Theoretical Studies on N^G-Hydroxy-L-arginine and Derived Radicals: Implications for the Mechanism of Nitric Oxide Synthase

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Nitric oxide synthases (EC 1.14.13.39, NOS) catalyze the conversion of L-arginine (**1**) to L-citrulline (**2**) and nitric oxide (NO) (Scheme 1), an important biological second messenger and effector molecule involved in various physiological functions.[1−2] An intermediate in the conversion of L-arginine to L-citrulline and NO has been identified as N^G-hydroxy-L-arginine (NOHA).[3] This intermediate has been depicted by various authors as the oxime tautomer **3**,[1,4−13] the imine tautomer **4**,[14,15] and protonated species **5** (Chart 1).[16,17] Recently, ENDOR spectroscopy was utilized to determine the position and structure of NOHA bound to holo-nNOS.[18] On the basis of pulsed ^{15}N and ^{1,2}H ENDOR measurements, two reasonable models of bound NOHA emerged: one involving structure **3** and the other involving either structure **4** or **5** (both of which possess the NHOH functionality). Neutral **4** and protonated **5** could not be differentiated by the ENDOR experiments; however, a planar, resonance-stabilized structure for **5** with significant double bond character about the C−NH(OH) bond was eliminated as a possibility due to the geometric constraints imposed by the ^{1,2}H ENDOR experiments.[18] The ENDOR-derived models based on structures **3** and **4**/**5** differ in the placement of the N−O bond relative to the heme iron; they may therefore be differentiated by X-ray crystallographic determination of the relative positions of the N−O bond and the heme iron in the NOS−NOHA complex. In a Note Added in Proof to the ENDOR paper,[18] it was revealed that Drs. C. S. Raman and H. Li in Professor Thomas Poulos' lab had determined the crystal structure of the oxygenase domain of nNOS; they suggest that the position of the N−O bond of NOHA corresponds to that predicted by the ENDOR experiments for structure **4** or **5**, not to that predicted for structure **3**. The ENDOR results also revealed that if structure **4** or **5** is bound at the active site, the N−H hydrogen, not the O−H hydrogen, of NOHA is closest to the heme iron.

Scheme 1

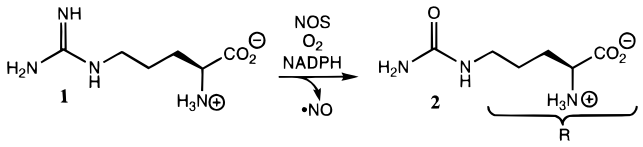
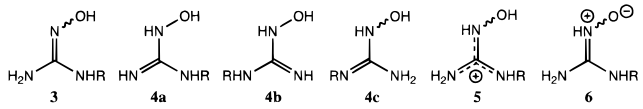


Chart 1



In this contribution we report density functional theory calculations of the structures and relative energies of models of **3**−**5** based on *N*-hydroxy-L-guanidine (NOHG) and the corresponding *O*- and *N*-radicals derived from them by H-atom abstraction (**7**−**11**, Chart 2). On the basis of these calculations, we predict the structures of the bound forms of NOHA and the radical intermediate derived from it in the NOS-catalyzed production of NO.

Figure 1 shows the structures and relative energies of several conformers of the NOHG models of **3**−**6** (optimized at the B3LYP/6-31G(d) level[19−21]). The models for **3** and **4** show a clear differentiation between significant double bond character in the C−N bonds where N is disubstituted (r_{CN} = 1.27−1.29 Å) and significant single bond character in the C−N bonds where N is trisubstituted (r_{CN} = 1.38−1.43 Å); the trisubstituted nitrogens are also significantly pyramidalized. Of these structures, the model for **3** is clearly the most stable, as a result of the additional oxime resonance and the stabilizing inductive effect exerted by the OH group on the imino-type nitrogen. Internal hydrogen bonds are observed in all cases, although it is unclear at this point if they will be disrupted (by the alkyl side chain) in the NOS-bound form; nonetheless, the loss of H-bonding should have a similar effect for each of structures **3** and **4**. The tautomer of **3** which is formally zwitterionic (**6**, Chart 1 and Figure 1) is highly unfavorable.

If NOS were to bind **4** selectively over **3**, it would have to provide selective noncovalent stabilizing interactions worth at least 8 kcal/mol. However, examination of the published X-ray structures of various NOS isoforms[5,22−23] suggests that binding

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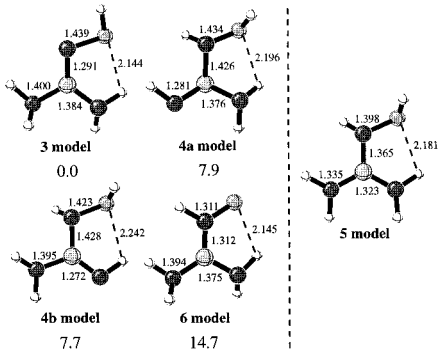
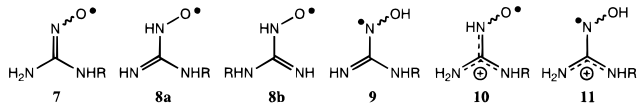


Figure 1. Structures and relative energies of potential hydroxyguanidine intermediates. Selected bond lengths (Å) and relative energies (kcal/mol) are shown. The energies of models **3**, **4**, and **6** are relative to that of model **3**.

Chart 2



of **4** would involve less hydrogen bonding interactions than binding of **3**. This suggests that the enzyme has not evolved to stabilize **4** preferentially. Additionally, the combination of ENDOR and X-ray results[18] effectively rule out structure **3** as the bound form of NOHA, thereby leaving structure **5** as the most likely candidate. This assertion is supported by the fact that the protonated form of *N*-hydroxyguanidine predominates in aqueous solution ($pK_a \approx 8$).[24]

The ENDOR results[18] suggest that the C−NH(OH) bond in bound **5** would have to have significant single bond character, yet the fully optimized structure (Figure 1) is significantly delocalized (although the C−NH(OH) bond is notably longer than the other two C−N bonds). A structure for **5** in which the N(OH)−H bond was constrained to be perpendicular to the guanidinium plane was optimized and found to be only 6.0 kcal/mol less stable than the fully optimized structure. A complex of protonated NOHG and a formate molecule was also computed as a model for the interaction between NOHA and a conserved active site glutamate residue that is necessary for activity.[5,22−23,25−26] For this complex, the optimized structure with the N−H bond constrained to be orthogonal to the guanidinium plane is only 3.6 kcal/mol less stable than the planar species. Taken together, the ENDOR, X-ray, and computational results suggest that structure **5** is the bound form of NOHA, and that this structure is perturbed from that of the highly delocalized unbound structure.

The generation of NO from NOHA by NOS requires further oxidation chemistry. Several labs have previously reported that bound NOHA undergoes a one electron oxidation or H-atom abstraction as a crucial step during catalysis.[8,27−29] Thus, we have

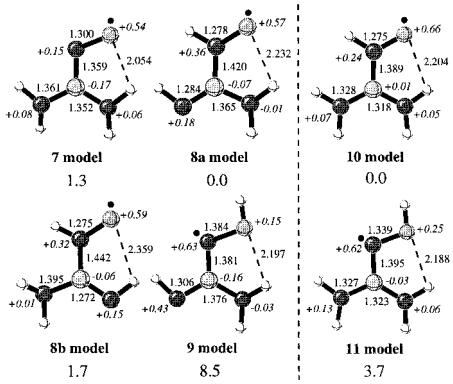
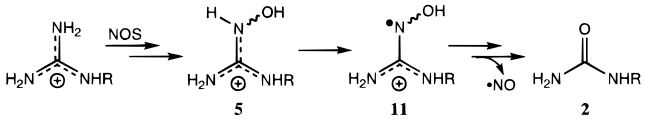


Figure 2. Structures and relative energies of potential radical intermediates. Selected bond lengths, (Å), Mulliken spin densities (italics), and relative energies (kcal/mol) are shown. The energies of models **7**−**9** are relative to that of model **8a**, and those of models **10**, **11** are relative to that of model **10**.

Scheme 2



performed further calculations to address the energetics of species that might be produced in this catalytic step. Figure 2 shows the structures and relative energies of several conformers of the NOHG analogues of radicals **7**−**11** (optimized at the UB3LYP/6-31G(d) level,[19−21,30] see also Chart 2) which could be produced by H-atom abstraction from **3**−**5**. A comparison of *O*-radicals **7**, **8a**, and **8b**, and *N*-radical **9**, derived from neutral NOHA models **3** and **4**, shows that *O*-radicals are significantly more stable than *N*-radicals for the neutral case. However, we have concluded that protonated NOHA (**5**) is the bound form of NOHA, and in contrast to the neutral case, the *O*- and *N*-radicals (**10** and **11**) derived from **5** are close in energy (Figure 2). Although *O*-radical **10** is slightly more stable than *N*-radical **11**,[31] the ENDOR results suggest that the N−H hydrogen atom in bound **5** is positioned more favorably than the O−H hydrogen for abstraction. Comparison of computed proton affinities[32] for models **3** and **9** suggests that the pK_a of *N*-radical **11** is comparable to that of protonated NOHA (**5**), and it is therefore likely that if the *N*-radical is formed, it will remain protonated. While *N*-radical formation would be highly unfavorable in the neutral case, it is clear that protonation in **5** provides a potential avenue for *N*-radical formation (Scheme 2).

On the basis of a combination of theory and experiment (ENDOR and X-ray), we conclude that structure **5** is the most likely form of N^G-hydroxy-L-arginine bound to NOS. In addition, *N*-radical formation is shown to be a plausible alternative to *O*-radical formation in the NOS-catalyzed conversion of **5** to L-citrulline and NO.

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(26) Reference 23 notes that arginine and various inhibitors of NOS all bind to the conserved active site carboxylate in a bidentate fashion, while NOHA bound to eNOS appears to interact with this carboxylate in a monodentate fashion. Further experiments are necessary, however, to clarify the details of NOHA binding by the various isoforms of NOS.

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(30) $\langle S^2 \rangle$ values for all radical species described are between 0.75 and 0.78.

(31) Both **10** and **11** are π-radicals.

(32) (a) The gas-phase proton affinities ((U)B3LYP/6-31G(d)) for **3** and **9** differ by only 0.3 kcal/mol (238.1 and 238.4 kcal/mol, respectively). Inclusion of aqueous solvation (SCI-PCM-(U)B3LYP/6-31G(d) single points[32b−d] with $\epsilon = 78.5$) has a negligible effect on this energy difference. (b) Wiberg, K. B.; Keith, T. A.; Frisch, M. J.; Murcko, M. *J. Phys. Chem.* **1995**, *99*, 9072−9079. (c) Wiberg, K. B.; Castejon, H. *J. Comput. Chem.* **1996**, *17*, 185−190. (d) Zheng, Y.-J.; Ornstein, R. L. *J. Mol. Struct. (THEOCHEM)* **1998**, *429*, 41−48.