Mechanism of Nitric Oxide Synthase. Evidence that Direct Hydrogen Atom Abstraction from the O−H Bond of N^G-Hydroxyarginine Is Not Relevant to the Mechanism

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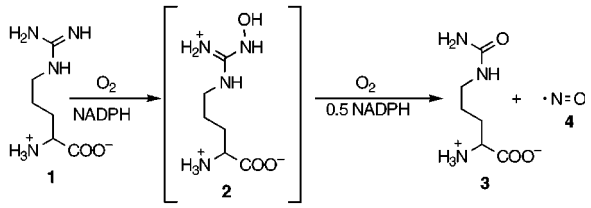
Mammalian nitric oxide synthases (NOS, E.C. 1.14.13.39) comprise a family of enzymes that catalyzes the conversion of L-arginine (**1**) to L-citrulline (**3**) and the second messenger molecule nitric oxide (**4**, Scheme 1).[1] There are two constitutive isozymes of NOS, neuronal nitric oxide synthase (nNOS), which is believed to generate NO in the brain[2] and is involved in neurotransmission and long-term potentiation,[3] and endothelial nitric oxide synthase (eNOS), which is important as it is involved in the regulation of smooth muscle relaxation and vascular tone.[4] A third, inducible, isoform in macrophage (iNOS) is important in the immune system defense against microorganisms and tumor cells.[5]

NOS is a complex enzyme that requires five cofactors for activity. The N-terminus is the oxygenase domain, to which heme, tetrahydrobiopterin, and the substrate bind. The C-terminus reductase domain binds molecules of FMN, FAD, and NADPH; the two domains are connected by a calmodulin-binding domain.[6] The active form of the enzyme exists as a homodimer in which the interaction occurs primarily between two oxygenase domains,[7] but forms an extended dimer interface.[8]

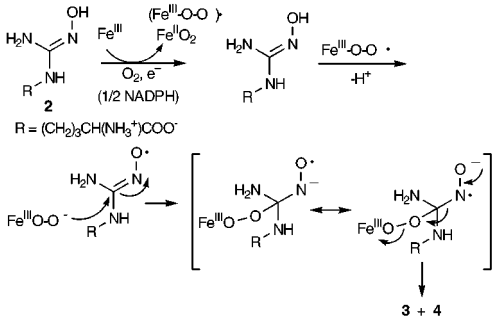
The mechanism of action of this family of enzymes is not yet clear, although it is known that the reaction proceeds from L-arginine to L-N^G-hydroxyarginine (**2**) and then to L-citrulline and NO.[9] N^{ω}-Hydroxy-L-arginine was shown to be a kinetically competent substrate for macrophage NO synthase that gives stoichiometric amounts of L-citrulline and NO.[10] [^{15}N]*N*-Hydroxy-L-arginine gives ^{15}NO, indicating that the hydroxylamine nitrogen becomes the N in NO.[10b] The first half-reaction consumes two electrons from NADPH and incorporates one atom of oxygen from molecular oxygen to give **2**. The second half-reaction oxidizes **2**, with the consumption of half an equivalent of NADPH and a molecule of molecular oxygen to give the final products. The simplest way to rationalize the conversion of **1** to **2** is via a standard heme-dependent hydroxylation mechanism; however, there is evidence that the tetrahydrobiopterin also may be involved in the electron-transfer process of this reaction.[11] The second half-reaction presents an even greater challenge to the mechanistic enzymologist, having no direct analogy in other systems. Consequently, a variety of mechanistic possibilities have been proposed over the years for this step. The early proposals, that **2** undergoes hydrolysis to **3** and NO,[12] cannot be correct because the oxygen atom in **3** was shown to come from O_2, not from H_2O.

Schemes 2−8 show some of the different mechanistic possibilities previously proposed for the conversion of **2** to **3** and **4** which use molecular oxygen as the oxygen source for both half reactions. The principal difference in these mechanisms involves the order of bond cleavage. In all cases the O−H bond of **2** must be cleaved, but in Schemes 2,[13] 3,[14] 4,[15] 5,[16] and 6[17] the O−H

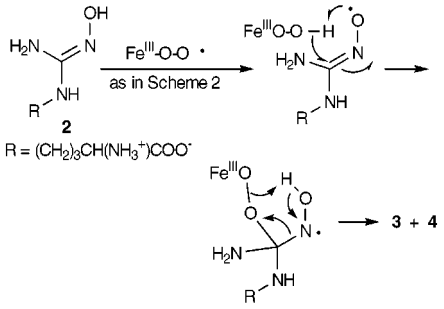
Scheme 1



Scheme 2



Scheme 3



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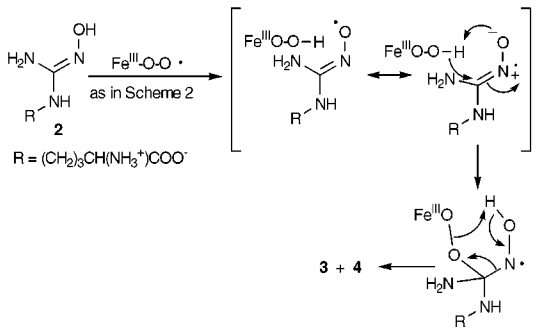
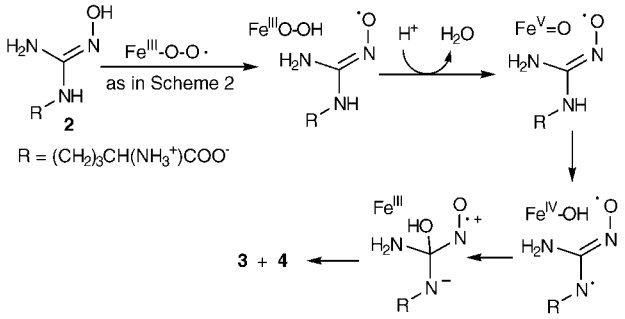
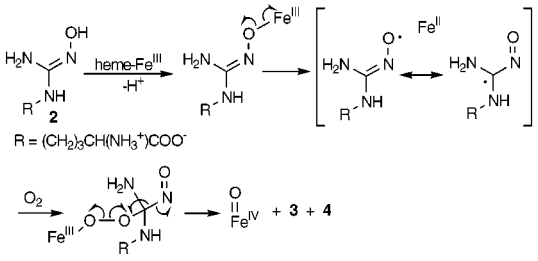
Scheme 4



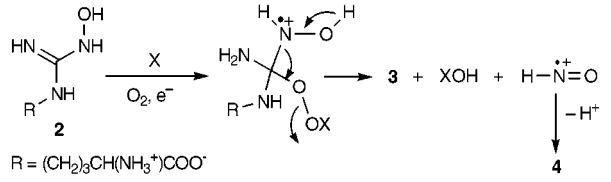
Scheme 5



Scheme 6

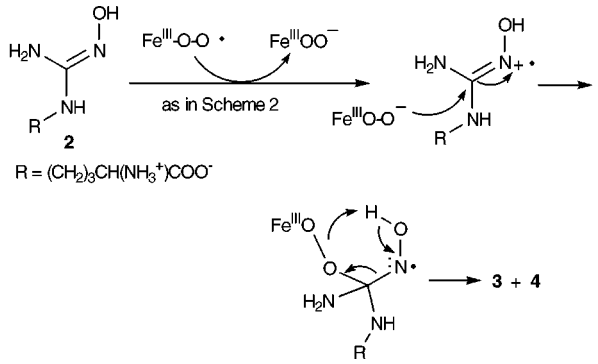


Scheme 7



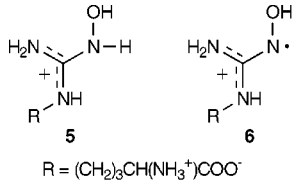
bond is broken during the one-electron oxidation step. In Schemes 7[18] and 8[19] the O−H bond is broken after an initial one-electron transfer to an unspecified group (X) and O_2 (Scheme 7) or to a heme peroxy radical (Scheme 8). In both cases, the peroxide that is formed then adds to the carbon−nitrogen double bond to give a species that either decomposes spontaneously to **3** and protonated NO (Scheme 7) or undergoes decomposition via a six-membered transition state involving simultaneous deprotonation and conversion to products (Scheme 8). On the basis of electrochemical model studies and thermodynamics, Korth et al.[14] suggested that the oxime radical cation in Schemes 7 and 8 would

Scheme 8



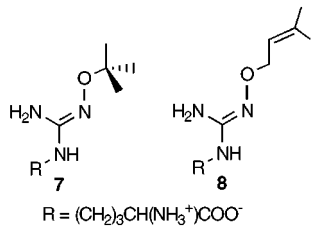
lose a proton to give the oxime radical much faster than nucleophilic addition of the ferric hydroperoxide could occur, and therefore the mechanism in Scheme 8 was not reasonable.

Recently, ENDOR spectroscopy of **2** bound to nNOS indicated that the N−H hydrogen of **2** was juxtaposed closer to the heme iron than was the O−H hydrogen.[20] From the crystal structure of **2** bound to the oxygenase domain of iNOS, Crane et al.[21] concluded that the hydroxyl hydrogen was not oriented correctly for hydrogen atom abstraction, but the N^G N−H was properly positioned. Density functional theory calculations of the structures and relative energies of various model forms of **2** showed that the most likely form of L-N^G-hydroxyarginine bound to NOS is the protonated form (**5**), and to rationalize the ENDOR results, it was found that a reasonable alternative to *O*-radical formation from **5** would be *N*-radical formation (**6**) during conversion of **2** to **3** and **4**.[22]



In this communication we describe experiments with analogues of L-N^G-hydroxyarginine (**2**) to test whether initial O−H or N−H bond cleavage occurs in the conversion of **2** to **3** and **4**. The results of these experiments suggest that initial O−H bond cleavage is *not* relevant to the reaction, providing chemical support for the ENDOR spectroscopic,[20] crystallographic,[21] and theoretical[22] proposals for how NOS catalyzes the degradation of **2**.

To test initial O−H bond cleavage of **2** by NOS, N^G-*tert*-butyloxy- (**7**)[23] and N^G-(3-methyl-2-butenyl)oxy-L-arginine (**8**)[24] were synthesized by the same route used to prepare **2**,[10b,25]



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(23) ^{1}H NMR (D_2O) δ 4.07 (t, 1 H), 3.28 (t, 2 H), 1.95 (m, 2 H), 1.72 (m, 2 H), 1.23 (s, 9 H); HRMS (M + 1) calcd for $C_{10}H_{22}O_3N_4$ 247.1715, found 247.1714.

(24) ^{1}H NMR (D_2O) δ 5.38 (t, 1 H), 4.24 (d, 2 H), 3.63 (t, 1 H), 3.05 (t, 2 H), 1.82 (m, 2 H), 1.74 (s, 3 H), 1.68 (s, 3 H), 1.59 (m, 2 H).

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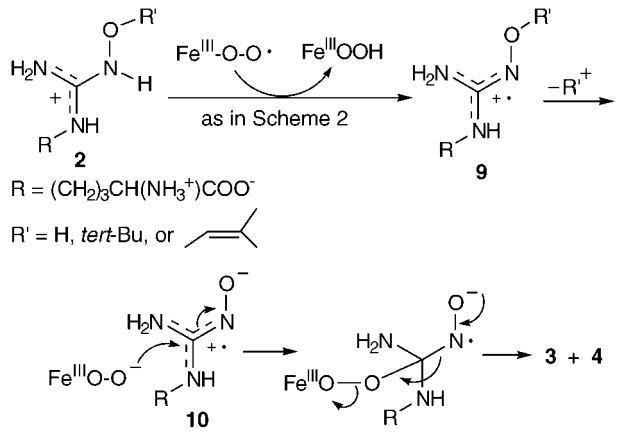
Scheme 9



substituting either *O*-(*tert*-butyl)hydroxylamine (Aldrich) or *O*-(3-methyl-2-butenyl)hydroxylamine[26] for hydroxylamine. The *tert*-butyl and 3-methyl-2-butenyl substituents would prevent a mechanism that requires an initial hydrogen atom or proton abstraction from the O−H bond. However, if initial N−H bond cleavage or electron transfer from that nitrogen atom occurs, the intermediate (**9**, Scheme 9) could readily decompose, with loss of either *tert*-butyl cation or 3-methyl-2-butenyl cation to give the same intermediate proposed after hydrogen atom abstraction from **2** (**10**, Scheme 9), and **3** and **4** would be produced.

Both **7** and **8** were shown to be substrates for nNOS,[27] producing both citrulline (data not shown) and NO (Figure 1, A and B, respectively) with Michaelis−Menten kinetics (Table 1). Both compounds were shown to be free of possible contaminating L-arginine (**1**) and L-N^G-hydroxyarginine (**2**) by treatment with *o*-phthalaldehyde reagent[28] and HPLC analysis. The limit of detection for **1** or **2** was 1 μM. Because neither **1** nor **2** was detectable in the incubation mixture, no citrulline produced from them would be detectable either (citrulline formation is detected by conversion to the *o*-phthalaldehyde derivative as well). The rate constants for **7** and **8** are similar to those for substrates **1** and **2**; the high K_m values for **7** and **8** reflect the steric hindrance

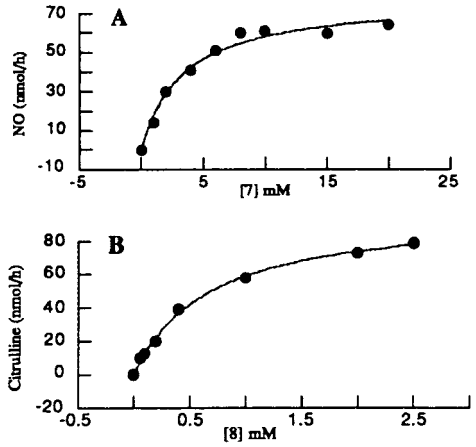


Figure 1. Michaelis−Menten plot of NO formation for **7** (A) and for **8** (B).

Table 1. Kinetic Constants for **2**, **7**, and **8**

compd (product assay)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
7 (NO)	3.27 ± 0.56	6.92 ± 0.36	2.12
7 (citrulline)	2.96 ± 0.73	6.82 ± 0.60	2.30
8 (NO)	0.82 ± 0.09	11.2 ± 0.5	13.66
8 (citrulline)	0.67 ± 0.06	8.92 ± 0.28	13.31
L-N^G-OHArg (**2**)	0.015 ± 0.002	21.2 ± 1.5	1.44 × 10^3

of the *tert*-butyl and dimethylallyl groups, respectively, toward binding. The fact that both act as substrates indicates that it is highly unlikely that initial O−H bond cleavage in the first step is relevant to the mechanism. This supports either initial N−H bond cleavage (Scheme 9, R′ = H) or electron transfer from the nitrogen atom to which the hydroxyl group of **2** is attached (Scheme 9 except electron transfer instead of hydrogen atom abstraction).

These results further support the density functional theory calculations[22] indicating that the product of hydrogen atom abstraction from the N−H of **5** is similar in energy to the product of hydrogen atom abstraction from the O−H. The results also are consistent with the crystal structure and mechanistic conclusions of Crane et al.,[21] who suggested that the peroxo-heme initially removes the N−H hydrogen. The mechanism shown in Scheme 9 would be consistent with all of these results, but the mechanisms in Schemes 2−6 are inconsistent.

Acknowledgment. The authors are grateful to Professor Michael A. Marletta for the recombinant *E. coli* cells that express murine macrophage iNOS and to the National Institutes of Health (GM49725) for financial support of this research.

Supporting Information Available: Syntheses of **7** and **8**, LC-MS for the control reactions to show the absence of contaminating L-arginine and L-N^G-hydroxyarginine, and assay procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(26) 1-Chloro-3-methyl-2-butene (2 g, 20 mmol) was treated with anhydrous K_2CO_3 (2.76 g, 20 mmol) and *N*-hydroxyphthalimide (3.3 g, 20 mmol) in DMSO (30 mL) at room temperature. After being stirred for 24 h the mixture was poured into cold water, and the white precipitate was collected; recrystallization from ethanol gave white prisms (3 g, 66%): ^{1}H NMR ($CDCl_3$) δ 7.85 (m, 2H), 7.75 (m, 2H), 5.53 (t, 1H), 4.72 (d, 2H, J = 7.4), 1.77 (s, 3H), 1.74 (s, 3H). This product (3 g, 13 mmol) was treated with hydrazine hydrate (3.6 mL, 20 mmol) in refluxing ethanol (60 mL) for 30 min. Following cooling, the phthalazine was dissolved in 3% Na_2CO_3, and the product was extracted with ether. After drying ($MgSO_4$), HCl gas was bubbled into the ether solution, and the white flakes (1.0 g, 76%) were collected: ^{1}H NMR (D_2O) δ 5.36 (t, 1H), 4.53 (d, 2H), 1.79 (s, 3H), 1.73 (s, 3H).

(27) A recombinant murine iNOS/calmodulin overexpression system in *Escherichia coli* was obtained, and the enzyme was purified (Hevel, J. M.; White, K. A.; Marletta, M. A. *J. Biol. Chem.* **1991**, *266*, 22789−22791). The enzyme was quantified according to the methods of Stuehr and Ikeda-Saito (Stuehr, D. J.; Ikeda-Saito, M. *J. Biol. Chem.* **1992**, *267*, 20547−20550) using ϵ_{397} = 71 mM^{-1} cm^{-1} and a molecular mass of 150 kDa.

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