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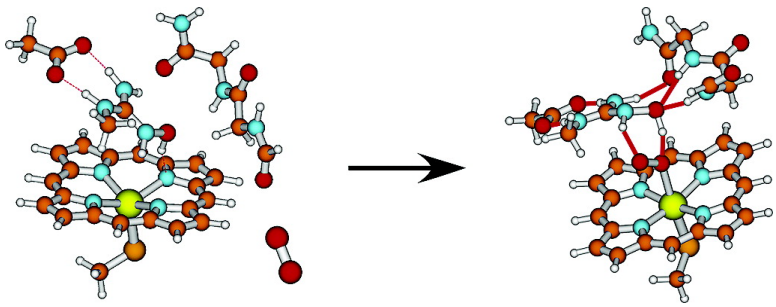

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Quantum Chemical Calculations of the NHA Bound Nitric Oxide Synthase Active Site: O_2 Binding and Implications for the Catalytic Mechanism

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Abstract: Density functional theory has been employed to model the binding of the intermediate substrate NHA, by nitric oxide synthases. In particular, the orientation and interactions of possibly catalytically important substrate hydrogens, with and without molecular oxygen bound to the active site heme group, are considered. Without O_2, three possible conformers have been found, with the energetically most favored structure being that in which both protons of the −NHOH moiety of NHA are directed toward the heme group. With oxygen bound, four different structures were found. The energetically lowest structure is again found to have both hydrogens of the −NHOH group pointing toward the heme group, thus forming hydrogen bonds between −NH− and the terminal oxygen, and between −OH and the inner oxygen of the heme−O_2 group. In addition, unprotonated structures of the substrate bound to the active site are considered and the proton affinity calculated.

1. Introduction

Nitric oxide synthases (NOSs) are a group of enzymes that catalyze the conversion of L-arginine to L-citrulline and nitric oxide (NO), an important biological messenger.[1−3] In mammals, three isoforms are known and are named according to where they are predominantly found: in endothelial cells (eNOS), in neuronal cells (nNOS), and in macrophage cells (iNOS). All forms require multiple cofactors such as heme, tetrahydrobiopterin, NADPH, FAD, and FMN. In the first half-reaction, L-arginine is converted to N^{ω}-hydroxy-L-arginine (NHA) by consumption of two electrons and O_2 by what is believed to be a P450-type reaction.[4] The second half-reaction, which has no known enzymatic analogues, is the reduction of NHA to L-citrulline and NO by consumption of an additional O_2. X-ray crystal structures with L-arginine or NHA bound to the substrate site have been solved;[5,6] however, questions still remain about the positions of the protons. Since the protons are proposed to be actively involved in the catalytic mechanism of the enzyme by means of hydrogen bonds and charge transfers, determining their internal orientations would provide valuable information toward solving the catalytic mechanism of these important enzymes.

In this study, we have employed density functional theory[7] to investigate binding modes of the intermediate substrate NHA and O_2 within an iNOS active site model to determine possible positions of the catalytically important protons involved and their mechanistic implications.

2. Computational Details

All calculations were performed using the density functional theory (DFT) functional B3LYP,[8−11] as implemented in Jaguar 5.5.[12] For all geometry optimizations and frequency and solvent effect calculations, the LACVP basis set was used, except on the sulfur atom for which the LACV3P** basis set was used. Relative energies were calculated by performing a single-point calculation at the B3LYP/LACV3P** level on the above optimized geometries. Zero-point vibrational energies (ZPVE) including internal energies at 298.15 K were taken from the frequency calculations. Environmental electrostatic effects (solvent) were estimated at the optimization level of theory using a self-consistent reaction field method with a dielectric medium of $\epsilon = 4$. This value has been used previously in investigations on related enzyme models[13] and has been found to be a suitable compromise between $\epsilon = 3$ for the protein itself and $\epsilon = 80$ for the surrounding water. It is not expected to dramatically influence the outcome of the calculations but is included for a more complete model of the influence of the active site and protein environment on the substrate.

The chemical model used for the iNOS active site was a porphyrin−iron ring for the heme, thiolmethyl anion for Cys194 (iNOS numbering) and ethanoate (acetate) ion for Glu371 (iNOS numbering). In addition,

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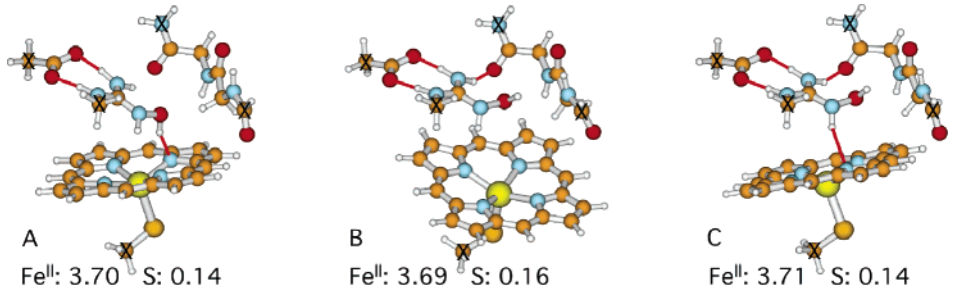
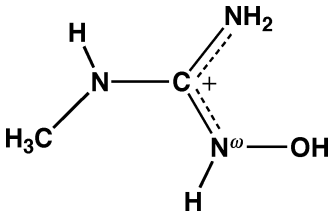


Figure 1. Three lowest energy conformers of the model iNOS active site with bound NHA. The fixed atoms, marked with "X", are aligned in the three conformers to facilitate spatial comparisons. Hydrogen bonds are indicated by red lines. Spin densities on the iron and sulfur atoms are shown for each structure.

Scheme 1. Schematic Illustration of 1-Methyl-2-hydroxy-guanidinium as Used to Model N$^{\omega}$-Protonated NHA



the backbone chain between the carboxylate carbon of Asn364 and the peptide amide of Tyr367 was also included. The substrate NHA was modeled using 1-methyl-2-hydroxy-guanidinium (Scheme 1), hereafter referred to as NHA for simplicity. Unless otherwise noted, the substrate was modeled as protonated at the NHA N$^{\omega}$-position, as proposed in several recent studies.[14,15] The resulting total charge on the model was −1, with the initial iron oxidation state set as Fe(II).

The starting coordinates for the active site model were taken from the crystal structure (PDB: 1DWX),[6] with particular care taken for the added NH and OH protons and O_2 to ensure that all of the most probable binding orientations were considered. The optimized results should represent typical possible binding modes. To mimic protein constraints, five atoms were chosen to be held fixed in their starting positions during the optimizations, all corresponding to cutoff points when constructing our model from the full crystal structure. It has previously been shown that if the cutoff, and hence frozen, points are kept to an essential minimum and well-chosen, such an approach does not cause significant energy differences when compared to unconstrained models.[16] Indeed, it may in fact give a more correct representation of steric constraints placed upon the active site by the larger enzyme. For recent reviews on related enzymatic systems using these approaches, see for example, refs 13 and 17. Optimized structures for all species in this present study are given in xyz-format in the Supporting Information. All relative energies are in kcal mol^{-1} and bond lengths in angstroms.

3. Results and Discussion

Structures without O_2. For this system, the total spin multiplicity of the model investigated, N$^{\omega}$-protonated NHA (N$^{\omega}$-proton) within the iNOS active site, was set to 5, consistent with four unpaired electrons on the Fe(II) center. A variety of possible conformations for the binding of N$^{\omega}$-proton were examined. Three conformations were found, shown in Figure 1, all lying within 5.5 kcal mol^{-1} of each other (Table 1). The lowest energy conformer, **1A**, has both the −NH− and −OH protons of the −NHOH moiety of N$^{\omega}$-proton directed toward the heme group (see Figure 1). Indeed, the −OH proton forms a hydrogen bond (1.739 Å) with a heme−pyrrole group nitrogen, while the −NH− proton is directed toward another heme−pyrrole nitrogen (2.594 Å). In the next lowest energy conformer, **1B**, lying just 5.0 kcal mol^{-1} higher in energy than **1A**, the −NH− proton remains directed toward the heme (see Figure 1). Now, however, the −OH proton is directed away from the heme group, toward the adjacent peptide backbone fragment. Conformer **1C**, lying just 0.5 kcal mol^{-1} higher in energy than **1B**, is structurally the same as **1B** except that the heme group has tilted slightly.

Table 1. Relative Energies (kcal mol^{-1}) for All Optimized Conformers

structure	relative energy[a]	solvent correction	total relative energy[b]
1A	0.00	0.00	0.00
1B	9.47	−4.53	4.95
1C	7.88	−2.46	5.42
3A	0.00	0.00	0.00
3B	6.12	−2.11	4.01
4A	0.00	0.00	0.00
4B	4.24	−1.52	2.72
4C	15.27	−4.02	11.25
4D	18.91	−1.48	17.42

[a] Large basis set single-point with ZPVE (see text). [b] Relative energy + solvent correction.

Comparisons with Experiment. The calculated structures **1A**, **1B**, and **1C** are overlayed on the corresponding crystal structure (PDB: 1DWX) in Figure 2. Reasonable agreement is seen in all three comparisons. However, some significant differences exist. For example, in all three comparisons, the calculated positions of the enzyme amino acid residues are in good agreement with the crystal structure coordinates. For **1A**, however, while there is close agreement with respect to the position of the substrate, NHA, the heme group has essentially tilted slightly at the Fe center. In contrast, while the calculated positions of the substrate are quite similar for **1B** and **1C**, they are no longer in as good agreement with the crystal structure as observed for **1A**. In addition, in **1B** the heme essentially remains in the same plane as in the crystal structure, while, in **1C**, it has tilted considerably. It is noted that the relative energies **1B** and **1C** differ by just 0.5 kcal mol^{-1}, suggesting that, within the model used, the heme group has a relatively flexible degree of rotational freedom. In addition, we also note that a variant of **1A** was optimized such that select heme carbon atoms

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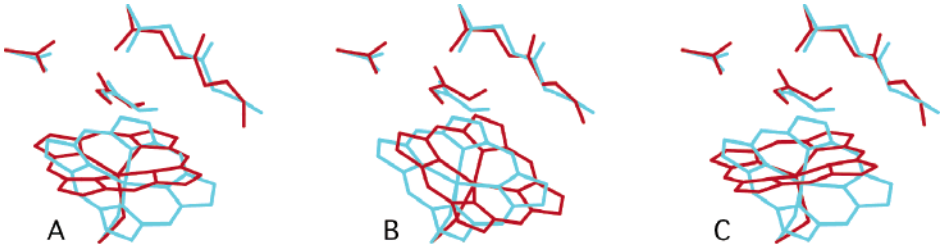

Figure 2. Comparison of the calculated structures **1A**, **1B**, and **1C** (red) with the crystal structure (light blue; PDB: 1DWX). Hydrogens have been omitted for clarity.

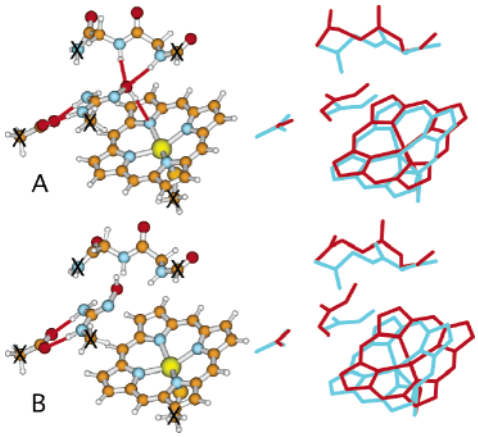

Figure 3. Two possible binding modes, **3A** and **3B**, of the N^{ω}-unprotonated substrate NHA and an overlay with the crystal structure. Red lines indicate hydrogen bonds, and "X" are as in Figure 1.

corresponding to the point of attachment of the propionate chains were fixed in their crystal structure positions, thus constraining the heme group. The energy of the resulting optimized species was just 2.9 kcal mol^{-1} higher than that of **1A** (data not shown). Thus, the present DFT calculations appear to support **1A** as the preferred conformation of NHA within the iNOS active site.

In contrast, none of the currently calculated structures are in complete agreement with those proposed from ENDOR measurements (not shown):[14] all three protonated structures have more than one proton within 5.3 Å from the Fe center as opposed to that suggested by the ENDOR results. For example, **1A** has an Fe···N^{ω} distance of 3.839 Å in good agreement with the ENDOR value of 3.8 Å; however, the proton in closest agreement with the ENDOR suggested Fe···H—N^{ω} distance of 4.8 Å is in fact a proton in the far side amino group of NHA (4.917 Å). The other structures (including the nonprotonated structures, see below) have Fe···N^{ω} distances that are too long and with protons at various distances from the Fe center, none being in close agreement with the ENDOR suggested distance of 4.8 Å. Similar disagreements between the structures obtained by X-ray crystallography and as proposed by ENDOR measurements have been previously noted and discussed.[6]

Protonation State of Bound NHA. Calculations have also been performed on the unprotonated substrate 1-methyl-2-hydroxyguanidine, i.e., no proton on $-N^{\omega}-$, to gain greater insight into the likely protonation state of NHA. Two conformers were found and are shown in Figure 3. In one (**3A**), the −OH proton is directed toward the heme while in the other (**3B**), lying just 4.0 kcal mol^{-1} higher in energy, it is directed away from the heme. The proton affinity (PA) of $-N^{\omega}-$, and the structural consequences of protonation, can be determined by comparing the appropriate corresponding systems, i.e., **1A** and **3A** (cf. Figures 1 and 3). Structure **3A** lies almost 306.2 kcal mol^{-1} lower in energy than **1A**. This difference in energy is expected to approximately equal the PA of NHA at the $-N^{\omega}-$ center because, although **3A** and **1A** differ structurally in particular with respect to the position of the backbone, this reorientation energy should only account for a fraction of the calculated energy difference. In comparison, the PA of OH^- and H_2O are approximately 349.7 and 233.7 kcal mol^{-1}, respectively, at the same level of theory in solvent. The influence of the active site and enzyme on the PA of the $-N^{\omega}-$ center of NHA was also considered by calculating the corresponding PA in aqueous solution ($\epsilon = 80.37$), i.e., without the active site. In solution it is estimated to be 282.7 kcal mol^{-1}, approximately 23.5 kcal mol^{-1} lower than when within the active site. Thus, the active site appears to increase the PA of the substrate slightly, and the relatively high PA of $-N^{\omega}-$ suggests that, in vivo, it is quite possibly protonated. It is also noted that structurally **3A** and **3B** differ more from the crystal structures than observed for **1A**, **1B**, and **1C** (cf. Figures 2 and 3), further suggesting that the NHA is protonated (see above). However, without further calculations on the full catalytic mechanism, no definitive conclusions can be drawn on the protonation state of NHA. In all further calculations, we have chosen to model it as protonated.

Structures with O_2. Upon binding O_2, four possible conformers were obtained, shown in Figure 4, all having a spin density on Fe consistent with that of high-spin Fe(III) (Table 2) given the chosen multiplicity of 7 for this structure.

In the lowest energy conformer, **4A**, two strong hydrogen bonds are formed between the −NH− and −OH groups of the −NHOH moiety and the terminal (O_{out}) and inner (O_{in}) oxygen atoms of the heme−O_2 group, respectively; see Table 2. This structure has also been investigated with various multiplicities (3, 5, and 9); however, only very minor structural changes were observed. Conformer **4B**, lying just 2.7 kcal mol^{-1} higher in energy, has just one strong hydrogen bond; between −OH of the substrate and O_{out}. Now, however, the −NH− of the −NHOH moiety forms a weak hydrogen bond with O_{in} (Table 2). Weak interactions are also observed between −OH and the backbone amide −NH− group of Gly365 (≈2.1 Å) and between O_{out} and the backbone −NH− of Trp366 (≈2.5 Å). Conformers **4C** and **4D** are considerably higher in energy than **4A**, approximately 11.3 kcal mol^{-1} and 17.4 kcal mol^{-1}, respectively. Both contain just one hydrogen bond; between −NH− and O_{in}. In addition, in **4C**, O_{out} points away from the NHA substrate, while, in **4D**, it points inward toward the NHA guanidinium carbon (C_{guan}). However, the relatively high energies of **4C** and **4D** most probably make such binding modes uncompetitive.

Mechanistic Implications. It is generally proposed that during catalytic turnover, a tetrahedral intermediate is formed

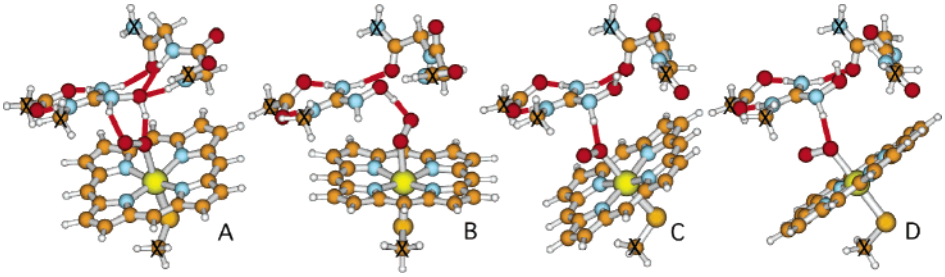

Figure 4. Optimized conformers for iNOS active site with bound substrate and O_2. Red lines indicate hydrogen bonds, and "X" as in Figure 1.

Table 2. Selected Important Spin and Structural Parameters of Figure 4

	spin density				key distances (Å)			
structure	Fe	O_{in}	O_{out}	S	NH···O[a]	OH···O[a]	Fe–O[a]	Fe···N[b]
4A	4.1	0.46	0.61	0.44	1.875	1.554	2.296	4.858
4B	4.1	0.59	0.49	0.44	2.656	1.490	2.280	5.004
4C	4.1	0.47	0.66	0.40	1.605	3.893	2.245	4.536
4D	4.1	0.48	0.64	0.40	1.713	4.305	2.222	4.844

[a] Distance to the closest oxygen of the O_2 moiety. [b] N of −NHOH moiety.

in which the O_2 bridges Fe and C_{guan}. The exact nature, however, of any such intermediate remains unclear. At some stage, any precursor structure must arrange itself as in **4D**, with O_{out} in close proximity to C_{guan}. Given the relatively high energy of **4D**, it is unlikely that such an intermediate is formed directly by attack of O_2 at the C_{guan} center of NHA. Indeed, optimizations did not result in any stable intermediates involving $C_{guan}-O-O-Fe^{III}$ but returned to the separate O_2-Fe^{III} and guanidinium state. Instead, an optimized structure for $C_{guan}-O-OH-Fe^{III}$ was obtained where the −NH− proton had moved to the inner oxygen. This structure was found to be 36.1 kcal mol^{-1} higher in energy than **4A**. Therefore, the repositioning of the −NH− proton upon binding O_2 such that it forms a hydrogen bond with O_{out} in **4A**, cf. **1A**, appears to support proposals[15,18] that the initial step in the mechanism of NOSs involves abstraction of the hydrogen from the −NH− group by the heme$-O_2$, giving a heme$-O_2H$ derivative possibly followed by OH hydrogen abstraction forming H_2O_2.

The spin distribution illustrates the importance of residue Cys194 (Table 2). Given its proximity to the iron, we have modeled it as an anion ligand. In all structures, the spin is substantially delocalized between the iron and sulfur (Table 2). This suggests that the sulfur is a possible initial electron donor to the system. However, cysteine radicals are known to be short-lived in enzymatic systems. Hence, it is likely that an electron from outside donors, e.g., tetrahydrobiopterin, quickly reduces it. Further calculations to fully map the catalytic mechanism of NHA oxidation by NOS are currently in progress.

4. Conclusions

In summary, it has been previously suggested in small model studies that if NHA is protonated, the resulting −NHOH group may be distorted somewhat from a planar arrangement around C_{guan}.[15] The present results suggest that such a nonplanar arrangement is in fact preferred by protonated NHA within the confines of the iNOS active site. In addition, comparison of calculated protonated and nonprotonated structures with available crystal structures further supports the proposed protonation of NHA within the active site. Furthermore, the present results also support suggestions that reexamination of previous ENDOR studies is warranted.

The structures presented in this paper suggest an initial complex in the catalytic mechanism of NOSs in which both protons of the −NHOH moiety of NHA are directed toward the heme group, with subsequent hydrogen bonding to both the oxygen atoms upon binding O_2. In addition, the −NH− proton interacts with the O_2 moiety in all four O_2-bound structures presented here, suggesting that the involvement of the −NH− proton in the initial stages of the reaction mechanism is plausible as noted above.

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Supporting Information Available: The coordinates of the structures of this paper are supplied in xyz-format, as well as the absolute energies (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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