Nitric Oxide Reduction by Heme-Thiolate Enzymes (P450nor): A Reevaluation of the Mechanism

Radu Silaghi-Dumitrescu[a]

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The details of the heme-thiolate nitric oxide reductase (P450nor) catalytic mechanism are still controversial. One theory, supported by computational results [D. L. Harris, *Int. J. Quantum Chem.* **2002**, *88*, 183–200], assumes two sequential one-electron transfers from NAD(P)H to an initial $[FeNO]^6$ complex. The $[FeNO]^8$ species thus formed would react with NO, eventually liberating the unstable $ONNO^{2-}$ anion (most probably in its protonated form), which decomposes to N_2O and water. However, more recent experimental results [A. Daiber et al., *J. Inorg. Biochem.* **2002**, *88*, 343–352] suggest the first committed step of the mechanism to be direct hydride transfer from NAD(P)H to $[FeNO]^6$, presumably resulting in an iron-bound HNO unit, $[Fe\text{-}(H)NO]^8$, that would be readily protonated to $[Fe\text{-}(H)NOH]^8$. Subsequent NO addition would yield the unstable HO-N(H)-N=O, which would dissociate from the heme and decompose to H_2O and N_2O. Here, the DFT geometry optimization of all previously proposed reaction intermediates is reported. The first step of the mechanism is predicted to be hydride transfer to $[FeNO]^6$, to produce $[FeNOH]^8$ or $[Fe\text{-}N(H)O]^8$. Subsequent addition of NO to $[Fe\text{-}NOH]^8$ (but not to $[Fe\text{-}N(H)O]^8$ or $[Fe\text{-}N(H)OH]^8$) is predicted to lead to immediate liberation of $HN_2O_2^-$, without any stable intermediates. Contrary to what would be predicted according to the "thiolate push effect" dogma, the *thiolate ligand at the heme active site is shown to obstruct NO reduction*, rather than facilitate it. It is in fact shown that replacement of the thiolate by a neutral nitrogen ligand (i.e., lysine, as found in the active site of cytochrome c nitrite reductase, an enzyme that can reduce NO) clearly favors, from a thermodynamic point of view, NO reduction at the heme site.

Introduction

The active site of fungal nitric oxide reductases (enzymes that belong to the superfamily of cytochromes P450, and are commonly referred to as P450nor) consists of a solvent-exposed, cysteinate-ligated heme.[1,2] The widely accepted mechanism of P450nor[3–9] (Figure 1) involves binding of NO to the ferric form of the active site heme, followed by reduction of this $[FeNO]^6$ complex by NAD(P)H. Subsequent attack of a second molecule of NO leads to formation of a nitrogen-nitrogen bond and liberation of the very stable N_2O. Besides the starting $[FeNO]^6$ species, only one intermediate, absorbing at 444 nm, has been experimentally observed.[3]

The details of the P450nor mechanism, and in particular the identity of the 444 nm intermediate, are still controversial. One theory[5] (top half, Figure 1), supported by recent computational results,[9] assumes two sequential one-electron transfers from NAD(P)H to the initial $[FeNO]^6$ complex. The $[FeNO]^8$ species thus formed would constitute the 444-nm intermediate, and would react with NO, eventually liberating the unstable $ONNO^{2-}$ anion (most probably in its protonated form), which decomposes to N_2O and water. However, more recent experimental results[8] support a theory originally put forth by Averill,[11] suggesting the first committed step of the mechanism to be direct hydride transfer from NAD(P)H to the nitrogen atom of $[FeNO]^6$, resulting in an iron-bound HNO unit, $[Fe\text{-}(H)NO]^8$, that would undergo protonation to $[Fe\text{-}(H)NOH]^8$ (444-nm intermediate). Subsequent NO addition would yield the unstable HO-N(H)-N=O, which would dissociate from the heme and decompose to H_2O and N_2O. The present study reports DFT geometry optimization results on the putative reaction intermediates implied by the P450nor mechanisms proposed so far. These results not only provide a reasonable account of the P450nor mechanism, but also provide a basis for better understanding key notions in hemoproteins, such as "the thiolate push effect". Implications for other nitric oxide-reducing proteins (such as cytochrome c nitrite reductase) are also noted.

Results and Discussion

Geometry optimization results are shown in Table 1 and Table 2. Some of the models ($[FeNO]^{6,7,8}$) have already been

[a] Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia,
Athens, GA 30602, USA
E-mail: silaghi@chem.uga.edu

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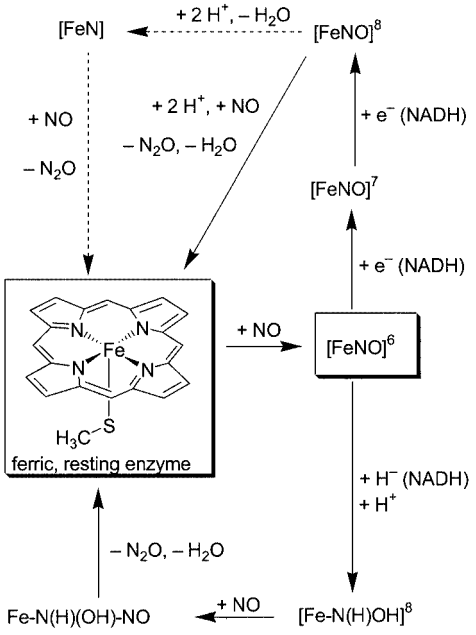

Figure 1. The two previously proposed mechanisms for P450nor (top half: supported by calculations,[9] bottom half: supported by experimental data[10]); species unambiguously known to be involved in the catalytic cycle are highlighted; species in the top and bottom right corners, respectively, have been proposed to constitute the experimentally-observed 444-nm intermediate;[9,10] an alternative pathway, based on partial DFT results, is marked with dashed arrows;[9] the Enemark−Feltham notation is used for iron-nitrosyl species $[FeNO]^n$, as well as for their protonated counterparts such as $[Fe\text{-}(H)NOH]^n$ (with the hydrogens counted as protons when summing up the electrons, "*n*", in the Enemark−Feltham formalism)

characterized elsewhere,[4,6,9,12] and are therefore not discussed in any detail here. It should be noted, however, that the predicted optimum geometries and spin states are in agreement with previously published data. Results on small molecules (i.e., NO, HNO, N_2O, H_2O, etc.), that would be useful as references when discussing overall energetics of the mechanism, are supplied as Supporting Information (see footnote on the first page of this article).

Regarding the first committed step of the P450nor mechanism, reduction of the $[FeNO]^6$ complex by NADH, two theories have been put forward to date.[9,11] Previous DFT results[9] have been used to support the idea that two electrons would be sequentially delivered to the active-site $[FeNO]^6$ complex by NADH in an outer-sphere mechanism, to generate $[FeNO]^8$ (which would be the experimentally observed 444-nm intermediate). According to this hypothesis, any one-electron reducing agent with a redox potential equal to or lower than that of NADH should yield the same $[FeNO]^8$ intermediate upon reaction with the $[FeNO]^6$ at the P450nor active site. Yet, this is known not to be the case: dithionite (redox potential lower than that of NADH by about 100 mV) can only reduce the active site $[FeNO]^6$ to $[FeNO]^7$.[3] Daiber et al.[8] have pointed out this shortcoming of the one-electron theory, and have instead shown kinetic isotope effects, using deuterium-substituted NADH, thereby concluding that hydride transfer from NADH to the nitrogen atom of $[FeNO]^6$ is the key step of the mechanism. The 444-nm intermediate would thus be $[Fe\text{-}N(H)OH]^8$, with the second proton provided by solvent and/or the protein matrix. These authors[8] were also able to generate the 444-nm intermediate by chemical reduction of $[FeNO]^6$ with borohydride, or by addition of pulse radiolysis-generated $NHOH^{\cdot}$ radicals to the ferric (resting) form of the active site heme. However, no evidence exists so far as to why a strong reducing agent like dithionite is not able to generate a $[FeNO]^8$ (protonated or not) species at the P450nor active site.

The energies listed in Table 1 suggest that a mechanism implying two sequential one-electron transfer steps for the reduction of $[FeNO]^6$ to $[FeNO]^7$ to $[FeNO]^8$ would be energetically unfeasible. That is, a $[FeNO]^7$ species would be generated as an intermediate, that is more than 200 kJ/mol more stable than the end-product $[FeNO]^8$, and some 150 kJ/mol more stable than $[FeNO]^6$. Thus, with one-electron reducing agents (such as dithionite) the P450nor site remains trapped in a $[FeNO]^7$ form that is too stable to be catalytically useful. By contrast, with a two-electron donor, such as NADH, reduction proceeds from $[FeNO]^6$ *directly*

Table 1. Results (energies in kJ/mol, distances in Å) for relevant P450nor models

Model	Energy	Fe−S	Fe−N	N−O	N−N	N−H/O−H	Fe−N−O
$[FeNO]^6$, $S = 0$	−7404902	2.30	1.67	1.18	–	–	163°[a]
$[FeNO]^7$, $S = 1/2$	−7405056	2.44	1.75	1.21	–	–	138°
$[FeNO]^8$, $S = 0$	−7404840	2.57	1.80	1.22	–	–	130°
$[FeNO]^8$, $S = 1$	−7404840	2.43	1.77	1.21	–	–	140°
$[Fe\text{-}N(H)O]^8$, $S = 0$	−7406619	2.37	1.81	1.26	–	1.06	135°
$[Fe\text{-}N(H)O]^8$, $S = 1$	−7406539	2.28	1.98	1.27	–	1.05	130°
$[Fe\text{-}NOH]^8$, $S = 0$	−7406522	2.42	1.74	1.40	–	0.98	116°
$[Fe\text{-}NOH]^8$, $S = 1$	−7406456	2.35	1.87	1.41	–	0.98	121°
$[Fe\text{-}N(H)OH]^8$, $S = 0$	−7407980	2.22	1.82	1.37	–	1.03/0.98	131°
$[Fe\text{-}N(H)OH]^8$, $S = 1$	−7407940	2.22	2.00	1.38		1.02/0.98	128°
Fe-N(O)-NOH, $S = 1/2$	−7747788	2.24	2.05	1.30/1.40[b]	1.29	1.03	123°
Fe-N(OH)-NO, $S = 1/2$	−7747788	2.24	2.01	1.39/1.31[b]	1.31	1.05	121°

[a] S−Fe−NO angle: 172°. This geometry accurately reproduces both previous DFT[9] results and the crystal structure of the P450nor $[FeNO]^6$ complex.[6] [b] Iron-bound nitrogen and non-iron-bound nitrogen, respectively, in this order.

Table 2. Charges and spin populations derived from NBO analyses (spin populations listed in parentheses) for relevant P450nor models

Model	Fe	S	N	O	L[a]	P[b]
$[FeNO]^6$, $S = 0$	1.27	−0.16	0.06	−0.16	−0.10	−0.91
$[FeNO]^7$, $S = 1/2$	1.28 (0.33)	−0.37 (0.13)	−0.07 (0.31)	−0.27 (0.20)	−0.34 (0.51)	−1.41 (0.04)
$[FeNO]^8$, $S = 0$	1.31	−0.49	−0.13	−0.33	−0.46	−2.03
$[FeNO]^8$, $S = 1$	1.30 (0.43)	−0.41 (0.11)	−0.09 (0.31)	−0.31 (0.19)	−0.40 (0.50)	−2.32 (0.96)
$[Fe\text{-}N(H)O]^8$, $S = 0$	1.29 (0.01)	−0.36	−0.27	−0.36	−0.29 (−0.01)	−1.39
$[Fe\text{-}N(H)O]^8$, $S = 0$	1.36 (0.96)	−0.25 (0.19)	−0.36 (0.39)	−0.37 (0.37)	−0.39 (0.76)	−1.63 (0.07)
$[Fe\text{-}NOH]^8$, $S = 0$	1.30 (0.01)	−0.38	−028 (−0.01)	−0.53	−0.33 (−0.01)	−1.43
$[Fe\text{-}NOH]^8$, $S = 1$	1.36 (0.81)	−0.31 (0.19)	−0.35	−0.56 (0.16)	−0.43 (0.16)	−1.48 (0.48)
$[Fe\text{-}N(H)OH]^8$, $S = 0$	1.32	−0.10	−0.42	−0.48	0.01	−1.12
$[Fe\text{-}N(H)OH]^8$, $S = 1$	1.35 (0.94)	−0.10 (0.26)	−0.41 (0.63)	−0.48 (0.21)	0.01 (0.84)	−1.17 (−0.04)
Fe-N(O)-NOH, $S = 1/2$	1.36 (0.87)	−0.20 (0.19)	−0.02/−0.04[c]	−0.54/−0.59[c]	−0.68 (0.04)	−1.36 (−0.10)
Fe-N(OH)-NO, $S = 1/2$	1.35 (0.87)	−0.20 (0.20)	−0.02/−0.04[c]	−0.59/−0.55[c]	−0.70 (0.04)	−1.35 (−0.10)

[a] Sum of partial atomic charges and spin populations over the NO ligand or its replacements. [b] Sum of partial atomic charges and spin populations over the porphyrin atoms. [c] Iron-bound nitrogen and non iron-bound nitrogen, respectively, in this order.

Table 3. Summary of geometry optimization results (energies in kJ/mol, distances in Å) for non-P450nor models; "X" is the axial ligand trans to NO

Model	X	Energy	Fe−N	N−O	Fe−N−O	Fe−X
X-$[FeNO]^6$, $S = 0$	−	−6253977	1.63	1.16	180°	−
X-$[FeNO]^7$, $S = 1/2$	−	−6254599	1.75	1.21	147°	−
X-$[FeNO]^8$, $S = 0$	−	−6254735	1.80	1.22	125°	−
X-$[FeNO]^6$, $S = 0$	CH_3NH_2	−6505813	1.65	1.15	177°	2.06
X-$[FeNO]^7$, $S = 1/2$	CH_3NH_2	−6506325	1.78	1.19	139°	2.21
X-$[FeNO]^8$, $S = 0$	CH_3NH_2	−6506425	2.33	1.81	125°	1.21

Table 4. Charges and spin populations derived from NBO analyses (spin populations listed in parentheses) for non-P450nor models; "X" is the axial ligand trans to NO

Model	X	Fe	X[a]	N	O	L[b]	P[c]
X-$[FeNO]^6$, $S = 0$	−	1.36	−	0.07	−0.10	−0.03	−0.33
X-$[FeNO]^7$, $S = 1/2$	−	1.33 (0.96)	−	−0.03 (0.04)	−0.20 (−0.01)	−0.23 (0.03)	−1.10 (0.01)
X-$[FeNO]^8$, $S = 0$	−	1.22	−	−0.08	−0.30	−0.38	−1.82
X-$[FeNO]^6$, $S = 0$	CH_3NH_2	1.35	−0.86	0.15	−0.07	0.08	−0.70
X-$[FeNO]^7$, $S = 1/2$	CH_3NH_2	1.33 (0.62)	−0.92 (0.03)	−0.01 (0.22)	−0.19 (0.14)	−0.20 (0.36)	−1.33 (−0.01)
X-$[FeNO]^8$, $S = 0$	CH_3NH_2	1.30	−0.93	−0.09	−0.31	−0.40	−1.95

[a] Sum of partial atomic charges and spin populations over atoms in "X". [b] Sum of partial atomic charges and spin populations over the NO ligand or its replacements. [c] Sum of partial atomic charges and spin populations over the porphyrin atoms.

to the (protonated or not) $[FeNO]^8$ stage. This observation also identifies the reason why the P450nor active site is solvent-exposed and allows NADH access to the heme,[2] unlike in any other member of the P450 protein[13] family. Thus, all other members of the P450 protein family have a heme active site buried inside the protein, and electrons are supplied to this heme by NADH in an indirect, outer-sphere, long-range manner, via flavin moieties, *in two sequential one-electron steps*.[13] Unlike P450nor, these other P450 proteins do not exhibit any nitric oxide reductase activity. Thus, the ability to perform the two-electron oxidation of NO at a heme-thiolate active site seems to depend on the possibility of performing the two-electron reduction *in a single step*.

Cytochrome c nitrite reductase (ccNirR) contains a heme at the active site, which can reduce NO to ammonia (with NO being, in fact, an intermediate product of nitrite reduction).[10] The electrons for this reduction are supplied by five neighboring heme units within the protein. Nitric oxide reduction by ccNiR may be deemed as unexpected, since no hydride donor appears to be required and the active site heme is actually not ligated by a thiolate, but by a lysine side-chain. Indeed, thiolate ligands are commonly believed to exert a certain "thiolate push effect" that would enable heme actives site to accomplish key reactions such as dioxygen activation or NO reduction. How can ccNiR perform the same chemistry as P450nor in the absence of a hydride donor *and* of a thiolate ligand? A simple answer is provided by calculations (cf. Table 3) on $[FeNO]^{6,7,8}$ models where the thiolate ligand was replaced by a methylamine unit to mimmick the ccNiR active-site heme. Unlike in P450nor, the ccNiR $[FeNO]^8$ complex is predicted to be more stable

than Fe[NO]^7. We propose this to be the reason why in ccNiR there is no need for a two-electron (hydride) donor. As shown in Table 3, the same situation as in the ccNiR models occurs if the axial ligand trans to NO is omitted altogether. A general observation may then be that nitrogenous heme axial ligands (such as lysine, and, most likely, histidine) favor easier reduction of NO (*thermodynamically speaking*) than anionic ligands such as thiolate. This raises an intriguing question: could the same type of argument ("thiolate obstruction") hold true for other heme enzymes, such as oxygen-activating (heme-thiolate) and oxygen-binding (heme-histidine) enzymes? The data in Table 1−4 clearly confirm that the "thiolate push effect" is a reality in the [FeNO]^{6,7,8} systems examined here. Indeed, in thiolate-ligated systems the electron density on the NO moiety is consistently higher (both charge-wise and spin-wise) than in their non-thiolate counterparts (Table 4). Nevertheless, the differences are not large enough (especially at the level of Fe−N and N−O distances) to warrant the assumption that this "push effect" significantly weakens the respective bonds to the extent that they would be easier to cleave.

With the one-electron theory now ruled out in P450nor by both DFT and experimental results, it follows that the first committed step in the P450nor mechanism can only be hydride transfer from NADH to [FeNO]^6, yielding [FeN(H)O]^8. Table 1 shows that the [Fe-N(H)O]^8 isomer is in fact more stable than its oxygen-protonated counterpart [FeNOH]^8. It is entirely conceivable that an [Fe-N(H)O]^8 species undergoes further protonation to [Fe-N(H)OH]^8 (presumably as a concerted process upon hydride addition), and Table 1 shows that such protonation would result in significant energetical stabilization. The active site has previously been shown to contain appropriate proton donors.[6] Thus, at this stage the Daiber mechanism has the full support of computational results, in that the [Fe-N(H)OH]^8 species appears the most likely candidate for the 444-nm intermediate.

The next step in the mechanism would then be addition of a second molecule of NO to the [Fe-N(H)OH]^8 intermediate, to form a nitrogen-nitrogen bond. Regardless of the exact mechanism of this step (see ref.[8]), this Fe-N(H)(OH)-NO complex would then decay to yield ferric heme, N_2O and H_2O, a process likely to occur by internal rearrangements of the $H_2N_2O_2$ moiety. Indeed, we found that geometry optimization of a putative $S = 5/2$ Fe-N(H)(OH)-NO model resulted in "cleavage" of the Fe−N bond, without an apparent energy barrier, leading to liberation of H_2N_2O. However, for $S = 1/2$ and $S = 3/2$ states of the same model, the Fe−N bond remained unperturbed and the N−N bond was "broken". Thus, Fe-N(H)(OH)-NO should not be conceived as a reaction intermediate, but rather as a transition state, with the observation that a spin crossover ($S = 1/2$ to $S = 5/2$) must occur in order for the interaction of the $S = 0$ [FeNHOH]^8 with $S = 1/2$ NO to be productive. However, for Fe-N(H)(OH)-NO models, $S = 1/2$ and $S = 3/2$ potential energy surfaces were more than 100 kJ/mol below $S = 5/2$, suggesting that, should the $S = 5/2$ pathway ever be active, it would have to be due to assistance from the protein matrix and/or solvent.[14] By contrast, if one assumes the 444-nm intermediate to be [FeNOH]^8 or even [FeNO]^8, addition of a second molecule of NO would result in formation of a strong nitrogen-nitrogen bond (cf. Table 1) and a significant weakening of the Fe−N bond, so that liberation of $HN_2O_2^-$ or (with further assistance from the protein) $H_2N_2O_2$, are entirely feasible reaction pathways. We note that in models where iron-nitrogen *and* nitrogen-nitrogen bonds exist, both electrons originating from NAD(P)H are located on the newly formed $N_2O_2H_x$ ($x = 1,2$) moiety, which is only loosely coordinated to Fe and is therefore likely to be readily displaced by solvent. Finally, it should be noted that attempts to dock an NADH molecule onto the P450nor active site using the Dock module of the Sybyl software package have consistently yielded conformations that would be appropriate for hydride transfer towards the oxygen — but not nitrogen — atom of the [FeNO]^6 unit. Thus, the above results suggest that [Fe-NOH]^8 and not its N-protonated versions is the productive intermediate that reacts with the second NO molecule. This conflict between theory and the Averill/Daiber mechanism may conceivably be solved by invoking active-site dynamics and solvation, which would then provide the extra energy required for the spin transition in the {[FeN(H)OH]^8-NO} system, to render the [FeN(H)OH]^8 + NO interaction productive.

Conclusion

A detailed account of the P450nor mechanism has been provided via geometry optimization of all possible reaction intermediates. The experimentally observed 444-nm intermediate is proposed to be a [FeNOH]^8 species, with [FeN(H)O]^8 or [FeN(H)OH]^8 not entirely ruled out as productive intermediates for reacting with the second NO molecule. Contrary to what would be predicted according to the "thiolate push" dogma, the *thiolate ligand in P450nor is shown to obstruct NO reduction*, rather than facilitate it. It is in fact shown that replacement of the thiolate with a neutral nitrogenous ligand (i.e., lysine, as found in the active site of cytochrome c nitrite reductase, an enzyme that can reduce NO) clearly facilitates (from a thermodynamic point of view) NO reduction at the heme site.

Experimental Section

Geometries of all models were optimized in the Spartan 02 (Windows)[15] package at the University of Georgia, on a Windows 2000-operated Pentium 4 PC (1.7 GHz, 1024 MB RAM). The UBP86 functional, which uses the gradient-corrected exchange functional proposed by Becke (1988) and the correlation functional by Perdew (1986), and the 6-31G** basis set were used as implemented in Spartan.[15] For the SCF calculations, a fine grid was used and the convergence criteria were set to 10^{-6} (for the root-mean square of electron density) and 10^{-8} (energy). For geometry optimization, convergence criteria were set to 0.001 au (maximum gradient criterion) and 0.0003 (maximum displacement criterion). Reported charges and spin densities were derived from NBO popu-

lation analyses. Energy differences between models of different sizes are fairly large, and therefore overall trends and relative energies are unlikely to be modified upon applying theoretically sophisticated and time-consuming corrections such as BSSE. Such corrections were therefore omitted from the present work.

All models were subjected to full geometry optimization, without any geometry or symmetry constraints. All models reported in Table 1 and 2 consisted of an unsubstituted heme coordinated axially by a methylthiolate unit (i.e., the species named "ferric, resting enzyme" in Figure 1), while the sixth coordination position was occupied by various ligands (see Table 1). All results reported in Table 1 for $[FeNO]^{6,7,8}$ models were obtained for end-on (monodentate) coordination, since $[FeNO]^6$ and $[FeNO]^7$ have already been characterized experimentally as being end-on complexes; a detailed theoretical treatment of $[FeNO]^{6,7,8}$ can be found elsewhere.[9] Attempts to model $[FeNO]^8$ as *bidentate* led to a *monodentate* geometry for the $S = 0$ state, and to a true bidentate geometry for $S = 1$ (details of this bidentate geometry are only listed as Supplementary Information; its energy was significantly higher than that of its monodentate isomer). Models reported in Table 3 and 4 consisted of an unsubstituted heme-coordinated NO, while the other axial coordination position was either empty, or occupied by a methylamine unit (see Table 2). Low-spin states ($S = 0$, 1/2) were chosen, for reasons detailed in ref.[10]

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[14] The $S = 2$ state of the [Fe-N(H)-OH]^8 "complex" itself is, in fact, not a proper energetical minimum, as its geometry optimization leads to "dissociation" of the NH-OH moiety from iron, with the energy of the resulting "structure" more than 100 kJ/mol higher than that of the ground state, $S = 0$ [Fe-N(H)-OH]^8.
[15] SPARTAN '02 for Windows, *Wavefunction Inc.,* 18401 Von Karman Avenue, Suite 370 Irvine, CA 92612; *Q-Chem. Inc.* Four Triangle Drive, Suite 160 Export, PA 15632.

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