This detoxification pathway is essentially an NO reduction process catalyzed by the bacterial NO reductase (bacNOR) enzymes [1-5], and it forms part of a larger denitrification pathway that is an important component of the global nitrogen cycle [6]. The bacterial NO reduction reaction, utilizing a bimetallic heme/non-heme active site, is represented by equation 1.

A complementary fungal NO reduction pathway exists that, in contrast to the bacterial process, involves the direct participation of NADH in NO reduction in a monometallic single-heme active site [7-11].

Crystal structures of bacNORs were not available until recently [2, 12]. The active site of the bacNOR enzyme from *P. aeruginosa* contains a heme *b*₃ and a non-heme Fe_B center that is coordinated by three His sidechains and a Glu211 sidechain [2]. Several proposals have been put forward regarding plausible mechanisms for bacterial NO reduction, but all share the intermediacy of a hyponitrite intermediate along the path to N₂O formation [13-16]. The likely hyponitrite intermediates from the three most common proposed mechanisms are shown in Figure 1 [13]. In the *cis*-heme *b*₃ mechanism, the first NO ligand binds to the heme and is subsequently attacked by a second NO molecule. In the *trans* mechanism, NO binds to both heme and non-heme Fe prior to N–N bond formation. The *cis*-Fe_B mechanism involves the formation of an Fe_B-dinitrosyl compound prior to N–N bond formation. Interestingly, recent

reports describe the spectral characterization of protonated hyponitrite intermediates of the form $[\text{Fe}]\text{-}\{\text{N}(\text{OH})\text{N}(\text{O})\}\text{-}[\text{M}]$ ($\text{M} = \text{Fe}, \text{Cu}$) in a bacterial NO reductase [17] and in a *caa*₃ oxidoreductase [18].

Figure 1. Proposed hyponitrite intermediates in NO reduction by bacNORs.

Regardless of the mechanism that is operative in bacNORs, the overall reaction represented in eq. 1 requires two electrons and two protons, and involves the formation of an N–N bond (to eventually form N_2O) and the cleavage of an N–O bond (to form H_2O). It is currently not exactly clear at which stage of the reaction the N–N bond formation event occurs.

We are interested in the role of electrons and protons in enhancing N–N bond formation from two NO molecules at the heme site. Several computational [16, 19, 20], spectroscopic [14, 17, 18, 21, 22], and model [23–27] studies have been reported that examine the NO reduction mechanism by bacNOR and related enzymes. In the absence of experimental data that clearly and unambiguously define the N–N bond formation step, we have approached the issue using density functional theory focusing on the role of a single-heme site, and have considered various scenarios by altering the sequence of addition of electrons and protons, and then analyzing the resulting structures that may shed some light into the important N–N bond forming reaction and eventual N_2O formation. In a previous communication of limited scope [28], we reported our initial studies on the first protonation event for a porphine system and showed that significant N–

Scheme 1. Overview of the reaction pathways investigated in the NO reduction reaction to yield N₂O and H₂O mediated by a single Fe porphine system.

2. Computational methodology

The active site of bacNOR contains an axially coordinated His ligand to Fe in a heme *b* macrocycle, hence we utilized the six-coordinate imidazole (Im) bound Fe porphines as models. Density functional theory (DFT) calculations were carried out with the DFT functional BP86 implemented in the Gaussian-09 suite either via the stand-alone program or through the WebMO interface (www.webmo.net). The triple- ζ valence polarization basis set (TZVP) was used for all atoms. Unrestricted open-shell calculations were performed. Based on our earlier results [28], the NONO and Im planes in the models were initially set to be mutually perpendicular. Geometry optimizations were performed for the six-coordinate porphine compounds **1-10** in their low-spin Fe configurations, followed by vibrational frequency calculations on the optimized structures. No imaginary frequencies were observed for the geometry-optimized structures, confirming that compounds **1-10** are minima along the potential energy curves. We also used the hybrid functional B3LYP/TZVP for the geometry optimizations (to explore its effect on the N–N bond) due to its ubiquity in computational chemistry. To explore the effect of macrocycle unsaturation on the N–N distances in the compounds **2-5**, we also utilized the chlorin macrocycle; the chlorin model was built using the porphine macrocycle as the starting point, modifying one of the pyrrole rings by defining a C–C single bond and satisfying the valence count of the two C atoms with H atoms.

A listing of the B3LYP/TZVP results and those from BP86/TZVP for the porphine and chlorin compounds **2-5** are shown in Table 1. Spin multiplicities (singlet (*s*), doublet (*d*), triplet (*t*)) are designated as shown in the compound labelings in the Schemes. Schematic figures were drawn using ChemDraw[®] and labels added using Adobe Photoshop[®]. Ball-and-stick structures were generated in GaussView and labeled in Adobe Photoshop[®].

Table 1. Selected bond distances (in Å) and bond angles (in deg.) of geometry optimized complexes using BP86 and B3LYP methods.^a

Compounds	Method	Fe–N(Im)	Fe–N1	N1–N2	N1–O1	N2–O2	N2–N1– N(Im)–C(Im)
[(X)Fe{N(O)NO}(Im)] ⁰	BP86	2.080 [2.083]	1.789 [1.793]	1.957 [1.965]	1.192 [1.191]	1.158 [1.161]	91.1 [125.4]
	B3LYP	2.090 [2.087]	1.820 [1.818]	1.901 [1.900]	1.173 [1.172]	1.145 [1.146]	90.4 [80.1]
[(X)Fe{N(O)NO}(Im)] [–]	BP86	2.114 ^b [2.111]	1.886 ^b [1.888]	1.708 ^b [1.691]	1.217 ^b [1.202]	1.207 ^b [1.212]	98.2 ^b [89.5]
	B3LYP	2.100 [2.094]	1.978 [1.978]	1.426 [1.425]	1.239 [1.238]	1.234 [1.236]	89.9 [90.9]
[(X)Fe{N(O)NOH}(Im)] ⁰	BP86	2.079 ^b [2.074]	1.981 ^b [1.983]	1.292 ^b [1.291]	1.249 ^b [1.248]	1.427 ^b [1.432]	92.0 ^b [99.5]
	B3LYP	2.119 [2.109]	1.996 [1.994]	1.266 [1.267]	1.245 [1.244]	1.415 [1.417]	93.4 [94.2]
[(X)Fe(N ₂ O)(Im)] ⁺ + H ₂ O	BP86	1.949 [1.955]	1.989 [1.962] (Fe–N2)	1.135 [1.136]	1.183 [1.188]	3.164 [3.563] (to H ₂ O)	
	B3LYP	1.953 [1.947]	2.205 [2.279] (Fe–N2)	1.119 [1.120]	1.172 [1.174]	3.113 [3.345] (to H ₂ O)	

^a X = porphine or chlorin. The data for the chlorin analogues are shown in brackets.

^b Reference 28.

3. Results

We examined the complete NO-to-N₂O reduction pathway shown in eq. 1 facilitated by a single model heme. An overview of the BP86/TZVP computed structures and compound numbering is shown in Scheme 1. In an earlier communication [28], we presented our initial results from the first electron addition and protonation of the "(porphine)Fe(NO)(Imd) + NO" system, namely the processes **1**→**2**→**3**→**4** and **3**→**6** in Scheme 1. We have expanded on this work to include (i) both the first and second protonation events to generate the final Fe–N₂O product, (ii) alternate ordering of additions of electrons and protons to the heme system that enabled N–N bond formation and N₂O/H₂O production, (iii) a comparison between the porphine and chlorin (i.e., reduced porphine) systems (e.g., Table 1 and Supporting Information), using both the BP86 and B3LYP functionals.

3.1 Addition of NO and an electron to the six-coordinate [(P)Fe(NO)(Im)] (**1**) precursor to give [(P)Fe{N(O)NO}(Im)][–] (**3**)

The effect of the order of addition of NO and an electron to the {FeNO}⁷ precursor [(P)Fe(NO)(Im)] (**1**) is shown in Scheme 2. Addition of NO to the bound nitrosyl in the six-coordinate **1** results in a very weak interaction of the two NO moieties in **2** (N...N distance of 1.957 Å), accompanied by a lengthening of the Fe–NO distance and a shortening of the *trans* Fe–N(Im) bond. Addition of an electron to **2** generates **3** with an N–N distance of 1.708 Å [28]. Alternatively, addition of an electron directly to the precursor **1** generates the anionic {FeNO}⁸ derivative **7** in which both the Fe–NO and *trans* Fe–Im bonds are lengthened. Addition of NO to the bound nitrosyl in anionic **7** then generates **3**.

3.2 The first protonation step: protonation of anionic [(P)Fe{N(O)NO}(Im)][−] (**3**) and neutral [(P)Fe{N(O)NO}(Im)] (**2**)

We explored the O-protonation of the neutral and weakly N–N coupled compound **2**. O-protonation was considered with concomitant electron transfer resulting in a formal triplet state with unpaired electrons on both the ligand and the metal. This protonation resulted in the

Scheme 2. Optimized geometries for the addition of NO and an electron to the precursor complex **1**. Bond distances are in Å.

generation of a reasonable N–N bond (1.300 Å distance) in cationic **8**. Subsequent addition of an electron to **8** then gives neutral **4**.

Protonation of the O-atom of the terminal NO group in anionic [(P)Fe{N(O)NO}(Im)][−] (**3**) generates the neutral derivative **4** with an N–N bond length of 1.292 Å [28], as shown at the bottom of Scheme 3. We also considered the effect of N-protonation of the anion **3** and neutral **2** as shown in Scheme 4. Protonation of the N-atom of the terminal NO group in **3** generates the

Scheme 3. The O-protonation of the neutral compound $\underline{2}$ and the anionic compound $\underline{3}$. Bond distances are in Å.

Scheme 4. The N-protonation of the neutral compound $\underline{2}$ and the anionic compound $\underline{3}$. Bond distances are in Å.

compound $[(P)Fe\{N(O)N(H)O\}(Im)]$ ($\underline{6}$) with an N–N distance of 1.400 Å [28]. The analogous N-protonation of neutral $\underline{2}$ gives the cation $\underline{9}$ with an N–N distance of 1.436 Å. Addition of an electron to $\underline{9}$ then generates neutral $\underline{6}$.

3.3 The second protonation step: protonation of neutral [(P)Fe{N(O)NOH}(Im)] (**4**)

In examining the calculated geometries of the first protonation products **4** (from O-protonation) and **6** (from N-protonation), we deduced that the second protonation step would likely involve **4** rather than **6**, in large part due to the following: (i) compound **4** shows a shorter N–N bond (primed for N₂O formation), (ii) compound **4** has a longer terminal N–O bond (primed for cleavage to form H₂O), and (iii) compound **4** displays a longer Fe–N bond (for eventual Fe–N cleavage and release of an N_xO_y product).

O-Protonation of [(P)Fe{N(O)NOH}(Im)] (**4**) can proceed via two paths, resulting in two separate products **5** and **10** as shown in Scheme 5.

Scheme 5. The O-protonation of compound **4**. Bond distances are in Å.

In one case, protonation of the terminal OH group in **4** from an external direction results in the fragmentation of this moiety into a bound linear N₂O ligand in cationic **5**, and H₂O. In another, and when the incoming proton is placed close to this terminal OH group in **4** but in a

direction facing the internal NO moiety, the resulting product is one that contains a bound hyponitrous acid, namely $[(P)Fe\{N(OH)NOH\}(Im)]$ (**10**) (see Discussion).

3.4 The chlorin model system

In order to explore the influence of macrocycle unsaturation on N–N bond formation and eventual N_2O generation, both the porphine and chlorin macrocycles were employed in our calculations for the compounds **2-5** in Scheme 1. Performing the calculations with the chlorin macrocycle rather than the porphine macrocycle yielded similar geometry results for these compounds. The data for the chlorin calculations are shown in brackets just beneath the related individual porphine data in Table 1. A significant difference is evident, however, in the torsion angles defined by the N-atoms of the "NONO" units and the Im N–C linkages, namely the N2–N1–N(Im)–C(Im) linkages, in the chlorin and porphine compounds **2** (see Figure S3 in the Supporting Information), where the terminal NO group points closer to the unsaturated five-membered ring of the chlorin, rather than pointing towards a *meso*-carbon in the porphine analog.

3.5 A comment on the BP86 versus the B3LYP results

In general, the trends of the geometrical data from the optimized structures using B3LYP parallel those obtained from the BP86 study, as shown in Table 1. A notable difference occurs with the determination of the N–N bond length of compound **3**, which is much shorter from the B3LYP study (1.426 Å) when compared with that from the BP86 study (1.708 Å). We note that the significant difference in N–N bond lengths for the BP86 and B3LYP geometries is beyond the generally accepted error of ~2 pm for intraligand bond lengths for DFT in bioinorganic chemistry [30]. It is common for pure functionals, such as BP86, to over-estimate bond lengths, while inclusion of exact Hartree Fock exchange is known to contract these values [31].

However, the N–N coupling for two NO units is anticipated to be difficult being a long range and weakly coupled bond, and this difference in bond lengths is likely a result of the difficulty of DFT to model these specific bonds [30]. The population of the π -fragment orbital (FO) of ONNO makes this fragment hyponitrite radical $(\text{ONNO})^-$ in character, and it is anticipated that this would result in an intermediate bond distance between loosely coupled $(\text{NO})_2$ and coordinated hyponitrite $(\text{ONNO})^{2-}$. Within both the BP86 and B3LYP single point results (BP86 optimized geometry), the spin density is primarily localized on the hyponitrite radical, consistent with this rationale.

Based on these results, including those from Section 3.4, and the fact that the enzyme bacNOR utilizes a porphyrin in its active site, we focused our further analyses on the porphine models using the BP86 functional.

4. Discussion

Coupling of two NO molecules, to generate N_2O and water, necessitates the formation of an N–N bond along the reaction pathway. In the absence of an O-atom abstracting agent, it is reasonable to assume that two molecules of NO approach each other to enable the coupling to occur, and that this is facilitated by a metal within the active site of the bacNOR enzyme. The neutral NO dimer, namely $(\text{NO})_2$, is weakly coupled with a long $\text{N}\cdots\text{N}$ distance of $\sim 2 \text{ \AA}$, and a very low dissociation energy of $\sim 2 \text{ kcal/mol}$ [32, 33]. It has been calculated previously that the successive addition of electrons to generate the hyponitrite radical and then the dianion strengthens the N–N interaction (*c.f.*, the formal $\text{N}=\text{N}$ double bond distance = $1.25 \text{ \AA}$) [32, 34].

NO reduction to N₂O requires two electrons and two protons (eq 1). It is thus reasonable to assume that the role of ferrous ion in heme in the reaction cycle is to provide an electron to assist in the favorable formation of the N–N bond. Consequently, and as mentioned above, we designed our calculations to examine the addition of the second electron and two protons to the "[P]Fe(NO)(Im)] + NO" system as might occur in the bacterial NO reduction pathway.

4.1 N–N bond formation

In our earlier work [28], we showed that addition of NO to the neutral [(P)Fe(NO)(Im)] (**1**) to give neutral [(P)Fe{N(O)NO}(Im)] (**2**) resulted in only a weak N...N interaction (Scheme 2). In contrast, addition of NO to the 1-electron reduced compound **7** gives a relatively more favorable N–N interaction (1.708 Å) in **3**. Importantly, six-coordinate {FeNO}⁸ complexes such as **7** have been generated electrochemically [35–37], and shown to have weaker Fe–N(Im) linkages, i.e., increased *trans* effect (e.g., Δ[Fe–N(Im)] = +0.26 Å in this work). In contrast, neutral porphyrin {FeNO}⁷ compounds **1** are unreactive with NO and do not give the NO-coupled products **2** [38, 39]. Addition of an electron to **2**, or addition of NO to **7**, both generate the compound **3**.

Both O-protonation and N-protonation were considered for the first protonation event, as reported earlier for the porphine system [28]. Protonation of neutral **2** at the terminal O-atom or N-atom gives the cationic derivatives **8** and **9**, respectively. Similarly, protonation of the anion **3** at either the O-atom or N-atom position results in derivatives **4** and **6**, respectively. As described in Section 3.3, compound **4** with the generated "ONNOH" ligand (with a relatively short 1.292 Å N=N bond length indicative of double-bond character) appears to be the likely precursor to N₂O and H₂O formation. In fact, this O-protonation of the terminal O-atom has been proposed in the reaction mechanism of the bacNOR enzyme [1, 3].

A simplified figure showing the relevant molecular orbitals associated with the conversion of the anion $[(P)Fe\{N(O)NO\}(Im)]^-$ (**3**) to neutral $[(P)Fe\{N(O)NOH\}(Im)]$ (**4**) are sketched in Figure 2. As shown on the left of Figure 2, the Fe valence orbital in the anion is doubly occupied, and the π bond of the hyponitrite radical is singly occupied. Thus, the unpaired spin density in the anion **3** is localized mainly on the ligand.

Figure 2. Simplified MO diagram showing the relative metal and ligand fragment orbital energies of compounds **3** and **4**. M is the metal fragment.

Upon protonation (right of Figure 2), the π orbital of the hyponitrite fragment is sufficiently low in energy that it is doubly occupied, and the SOMO electron is now localized on the metal fragment. Consequently, the unpaired spin density is localized mainly on the metal in the neutral protonated **4**. This is essentially a proton-coupled electron transfer event that involves the first proton shifting an electron from the metal to the ligand. Indeed, this formal Fe oxidation state change from ferrous to ferric, in going from **3** to **4**, is demonstrated further in

Scheme 6 which shows the calculated spin densities for the Fe and relevant heteroatoms of compounds **2-5**. Compound **2** is a singlet. The spin density in **3** localized on the ligand in this ferrous complex, whereas the spin density in **4** localized on the metal, revealing a shift of an electron to the ligand generating a formally ferric center in **4**.

Scheme 6. Calculated geometries (BP86/TZVP) and selected atomic spin densities (calculated using B3LYP/TZVP on the BP86-optimized geometries) for compounds **2-5**. Bond distances are in Å. Spin densities are in bold font, and bond distances are in italics-underline.

4.2 The second protonation leading to N₂O formation

We showed, in Scheme 5, that protonation of the compound $[(P)Fe\{N(O)NOH\}(Im)]$ (**4**) results in two separate products. The first is the desired and biologically productive cationic $[(P)Fe(N_2O)(Im)]^+$ product (**5**) with a linear N₂O ligand bound through the terminal N-atom with an Fe–N(N₂O) distance of 1.989 Å; we note that the gaseous N₂O molecule has a linear ground state structure [40]. Metal-coordinated N₂O complexes are rare, and successful coordination of

N_2O to a metal requires good overlap of the metal and ligand orbitals [41, 42][43]. Within our results (Figures S1 and S2 in the Supporting Information), little covalency is observed between the metal d orbitals and the N_2O orbitals.

The alternate product from the protonation in Scheme 5 is the complex $[(\text{P})\text{Fe}\{\text{N}(\text{OH})\text{NOH}\}(\text{Im})]^+$ (**10**) with a coordinated hyponitrous acid group in the *cis* configuration; this product forms when the second protonation occurs at the internal NO group. DFT calculations on the free hyponitrous acid also show the *cis* conformer to be more stable than the *trans* conformer [44].

Interestingly, the initial placement of the incoming proton for subsequent optimization was found to be critical in dictating the reaction outcome of Scheme 5. Placing the incoming proton in positions A, B, and C in Figure 3 (left) followed by geometry optimization resulted in the formation of the N_2O complex **5**. However, placing the incoming proton in position D (Figure 3), although closer to the O-atom of the terminal -OH group (e.g., 1.59 Å away) than the internal O-atom (e.g., 2.8 Å away), resulted in the proton being eventually attached to the internal O-atom to yield the HONNOH ligand in **10** as shown at the bottom of Scheme 5. This

Figure 3. Relative locations of the incoming (second) proton prior to geometry optimizations. The top view is shown on the left, and the side view shown on the right.

result suggests that there is more than one pathway for the second O-protonation step depending on the directionality of the incoming proton. Thus, delivery of the proton from positions A, B, or C in our model are the most productive for this calculated NO detoxification pathway by a single heme system to give N_2O . Curiously, we also note that the conserved Glu residue in the active site of the bacNOR enzyme has been proposed to serve as an important proton delivery shuttle from bulk water [1-3, 12]. This raises important questions regarding how directionality of proton delivery may influence productive NO detoxification in the bacNOR enzyme.

5 Conclusions

We have considered various scenarios for the addition of electrons and protons to a "heme-NO + NO" system of relevance to the bacNOR chemical mechanism of NO detoxification. In the bacNOR enzyme, a second (and non-heme) Fe site is conserved in the active site. Interestingly, we show in our work that N_2O can, in principle, be generated by the use of a single heme-NO system with the addition of NO, electrons, and protons. In future work, we will examine the role of electromers [10] of compounds **8** and **9** in the overall pathway for N_2O formation and factors that could lead to N_2O release from the heme-Fe center.

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