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Revisiting Heme Mechanisms. A Perspective on the Mechanisms of Nitric Oxide Synthase (NOS), Heme Oxygenase (HO), and Cytochrome P450s (CYP450s)

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ABSTRACT: Despite the essential biological importance of reactions that involve heme, mechanisms of heme reactions in enzymes like nitric oxide synthase (NOS), heme oxygenase (HO), and cytochrome P450s (CYP450s) are still not well-understood. This Perspective on NOS, HO, and CYP450 mechanisms is written from the point of view of the heme chemistry. Steps in the classical heme catalytic cycle are discussed based on the specific environment within each of these enzymes. Elucidation of the mechanisms of NOS inactivation by some substrate analogues provides important mechanistic clues to the NOS catalytic mechanism. On the basis of mechanistic studies of NOS inactivation by amidine analogues of L-arginine and other previous mechanistic results, a new mechanism for NOS-catalyzed L-arginine N^G-hydroxylation (the first half of the catalytic reaction) is proposed in this Perspective. The key step in the second half of the NOS catalytic reaction, the internal electron transfer between the substrate and heme, is discussed on the basis of mechanistic results of NOS inactivation by N^G-allyl-L-arginine and the structures of the substrate intermediates. Elucidation of the mechanism of NOS inactivation by amidines, which leads to heme degradation, also provides important mechanistic implications for heme oxygenase-catalyzed heme catabolism. Focusing on the *meso*-hydroxylation step during inactivation of NOS by amidines as well as the HO-catalyzed reaction, the essential nature of the heme-oxygen species responsible for porphyrin *meso*-hydroxylation is discussed. Finally, on the basis of the proposed heme degradation mechanism during NOS inactivation and the HO-catalyzed reaction, the mechanism for the formation of the monooxygenated heme species in P450-catalyzed reactions is discussed.

Iron-containing protoporphyrin IX, known as heme (**1**), is one of the more significant molecules in biochemistry. Heme plays a vital role in a wide range of biologically important reactions in humans, such as regulating the formation of the cell signaling small molecule, nitric oxide, in nitric oxide synthase (NOS) (*1*, *2*) and mediating oxidative metabolism of xenobiotics and drugs in cytochrome P450s (CYP450s[1]) (*3*). The degradation of heme itself, a process catalyzed by the non-heme-containing enzyme heme oxygenase (HO), displays both beneficial and deleterious effects depending on the circumstances (*4*). The mechanisms of heme-containing enzymes, such as NOS and P450s, have

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[1] Abbreviations: CYP450, cytochrome P450; HO, heme oxygenase; L-NHA, *N*-hydroxyl-L-arginine; L-NIO, N^5-(1-iminoethyl)-L-ornithine; NO, nitric oxide; nNOS, neuronal nitric oxide synthase.

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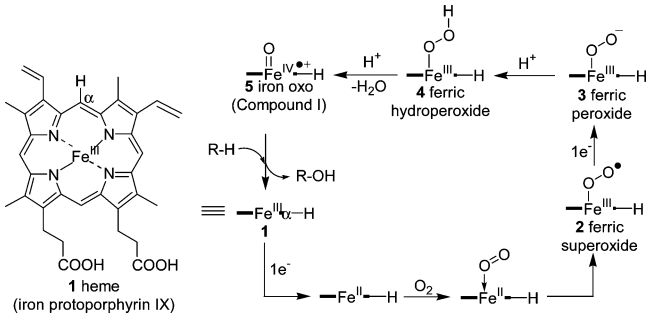
Scheme 1: The Classic Oxidative Catalytic Cycle of Heme



been studied extensively, while the mechanisms of heme regulation are still elusive despite the fact that the heme catalytic cycle (Scheme 1) has been known for a long time (*5*). As shown in Scheme 1, the Fe(III) atom of heme (**1**) is reduced to Fe(II), which then binds a molecule of O_2. The Fe(II)−O_2 complex is converted to ferric superoxide (**2**), which is further converted to ferric hydroperoxide (**4**) after one electron reduction to ferric peroxide (**3**) followed by protonation. The highly oxidative ferryl species (iron oxo species or Compound I, **5**), which is formed by ferric hydroperoxide dehydration, reverts to ferric heme after oxygen atom insertion.

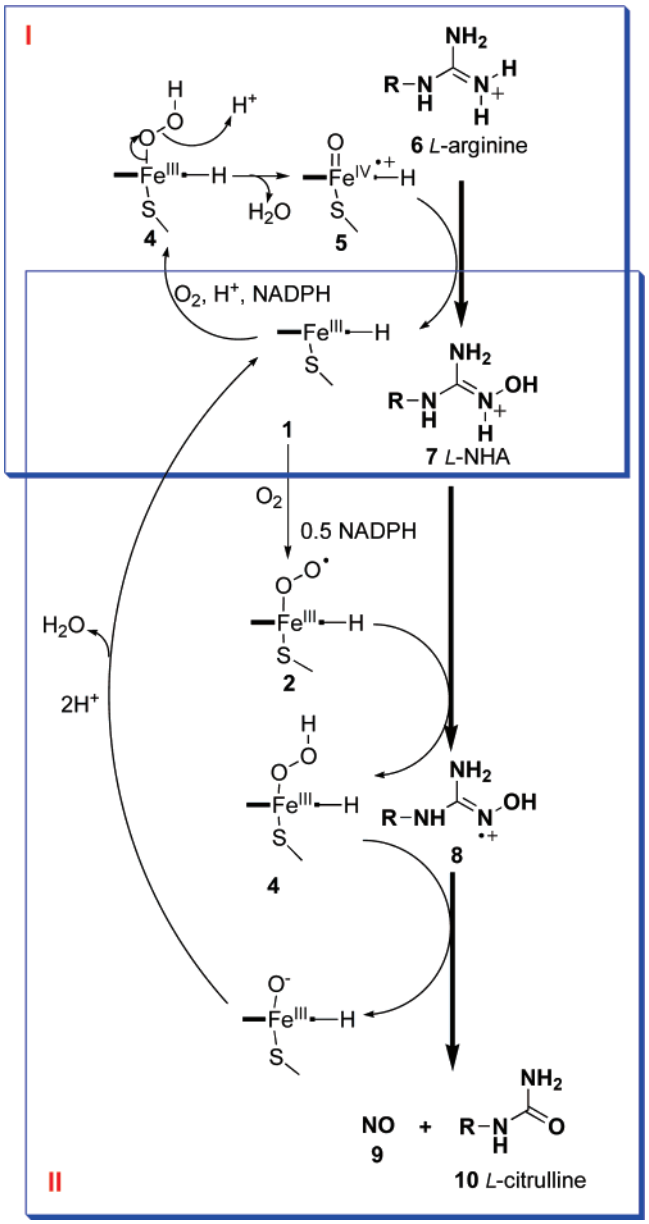
1. PERSPECTIVES ON THE CATALYTIC MECHANISM OF NITRIC OXIDE SYNTHASE (NOS)

As shown in Scheme 2, there are two distinct steps in the NOS reaction: the formation of N^G-hydroxy-L-arginine (L-NHA, **7**) from L-arginine (**6**) (the first half) and the conversion of L-NHA (**7**) to L-citrulline (**10**) and NO (**9**) (the second half). The heme cofactor in NOS is proposed to directly regulate these two steps using O_2, protons, and electrons from NADPH (*6*).

The first half of the NOS catalytic mechanism involves hydroxylation at the guanidine N^G-position of the substrate (Scheme 2, box **I**), whose mechanism was proposed to be similar to that of the reaction catalyzed by cytochrome P450, involving heme, O_2, and NADPH, with electron transfer through the flavoprotein reductase domain (*6*). The ferryl species (**5**), which is well established to be the active monooxygenation intermediate in the cytochrome P450-catalyzed *C*-hydroxylation reaction (*5*), was proposed to be directly responsible for the NOS-catalyzed *N*-hydroxylation reaction in the formation of L-NHA (**7**). However, there is no direct evidence supporting the involvement of **5** in this hydroxylation step. In fact, the existing evidence suggests that **5** is not directly involved in this half of the mechanism (see below). In this Perspective, a new mechanism is proposed for the heme-dependent NOS-catalyzed guanidine *N*-hydroxylation step that is consistent with all of the known mechanistic information.

The second half of the NOS catalytic mechanism involves a one-electron oxidation of L-NHA (**7**), followed by the conversion of the N^G-OH-guanidine group to NO (**9**) and L-citrulline (**10**) (*7*). There is evidence to support the involvement of the heme cofactor in this conversion. As shown in Scheme 2 (box **II**), it was proposed that the ferric hydroperoxide (**4**) is generated from a two-electron reduction of heme and O_2. This intermediate then undergoes nucleo-

Scheme 2: Proposed NOS Catalytic Mechanism[a]



[a] The fifth ligand of the heme iron in NOS is a cysteine residue.

philic attack by the peroxide on the guanidino carbon of L-NHA$^{\bullet+}$ (**8**) (*8*). Subsequent collapse of the tetrahedral intermediate generates NO (**9**) and L-citrulline (**10**). A stoichiometry of 0.5 equiv of NADPH (the first reducing equivalent) is needed for the second half of the mechanism. A key question in this half of the NOS catalytic reaction is the source of the second reducing equivalent delivered to the heme to reduce the ferric superoxide (**2**), formed after one-electron reduction from 0.5 equiv of NADPH and binding of O_2 to the nucleophilic ferric peroxide species (**4**). This has been proposed as the abstraction of the N−H hydrogen atom from L-NHA (**7**) by ferric superoxide (**2**), forming L-NHA$^{\bullet+}$ (**8**) and the ferric peroxide species (**4**) (*7*, *8*). The formation of this substrate radical sets the stage for the release of NO (**9**) after collapse of the tetrahedral intermediate. This proposed NOS mechanism predicts a stoichiometry of 1.5 equiv of NADPH when L-arginine (**6**) is the substrate and 0.5 when L-NHA (**7**) is the substrate, which is supported by experimental results (*9*).

Many aspects of the proposed NOS mechanism (Scheme 2) are still unclear, including the following: (1) In the first step, which residue in the active site donates the proton to dehydrate the ferric hydroperoxide (**4**), generating the ferryl species (**5**)? (2) If the ferryl species (**5**) is directly responsible for the oxygen atom insertion into the guanidine N−H bond of L-arginine (**6**) in the first step, why is it not supported by current experimental results (*16*, *17*) (see below)? (3) In the second step, is it a hydrogen atom abstraction from the O−H bond or from the N−H bond of the N^G-OH-guanidine group that functions as the source of the second electron for the formation of the ferric hydroperoxide (**4**)?

1.1. The Acidic Residue in the First Step. Crystallographic studies show that the active site region of all three isozymes of NOS around the L-arginine binding site is hydrophobic (*10*, *11*). The crystal structures of the NOS oxygenase domain with the amidine analogues of L-arginine, N^5-(1-iminoethyl)-L-ornithine (L-NIO; the methyl amidine of L-arginine) and *N*-(3-(aminomethyl)benzyl)acetamidine (1400W), bound show that both amidino methyl groups face inward toward the heme peroxide, making the active site even more hydrophobic. A water molecule that is observed between the guanidino group of bound L-arginine and the proline residue in the active site of endothelial NOS (eNOS) is not present in the complex between eNOS and L-NIO, presumably because of the increased hydrophobicity, when the amidine is bound (*10*). On the basis of crystallographic studies, Poulos and co-workers proposed that the protonated guanidine group of L-arginine acts as the proton source for the ferric peroxide species (*10*), which is supported by our mechanistic studies of inducible NOS (iNOS) inactivation by amidine analogues of L-arginine, L-NIO and 1400W (*12*, *13*). When the amidine replaces the guanidino group, there is no longer a proton source for the conversion of the heme hydroperoxide (**4**) or heme peroxyl anion (**3**) to the iron oxo species; consequently, the enzyme appears to resort to an alternative heme oxygenase-like reaction (Figure 1). This leads to the formation of α-*meso*-hydroxyheme, which then is converted to verdoheme and biliverdin by further oxidation. Thus, both the crystallographic studies and experimental data support the notion that the guanidine NH_2 group of L-arginine is both the site of metabolism and the proton source for catalytic turnover.

1.2. The Ferryl Mystery and a New Mechanism. The first half of the NOS catalytic reaction is monooxygenation of the hydrophilic guanidine N−H bond. The mechanism for this step is directly borrowed from another enzyme that catalyzes monooxygenation, cytochrome P450s (*5*, *6*), which catalyzes monooxygenation of C−H bonds, N−H bonds, as well as other functional groups (*14*). In the classic monooxygenation mechanism of cytochrome P450 chemistry (Scheme 3), the ferryl species (**5**) is formed after dehydration of the ferric hydroperoxide (**4**). The highly reactive ferryl species inserts an oxygen atom into the C−H bond of the substrate (R-H) to form R-OH (*5*,*15*).

Marletta and co-workers tested the existence (and the role) of the ferryl species (**5**) in the NOS mechanism with similar experiments that were used to establish the cytochrome P450 mechanism (*16*, *17*). First, iodosobenzene was shown to be able to replace NADPH, O_2, and H^+ to facilitate the cytochrome P450 monooxygenation reaction in the first step (Scheme 3, pathway a) (*18*). However, in the NOS−L-arginine system, L-NHA (**7**) formation is not supported by

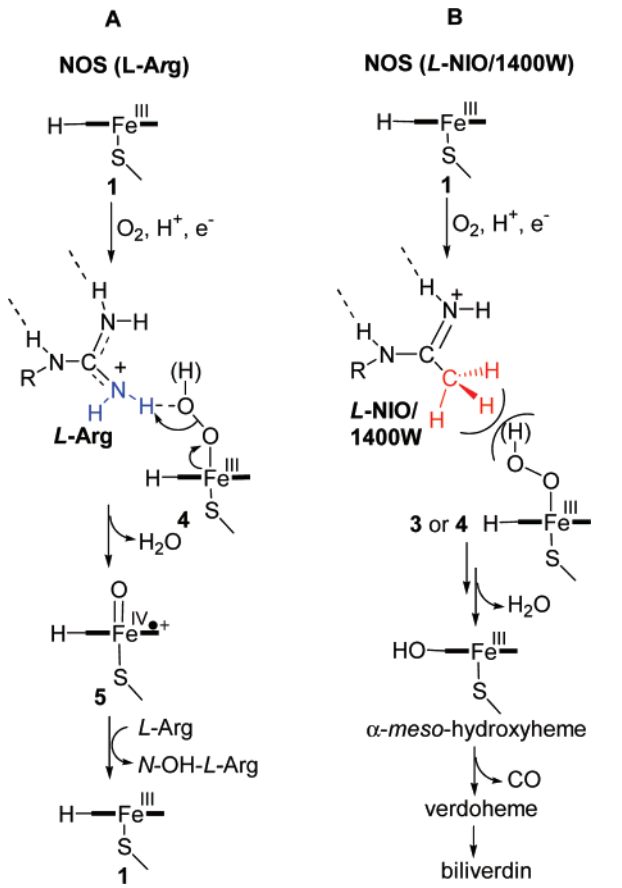
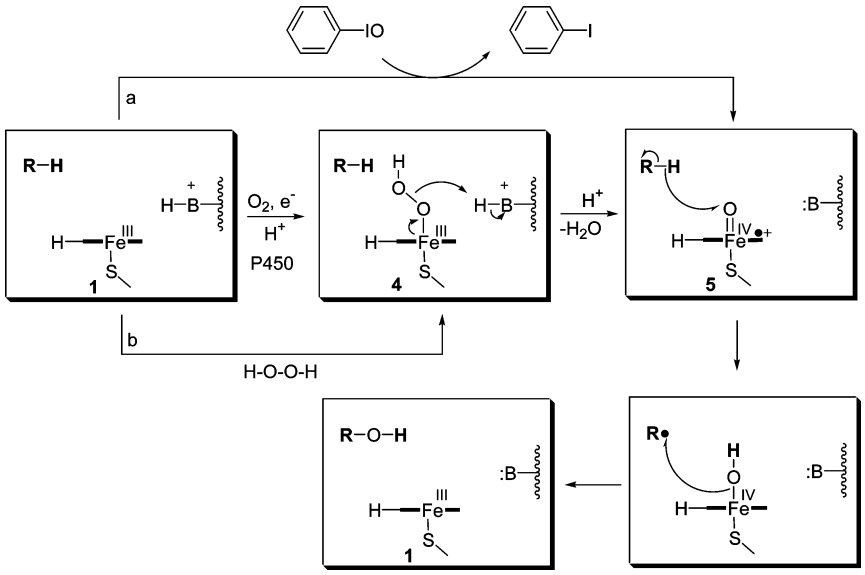


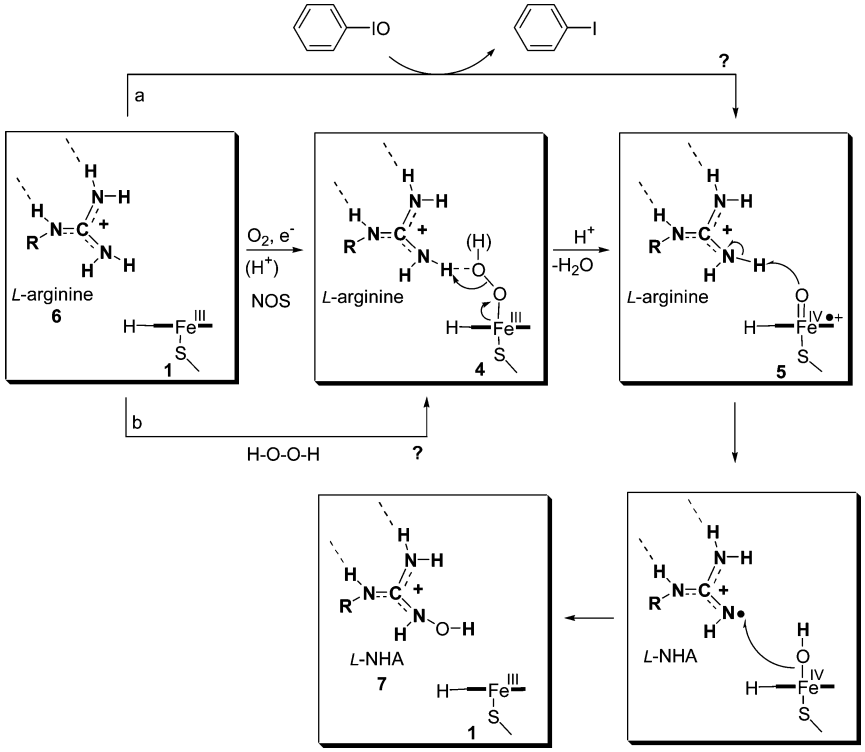
FIGURE 1: Comparison of the reaction of NOS with L-arginine bound (A) and the proposed mechanism when L-NIO or 1400W is bound (B). The guanidine NH_2 group of L-arginine is both the site of metabolism and proton source.

iodosobenzene (Scheme 4, pathway a) (*16*). Second, hydrogen peroxide was shown to channel the conversion of the ferric hydroperoxide from ferric heme directly with cytochrome P450 (Scheme 3, pathway b). However, the NOS−L-arginine system is completely unreactive with hydrogen peroxide (Scheme 4, pathway b) (*16*). One interpretation of this negative result for the hydrogen peroxide reaction is that H_2O_2 cannot gain access to the active site; however, the observation that H_2O_2 supports L-NHA oxidation (the second half of NOS reaction) (*17*) suggests that H_2O_2 can enter the active site. Furthermore, crystallographic studies show that there is a molecule of H_2O close to the center of the active site when L-arginine binds, which suggests the hydrophilic level of the active site is amenable for H_2O_2 to access the active site. The size of H_2O_2 should not be a problem because L-arginine and L-citrulline can get in and out of the active site as the catalytic reaction proceeds. Marletta and co-workers observed the formation of L-citrulline and N^G-cyanoornithine (CN-Orn), when L-NHA is the substrate for NOS using H_2O_2 as the oxidizing agent (*17*). It was proposed that H_2O_2 channels the generation of ferric hydroperoxide (responsible for the formation of L-citrulline), which can subsequently undergo dehydration to the iron oxo species (responsible for the formation of CN-Orn) directly from ferric heme. Therefore, H_2O_2 should also support the formation of these two active intermediates when L-arginine is the substrate, yet no turnover is observed. These mechanistic results lead to the following dilemma in the NOS mechanism: NOS is believed to be a hemoprotein that catalyzes a

Scheme 3: Proposed Mechanism for the Cytochrome P450-Catalyzed Reaction Is Supported by an Iodosobenzene Experiment (a) and a Hydrogen Peroxide Experiment (b)



Scheme 4: Proposed Mechanism for the First Step of the NOS-Catalyzed Reaction Is Not Supported by an Iodosobenzene Experiment (Pathway a) and a Hydrogen Peroxide Experiment (Pathway b)



monooxygenation reaction similar to that of cytochrome P450s with the involvement of the heme cofactor, but the existing experimental data do not support a direct involvement of the heme iron oxo species in the first N^G-hydroxylation step (*6*).

As implicated by the results of our mechanistic studies of iNOS inactivation by amidine analogues of L-arginine (*12*), the proton source around the heme iron (and iron peroxide) is crucial for heme regulation. Since the active site of NOS is very hydrophobic, a proton can only be directly donated from the substrate or substrate analogues (a H_2O is present between the carbonyl oxygen of a Pro residue and the guanidine nitrogen of substrate; this H_2O might be part of the proton delivery network) (*10*). As shown in Figure 2 for the complex between substrate (or inactivator) and heme, when there is only one proton available (from the protonated guanidine NH_2 group) for the ferric peroxide species to abstract, the catalytic pathway can proceed, but when there are two protons (one from H_2O_2 and one from the substrate), no substrate conversion occurs.

To reconcile all of the mechanistic information, we propose a new mechanism for NOS-catalyzed L-arginine N^G-hydroxylation, as inspired by the *N*-hydroxylation reaction of pyridine by organic peroxy acids. As shown in Scheme 5, the terminal oxygen anion of ferric peroxyl species **3** can remove the proton from the guanidine nitrogen atom.

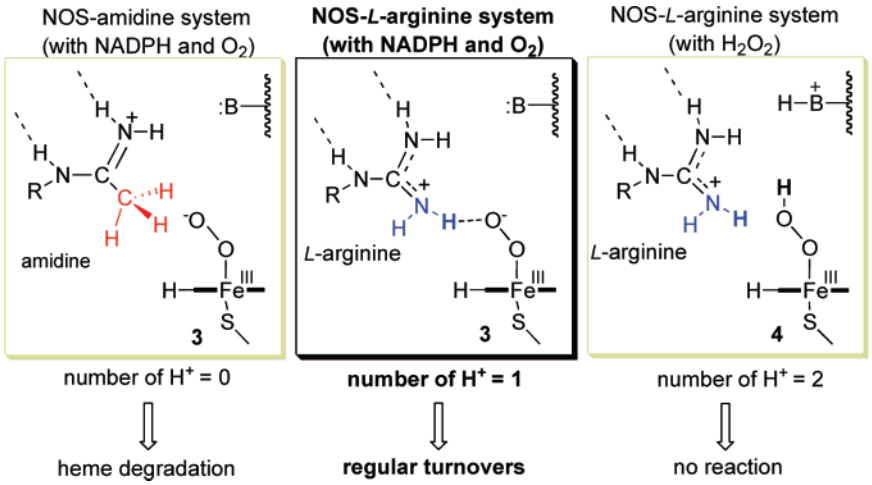
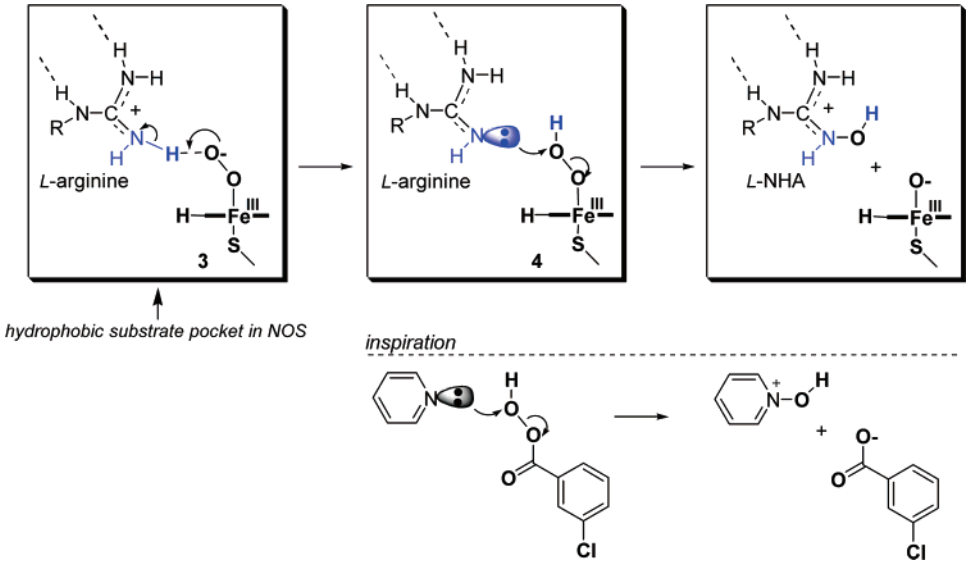


FIGURE 2: The number of protons available in the active site affects heme chemistry.

Scheme 5: Proposed New Mechanism for NOS-Catalyzed Guanidine N^G-Hydroxylation



Because of the unavailability of a proton source, this nitrogen atom will stay in the unprotonated state with the lone pair electrons available for attack on the O−O bond of the ferric hydroperoxide (**4**). This attack leads to N^G-hydroxylation.

Cytochrome P450s also catalyzes *N*-hydroxylation (*N*-oxygenation) of many other substrates (*14*). Mechanisms of cytochrome P450-catalyzed *N*-oxygenation reactions seem to involve different heme intermediates. Previous mechanistic studies suggest that iron oxo (*19*), ferric superoxide (*20*), and ferric hydroperoxide (*21*) can all serve as oxidizing species in different cytochrome P450-catalyzed *N*-oxygenation reactions. Mechanisms for individual *N*-oxygenation reactions are not fully elucidated. The new mechanism proposed for NOS-catalyzed guanidine *N*-hydroxylation provides insights applicable to cytochrome P450-catalyzed *N*-oxygenation reactions as well.

If cytochrome P450s and NOS both use a heme cofactor and catalyze the same reaction, why does the former class of enzymes have such broad specificity, but NOS has such narrow specificity? The new mechanism shown in Scheme 5 may answer this question. As depicted by this mechanism, the ferryl species (Scheme 3, **5**), which is directly responsible for the majority of cytochrome P450-catalyzed monooxygenation reactions, is not an intermediate in the NOS-catalyzed N−H monooxygenation reaction because of the lack of a second proton source to activate the ferric peroxyl species (**3**) and generate ferryl species **5**. The active catalytic heme species in the NOS-catalyzed guanidine N^G-hydroxylation reaction is ferric peroxide anion **3**, which is hydrogen bonded to a substrate guanidinium proton, thereby activating it to ferric hydroperoxide **4**. This intermediate undergoes a rapid *N*-hydroxylation of the free guanidine group because of the lack of an additional proton source required to allow dehydration of **4** to ferryl species **5**. In addition to the specific binding site for L-arginine, the essential basis for NOS chemistry is the hydrophobic nature of the active site and the hydrophilic guanidino NH_2 of the substrate.

1.3. The Internal Electron Transfer in the Second Step. The second step of the NOS reaction, the conversion from L-NHA (**7**) to NO (**9**) and L-citrulline (**10**) (Scheme 2, **II**), is more clearly understood. NADPH provides only one of two electrons needed for the formation of ferric hydroperoxide species **4** (*7*). The second electron was proposed to derive from L-NHA. It was shown that H_2O_2 can replace NADPH, O_2, and the proton source to support the formation of the ferric hydroperoxide species, which is believed to attack the guanidino carbon with its nucleophilic terminal oxygen, thereby leading to the final products of the L-arginine

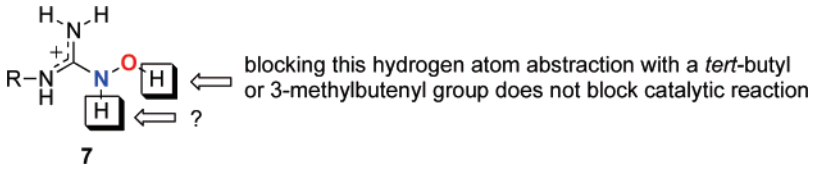


FIGURE 3: Replacement of the *N*-hydroxyguanidino O−H hydrogen by other groups does not affect the catalytic reaction

reaction (*16*, *17*). The H_2O_2-supported reaction gives L-citrulline and NO^- (instead of NO•), which is consistent with the proposed mechanism (Scheme 2, **II**), because L-NHA (**7**) is not oxidized to L-$NHA^{\bullet+}$ (**8**) by ferric superoxide (**2**) prior to nucleophilic attack by ferric peroxide **3**, and therefore the extra electron leads to NO^- formation instead of to NO (*16*). Besides L-citrulline and NO^-, N^G-cyano-L-ornithine (CN-Orn) is also found to be a product of the H_2O_2-supported L-NHA reaction. The formation of CN-Orn was proposed to support the existence of a ferryl species (**5**) in the NOS−L-NHA−H_2O_2 system (*17*).

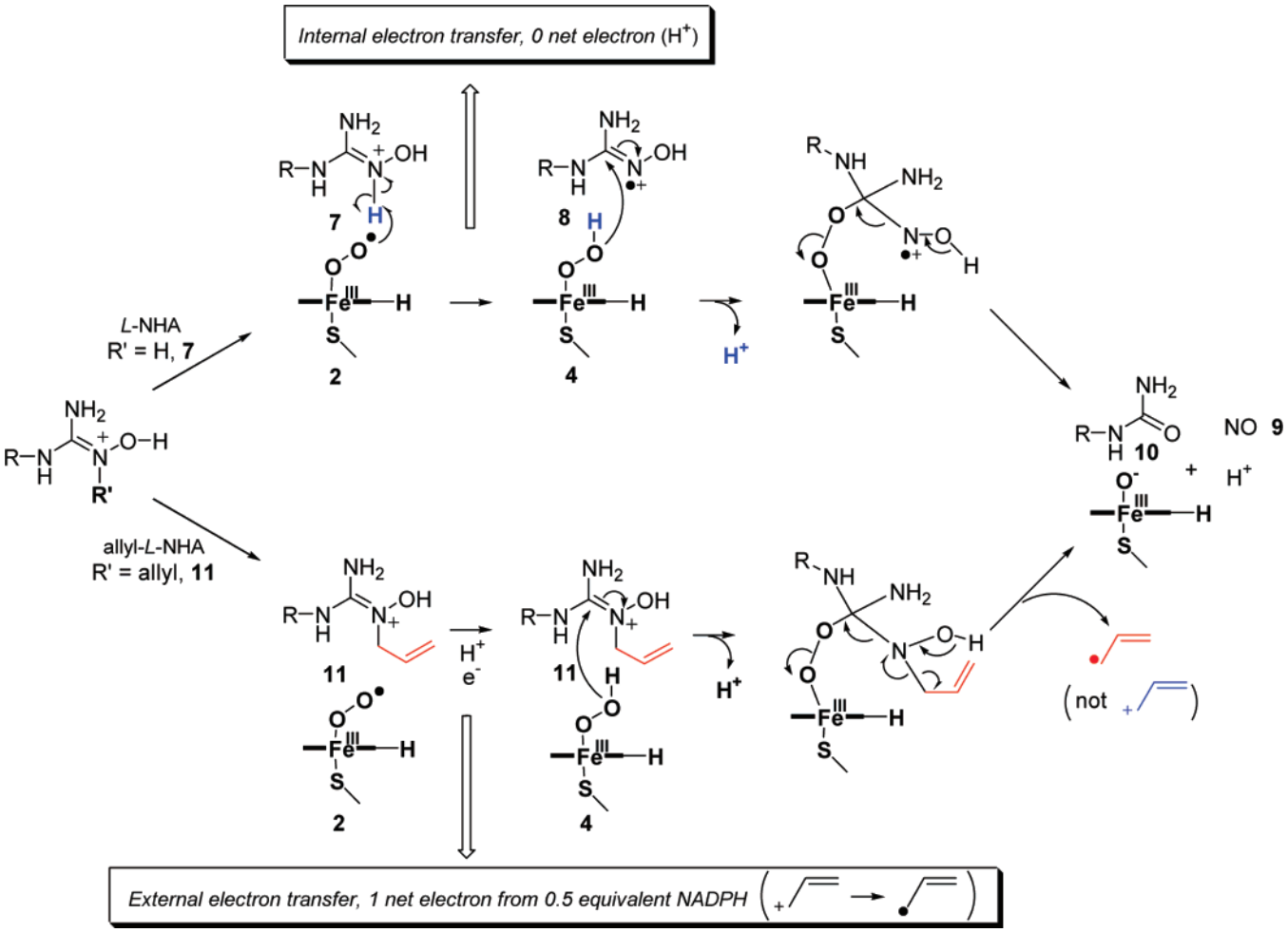
It has been proposed that direct hydrogen atom abstraction from the O−H bond or the N−H bond of L-NHA (**7**) by ferric superoxide **2** leads to an internal electron transfer with formation of reduced ferric hydroperoxide **4** and one electron-oxidized L-NHA (L-$NHA^{\bullet+}$, **8**), which sets the stage for the release of NO after collapse of the tetrahedral intermediate (Scheme 2, **II**) (*22*). Similar to the first half of the mechanism, the formation of ferric hydroperoxide **4** does not require an extra proton donor. Density functional theory calculations indicate that the product of hydrogen atom abstraction from the N−H of L-NHA (**7**) is similar in energy to the product of hydrogen atom abstraction from the O−H (*23*). However, crystallographic studies (*24*) and ENDOR spectroscopic studies (*25*) suggest that N−H cleavage is more favored than O−H cleavage in this internal electron transfer. Furthermore, mechanistic studies showed that direct hydrogen atom abstraction from the O−H bond is not relevant to this internal electron transfer (Figure 3) (*26*). Experimental support for the direct involvement of N−H cleavage in heme-regulated L-NHA degradation would be desirable.

Previous mechanistic studies of nNOS inactivation by N^G-allyl-L-arginine proposed that the inactivation results from irreversible heme modification by attack of an allyl radical (direct detection of the allyl radical has not been achieved) (*27*). Revisiting the previously reported mechanistic data provides support for direct involvement of N−H cleavage. The *N*-hydroxylation product of N^G-allyl-L-arginine, N^G-allyl-N^G-OH-L-arginine (allyl-L-NHA, **11**), is an analogue of L-NHA (**7**) with the N−H bond cleavage blocked by an allyl substituent. As shown in Scheme 6, when the hydrogen atom of the N−H bond is substituted by allyl, the substrate (**11**) cannot provide the hydrogen atom to the oxygen radical of ferric superoxide **2** for the internal one-electron transfer, which results in an external one-electron transfer from 0.5 equiv of NADPH needed for reducing the superoxide (**2**) to hydroperoxide (**4**). If the hydrogen atom of the O−H group were somehow abstracted, then the external electron would not be needed, and an allyl cation, not radical, would be transferred to the heme. This difference between the internal (top reaction when **7** is substrate; 0 net electron) and external electron-transfer mechanisms (bottom reaction when **11** is substrate; 1 net electron) is responsible for one important difference between the metabolic fates of the groups attached to the nitrogen: the hydrogen atom of the N−H in L-NHA (**7**) is ultimately lost as a proton, while the allyl group in *N*-allyl-L-NHA (**11**) is transferred to the heme as an allyl radical (instead of an allyl cation).

Previous mechanistic data show that substituting the hydrogen atom of the O−H bond with *t*-butyl or 3-methylbutenyl groups does not block the catalytic reaction (Figure 3) (*26*). These data support the hypothesis that N−H bond cleavage is more relevant to the catalytic mechanism, although one could argue that the blockage of the O−H bond cleavage forces the ferric superoxide to switch to the N−H bond for the hydrogen atom abstraction. Revisiting the mechanistic results for NOS inactivation by N^G-allyl-L-arginine reveals that the blockage of N−H cleavage prohibits hydrogen atom abstraction by internal electron transfer (ferric superoxide does not switch to O−H bond cleavage). Interpretation of the data from both the O−H and N−H blockage experiments, together with results from the density functional theory calculations (*23*), crystallographic studies (*24*), and ENDOR spectroscopic studies (*25*), clearly supports the mechanistic role for the N−H bond cleavage in the conversion of N^G-OH-L-arginine to L-citrulline and NO.

1.4. The Match-Up between the Oxygen Radical of the Heme-Oxygen Species and the N−H of the Substrates. Finally, another interesting aspect of the NOS mechanism concerns ferric superoxide **2**, which occurs in both steps of the NOS mechanism and appears to have two different mechanistic roles. In the first step, ferric superoxide **2** accepts an external electron to become ferric peroxide **3**, which abstracts a proton from the N−H bond of the substrate guanidino group, but in the second step, **2** abstracts a hydrogen atom from the N−H bond of the N^G-OH-guanidino group. This suggests that the heme-oxygen species prefers to take an electron from the substrate (or intermediate) preferentially to exogenous electron donors, such as tetrahydrobiopterin and NADPH. Therefore, in the first half of the catalytic reaction, when L-arginine is the substrate, two electrons from NADPH are needed for the conversion to product; in the second half-reaction, when N^G-OH-L-arginine is present, only one electron from NADPH is needed because ferric superoxide **2** takes an electron from the intermediate (*6*). An explanation for this difference in mechanism for the two steps is that the OH group on the guanidine impacts the N−H scission pattern. Density functional calculations indicate that the positive charge on the nitrogen of N^G-OH-L-arginine that contains the hydroxyl group is not delocalized into the entire guanidine group, but is localized on that nitrogen (*23*). As shown in Figure 4, when the guanidine is not hydroxylated (L-arginine), the positive charge of the protonated guanidine is delocalized through the guanidine group. In this highly delocalized protonated guanidine, homolytic scission to the *N*-centered radical by superoxide abstraction is disfavored because attachment of carbonyl groups is known to destabilize the amino radical center (*28*).

Scheme 6: Formation of the Allyl Radical in nNOS Inactivation by N^G-Allyl-L-arginine



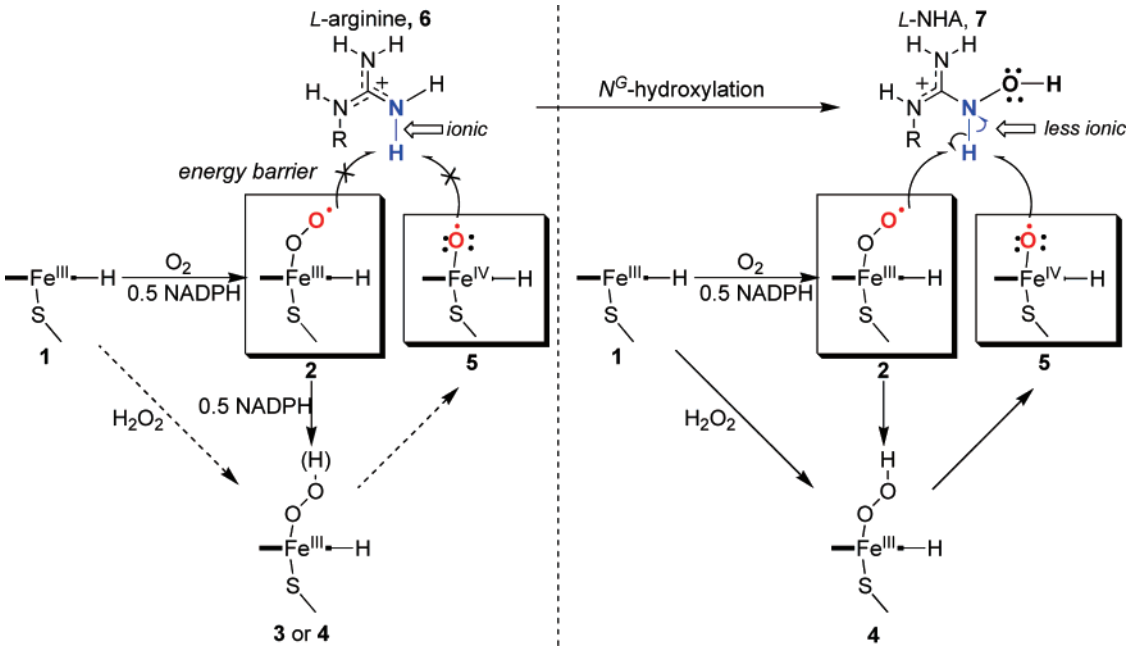
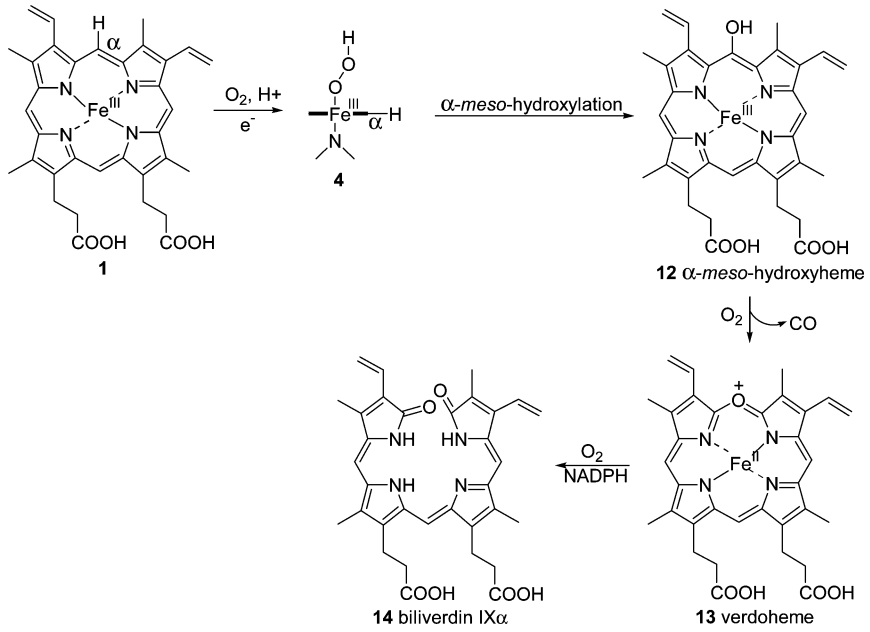


FIGURE 4: Comparison of the hydrogen atom abstraction by the oxygen radical of iron oxo and ferric superoxide. The OH group on the guanidine nitrogen atom impacts the N−H scission pattern.

Therefore, in the first half of the NOS reaction, the superoxide cannot abstract the hydrogen atom from the guanidine N−H bond, and it has to get the next electron from NADPH (just like the ferric superoxide does with N^G-OH-L-allylarginine when the allyl group blocks N−H bond cleavage; Scheme 6, bottom). After the guanidino group is hydroxylated (in L-NHA), the OH group lowers the pK_a of the adjacent nitrogen and provides a radical-stabilizing group

Scheme 7: Ferric Hydroperoxyheme-Facilitated Heme α-*meso*-Hydroxylation



(*28*), which leads to a less ionic N−H bond that can more easily undergo homolytic scission to provide a hydrogen atom to the ferric superoxide (the second step in NOS reaction). Previous computational results (*23*), X-ray studies (*24*), and ENDOR spectroscopy (*25*) suggest the protonated form of L-NHA is the bound form in the NOS active site, and the C−NH(OH) bond is notably longer than the other two C−N bonds with significant single bond character (little or no resonance with the other nitrogens), which supports our hypothesis that the NH bond in L-NHA can more readily undergo homolytic cleavage and is less ionic than the NH bond in L-arginine.

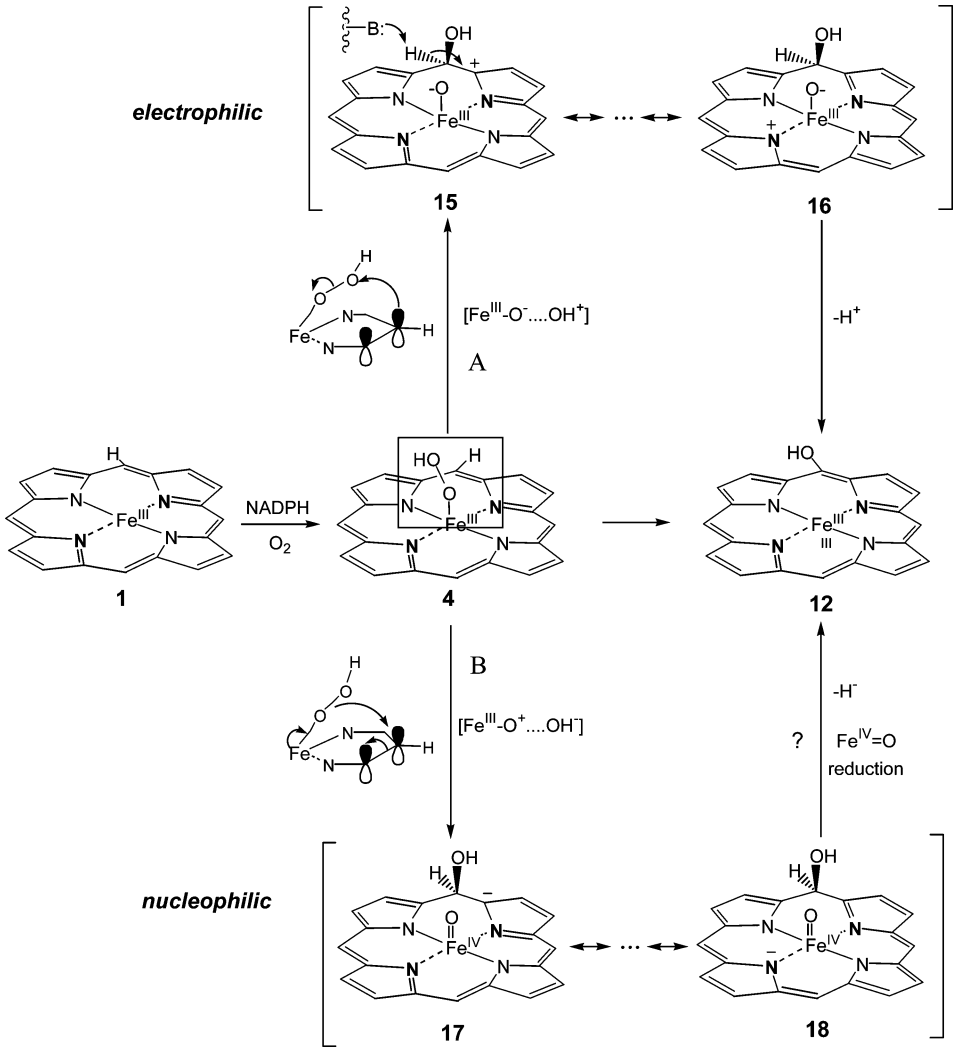
Previous studies suggest that the H_2O_2 shunt experiment with NOS leads to the formation of the iron oxo species (*17*). When L-NHA (**7**) is the substrate, N^G-cyano-L-ornithine (CN-Orn) is formed from the reaction of the iron oxo intermediate and L-NHA (**7**). As discussed earlier, when L-arginine is the substrate, the iron oxo intermediate also forms. The unreactive NOS−H_2O_2−L-arginine system suggests a "mismatch" of the iron oxo intermediate and the guanidine N−H bond, which implicates a high-energy barrier between the protonated guanidine N−H bond and the oxygen radical of the iron oxo intermediate, despite the high reactivity of the iron oxo species. This mismatch further supports the hypothesis that the OH group on the guanidine nitrogen atom impacts the N−H scission pattern (Figure 4). It also disfavors a cytochrome P450-like NOS mechanism for guanidine N^G-hydroxylation, in which hydrogen atom abstraction of the protonated guanidine N−H bond by the oxygen radical of the iron oxo species followed by OH radical rebound leads to *N*-hydroxylation (Scheme 4). As shown in Scheme 5, the proposed new N^G-hydroxylation mechanism does not involve the iron oxo species, which is consistent with the hypothesized "mismatch" between the iron oxo intermediate and the guanidine N−H bond. An interesting, but partial, test of this hypothesis and the new mechanisms (Scheme 5) would be to use EPR spectrometry in an attempt to detect the formation of the iron oxo intermediate in the NOS−L-arginine system when iodosobenzene or H_2O_2 replaces NADPH and O_2. It is known that NOS is inactive with L-arginine when iodosobenzene and H_2O_2 are the oxidants, although heme is likely to form the iron oxo species with iodosobenzene and H_2O_2 (*16*). If EPR spectrometry detects the formation of the iron oxo species, then it confirms the proposed mismatch between the guanidine N−H bond and the iron oxo species, which will disfavor the old mechanism (Scheme 4) and favor the new mechanism (Scheme 5). As the failure to see the iron oxo species could have many causes, probably a more robust strategy would involve extensions of the cryoreduction EPR/ENDOR approach (*29*).

2. PERSPECTIVES ON THE HEME *MESO*-HYDROXYLATION REACTION IN HEME OXYGENASE AND DURING NITRIC OXIDE SYNTHASE INACTIVATION BY AMIDINES

Heme oxygenase exists as two isoforms, HO-1 and HO-2 (*30*). In both isoforms, the fifth ligand to the heme is a histidine, and the distal ligand is a water molecule (*31*). Heme oxygenase is very unusual in that it uses heme as both its substrate and prosthetic group. The heme oxygenase-catalyzed reaction consumes O_2 and reducing equivalents provided by NADPH-cytochrome P450 reductase. The first step is a regiospecific hydroxylation of the porphyrin α-*meso* carbon (Scheme 7), which leads to the well-established heme degradation intermediate, α-*meso*-hydroxyheme (**12**) (*32*). The α-regiospecificity of this *meso*-hydroxylation step accounts for the regiospecificity of the product (biliverdin IXα, **14**) (*33*).

2.1. The Controversy Regarding the Polarity of the Heme Hydroxylation Species. As shown in Scheme 7, this *meso*-hydroxylation reaction is carried out by ferric hydroperoxyheme (**4**), which undergoes reaction with the aromatic porphyrin *meso* carbon to substitute the hydrogen atom with a hydroxyl group. Porphyrin is a macrocyclic aromatic molecule known to be versatile in accommodating different attacking species at its *meso*-positions (*34*). In the porphyrin center cavity, the iron-bound peroxide is also capable of catalyzing very different reactions (*35*). Therefore, the

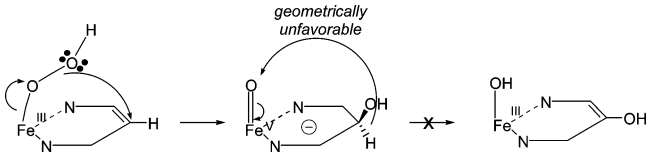
Scheme 8: Possible Mechanisms for Heme α-*meso*-Hydroxylation[a]



[a] A: Electrophilic aromatic substitution. B: Nucleophilic aromatic Substitution. The radical mechanism is not shown.

electronic interaction between the iron-bound peroxide and the porphyrin *meso*-position, resulting in porphyrin *meso*-hydroxylation, is unclear. The direction of electron flow in this hydroxylation step could occur in three ways: (1) the π-electrons of the porphyrin α-*meso*-carbon could attack the electrophilic O−O bond of the ferric peroxide, leading to a positively charged porphyrin intermediate with a [Fe^{III}−O^-] species in its central cavity (Scheme 8A); (2) the lone pair electrons of the terminal oxygen of the ferric peroxide could attack the p-orbital of the porphyrin α-*meso*-carbon, leading to a negatively charged porphyrin intermediate with a [Fe^{IV}═O]$^{+\bullet}$ species in its central cavity (Scheme 8B); (3) homolysis of the O−O bond, leading to a radical reaction (not shown). Therefore, for the ferric hydroperoxyheme-facilitated heme α-*meso*-hydroxylation, regardless of whether it is a stepwise or concerted mechanism, the ferric hydroperoxide could undergo either a heterolytic or a homolytic cleavage to generate three different species (OH^+, OH^-, $OH^\bullet$), which could attack the porphyrin *meso* carbon. The different mechanisms for this hydroxylation step depend on the polarities of the attacking species: (1) electrophilic aromatic substitution (electrophilic mechanism) features [Fe^{III}−O^-···OH^+]; (2) nucleophilic aromatic substitution (nucleophilic mechanism) features [Fe^{III}−O^+···OH^-]; (3) radical aromatic substitution (radical mechanism) features [Fe^{III}-$O^\bullet$···$^\bullet$OH].

Scheme 9: The Steric Issue for Internal Reduction of the Nucleophilic Mechanism



2.2. The Electrophilic Mechanism. In the electrophilic mechanism, the π-electrons of the porphyrin α-*meso*-carbon attack the O−O bond of the ferric hydroperoxide (Scheme 8A). In the porphyrin central cavity, Fe^{III}−O−OH is reduced to Fe^{III}−O^-, which can be easily protonated to recover the fairly stable Fe^{III}−OH coordination state. A positively charged porphyrin intermediate (**15**) is also formed. The positive charge of this key intermediate can be distributed over the porphyrin macrocyclic conjugated system; however, in **16** the positive charge is on a six-electron nitrogen atom. This makes **16** a highly unstable resonance structure. This is similar to electrophilic aromatic substitution with pyridine, which is highly disfavored (*36*). Therefore, the electrophilic mechanism seems to be disfavored in that it leads to a positively charged high-energy porphyrin intermediate, which minimizes resonance stabilization. A basic amino acid residue nearby can remove the proton from the α-*meso*-carbon in

15 to recover the porphyrin aromaticity.

The electrophilic mechanism was implicated by the finding that ethyl hydroperoxide (EtOOH) can mimic hydrogen peroxide (H_2O_2) in HO-1 heme reactions (*37*). Two previous mechanistic studies using porphyrin analogues with electronically diverse *meso*-carbons that favor the electrophilic mechanism were based on either a controversial enzyme model study (in which it is hard to attribute the observed regioselectivity to steric effects within the enzyme active site or to the electronic difference of the porphyrin *meso*-positions) (*38*) or close theoretical calculations of the electronic properties of the porphyrin *meso*-carbons (*39*), which led to an inconclusive heme *meso*-hydroxylation mechanism.

We recently reported a nonenzymatic model study with the porphyrin macrocyclic system (*40*). Iron-bound 2,4-diacetyldeuteroporphyrin, whose four *meso*-carbons are dramatically different in their electronic effects (the β- and γ-*meso* positions are much more nucleophilic than the α- and δ-*meso* positions), and coupled oxidation conditions that avoid the complications of steric effects of the heme oxygenase active site were employed to elucidate the mechanism. Mass spectral and deuterium-labeling experiments indicated that the porphyrin *meso*-positions that are at higher π-electron densities in ferric 2,4-diacetyldeuteroporphyrin are selectively attacked by the ferric hydroperoxide, which supports an electrophilic aromatic substitution mechanism for heme oxygenase-catalyzed porphyrin *meso*-hydroxylation (*41*).

2.3. The Nucleophilic Mechanism. In the nucleophilic mechanism, the lone pair electrons of the terminal oxygen atom of the ferric hydroperoxide attacks the p-orbital of the porphyrin α-*meso*-carbon (Scheme 8B), which leads to a negatively charged porphyrin intermediate (**17**) with a $[Fe^{IV}{=}O]^{+\bullet}$ species in its central cavity. The negative charge can be distributed over the porphyrin macrocyclic conjugation system and is further stabilized on the electronegative nitrogen atom in **18**, which has an octet of electrons. The negatively charged porphyrin system in **17** is, therefore, much more stable than the positively charged counterpart in **15**.

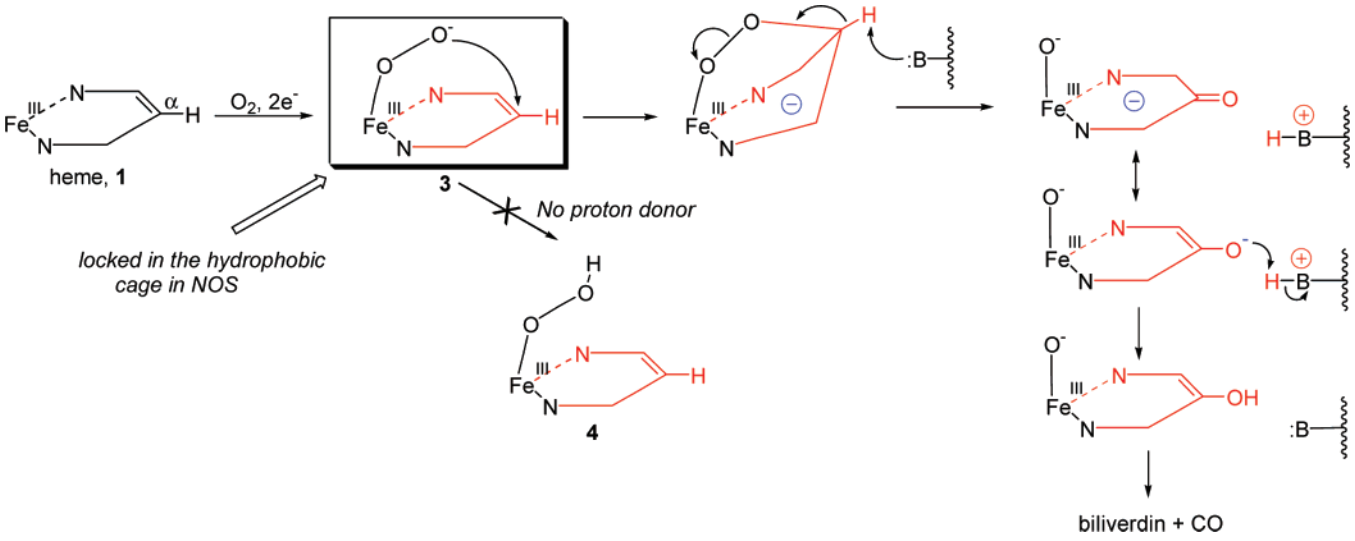
The controversy in the nucleophilic mechanism comes from the fate of key intermediate **17**, which has a $[Fe^{IV}{=}O]^{+\bullet}$ species in the porphyrin central cavity and a negatively charged porphyrin system. The $[Fe^{IV}{=}O]^{+\bullet}$ species (iron oxo intermediate or Compound I) is a strong oxidant, which is responsible for many monooxygenation reactions (*5*). When a monooxygenation substrate is not available in the heme oxygenase system, two extra electrons are needed to reduce this highly reactive $[Fe^{IV}{=}O]^{+\bullet}$ species to a more stable $[Fe^{III}{-}O^{-}]$ species, which is not consistent with the nature of the heme oxygenase reactions. The negatively charged porphyrin system needs to eliminate a hydride ion (H^-) to recover its aromaticity, which is not very common in enzymatic reactions (although NAD(P)H is known to lose H^- to give aromatic $NAD(P)^+$). It is tempting to propose that the hydride at the α-*meso*-carbon reduces the $[Fe^{IV}{=}O]^{+\bullet}$ in the central cavity, but the geometric improbability of this reduction is obvious (Scheme 9).

Above we discussed the versatility of the heme peroxyl species ($[Fe^{III}{-}O{-}O{-}H]$ or $[Fe^{III}{-}O{-}O^{-}]$) in NOS mechanisms: it can (1) get protonated and dehydrate to the iron oxo species (*5*), (2) attack the electron-deficient carbonyl carbon as a nucleophile (in the NOS-catalyzed decompositions of N^G-OH-guanidine) (*6*), or (3) add onto the porphyrin α-*meso*-carbon, which leads to heme α-*meso*-hydroxylation (in NOS inactivation by amidines) (*12*). This suggests that the terminal hydroxyl group of the heme hydroperoxide ($[Fe^{III}{-}O{-}O{-}H]$) is nucleophilic (because it is attracted by protons and carbonyl carbons). The porphyrin α-*meso*-carbon resembles the electronic properties of the other two competitors (H^+, carbonyl carbon) and is electrophilic. Therefore, the heme α-*meso*-hydroxylation that occurs during iNOS inactivation by amidines should favor a nucleophilic mechanism. Furthermore, as we observed from NOS inactivation by N^G-allyl-L-arginine, a reduced heme−allyl adduct (λ_{max} 280 nm) forms that was proposed to result from allyl radical attack on heme (λ_{max} 400 nm) followed by a one-electron reduction (*27*). This heme porphyrin reduction supports the electrophilic properties of the porphyrin aromatic conjugation system, which also favors nucleophilic attack on the porphyrin. We hesitate to use these implications to argue against an electrophilic mechanism in heme oxygenase-catalyzed heme *meso*-hydroxylation or, conversely, in borrowing an electrophilic mechanism from heme oxygenase for heme *meso*-hydroxylation during NOS inactivation by amidines because, although heme degradation during NOS inactivation by amidines shares the same general pathway as the heme oxygenase reactions (*12*, *32*), the mechanisms may differ in the two enzyme systems. Enzymologists tend to employ the mechanism of a well-known enzyme with a less studied enzyme that catalyzes a related reaction as a starting point to study the new mechanism. The relationship between NOS (the lesser known mechanism) and heme oxygenase (with respect to the heme α-*meso*-hydroxylation mechanism) might resemble the relationship between NOS (the lesser known one) and cytochrome P450s (with respect to the *N*-hydroxylation mechanism of the substrates). However, NOS may not invoke mechanisms from either of these well-known enzymes.

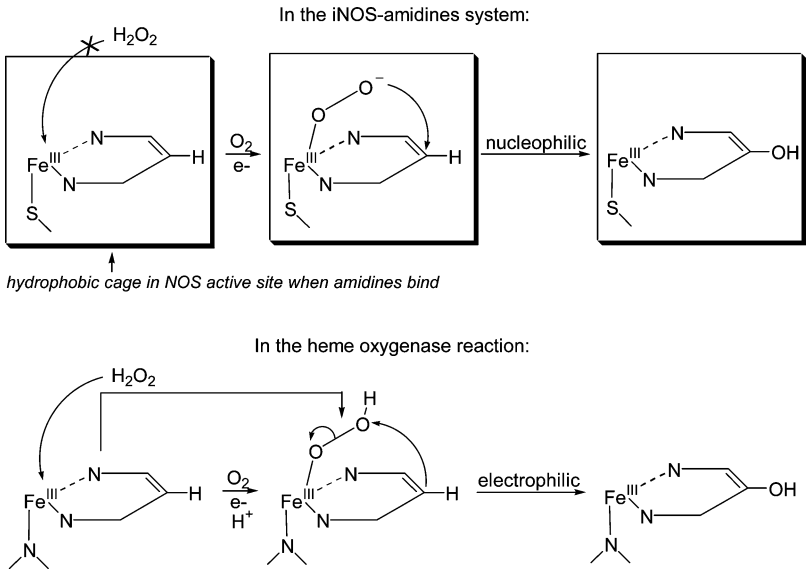
As we discussed above, the highly hydrophobic active site of NOS when amidines bind leaves the ferric peroxide unprotonated. Although the electrophilic mechanism appears to be convincingly established for α-*meso*-hydroxylation in heme oxygenase, it seems to be inappropriate for the similar reaction that occurs during NOS inactivation by amidines, because the terminal oxygen anion of ferric peroxide ($[Fe^{III}{-}O{-}O^{-}]$) is an electron-rich nucleophile, and it cannot be attacked by another nucleophile, such as the porphyrin α-*meso* p-electrons.

On the basis of the above considerations, an alternative nucleophilic mechanism is proposed for heme *meso*-hydroxylation during NOS inactivation by amidines. Hydroxylation is initiated by nucleophilic attack of the ferric peroxyl anion on the porphyrin α-*meso*-carbon (Scheme 10; nucleophilic aromatic addition). The negative charge can be stabilized by the porphyrin aromatic system (Scheme 8, bottom), and the α-*meso*-hydrogen of the bicyclo intermediate can be removed by a nearby basic amino acid residue (the crystal structure of iNOS shows that a tryptophan residue is near the α-*meso*-carbon (*11*)) followed by cleavage of the O−O bond. The negatively charged keto intermediate can then undergo "keto−enol" tautomerization followed by protonation to produce α-*meso*-hydroxyheme. In this mechanism the pair of electrons of the α-*meso*-C−H bond cleaves

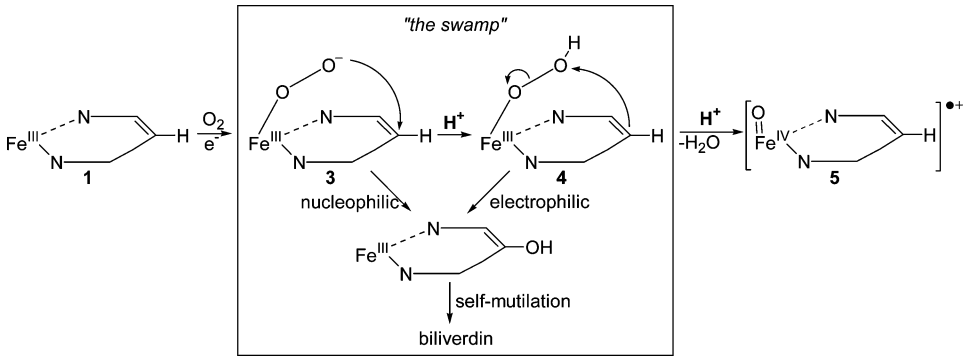
Scheme 10: Proposed Mechanism for the Formation of α-*meso*-Hydroxyheme during Amidine Inactivation of NOS



Scheme 11: Comparison of the Heme α-*meso*-Hydroxylation in the NOS−Amidine System and the Heme Oxygenase Reaction



Scheme 12: Formation of the Ferryl Species in Heme-Dependent Enzymes Has To Survive the "Self-Mutilation Swamp"



the O−O bond of the peroxide. This proposed mechanism is just a modification of well-known nucleophilic mechanisms of peroxides, such as ferric peroxide-facilitated deformylation by P450s (*42*) and peroxy acids or hydroperoxide-facilitated Baeyer−Villiger oxidations of ketones (*43*).

2.4. Coexistence of the Electrophilic and the Nucleophilic Mechanisms. For the same heme *meso*-hydroxylation step, an electrophilic mechanism is supported for the heme oxygenase reaction while a nucleophilic mechanism is favored for amidine inactivation of NOS. The active hydroxylating intermediates in the two systems are actually the same ferric peroxides, but at different protonation states (with different distal residues), as determined by the hydrophilic level in the heme environments.

A study that supports this mechanistic difference is the observation that H_2O_2 drives heme degradation in heme

oxygenase (Scheme 11, bottom) (*37*), but not in the inactivation of iNOS by amidines (Scheme 11, top) (*44*). This difference is consistent with the proposed difference in the hydrophilic level of the heme binding sites: the highly hydrophobic active site of the NOS−amidine complex prevents the access of H_2O_2. The different fifth ligands bound to heme might also contribute to the different mechanisms. In NOS, the fifth ligand of the heme iron is a cysteine residue, while in heme oxygenase the fifth ligand is a histidine residue. The electronic properties of the ferric peroxides are proposed to be affected by the fifth ligands (*45*). The electronic properties of the porphyrin conjugation system are also likely to be influenced by the fifth ligand. Therefore, the fifth ligand is expected to play a significant role in dictating the different mechanisms for the two enzyme systems, besides the impact from the hydrophilicity levels of the enzyme active sites. Theoretical calculations and nonenzymatic model studies of heme coordinated with different ligands hopefully can lead to a better understanding of these mechanisms.

3. PERSPECTIVES ON THE FORMATION OF COMPOUND I IN CYTOCHROME P450S

The most common reaction catalyzed by the cytochrome P450 superfamily is the monooxygenation of xenobiotics and drugs (*3*, *5*). The key intermediate is the highly reactive iron oxo species (Compound I, **5**), which is formed in several steps from ferric heme with O_2, electrons (normally from NADPH), and protons. Although the importance of O_2 and electrons is well appreciated, the necessity of a proton source for the formation of Compound I in CYP450 reactions is very often ignored.

As already discussed, the two precursors of Compound I (**5**), ferric peroxy anion **3** and ferric hydroperoxide **4**, can both lead to *meso*-hydroxylation (the coexistence of electrophilic and nucleophilic mechanisms), which functions as a self-destructive reaction against the classical monooxygenation catalytic mechanism. Scheme 12 shows that the rapid reaction of ferric peroxides to give α-*meso*-hydroxyheme highlights a fundamental property of hemoenzyme mechanisms that involve a ferryl species ($[Fe^{IV}{=}O]^{+\bullet}$, Compound I, **5**): The precursors of the ferryl species (**5**), the ferric peroxy anion (**3**) and the ferric hydroperoxide (**4**), must be provided with a proton source for decay to the ferryl species to occur fast enough to avoid self-mutilation of the heme prosthetic group, i.e., the formation of the ferryl species has to survive the "self-mutilation swamp" (Scheme 12). As we have found, in addition to the proton source, this survival in "the swamp" is dependent on the hydrophobic/hydrophilic nature and appropriate geometry of the active site.

CONCLUSIONS

The perspectives presented here are focused on the heme regulation mechanisms in NOS, HO, and CYP450s. The classical oxidative catalytic cycle of heme shown in Scheme 1 is the "ultimate" heme mechanism. Depending on the nature of the heme binding environments (different enzyme pocket structure, different fifth ligands, hydrophobicity/hydrophilicity, and different substrates), the heme reactions could deviate at almost every point in the "ultimate" cycle (Scheme 1), leading to different catalytic mechanisms and different inactivation mechanisms.

We also have tried to emphasize the value of known tenets regarding studies of enzyme mechanisms: (a) elucidation of an inactivation mechanism of an enzyme can often provide important insights regarding its catalytic mechanism; (b) crystallographic studies are important tools for elucidation of enzyme mechanisms; (c) seemingly different enzymes may utilize very similar reactions, although seemingly similar enzyme-catalyzed reactions may have different mechanisms.

As always, enzyme mechanisms are rarely, if ever, proven. Reasonable mechanisms are proposed to explain new experimental data and can often lead to new experiments to test hypotheses. It is hoped that the insights and proposed new mechanisms presented here will promote the design of new experiments to explore heme regulation mechanisms in a wide range of enzymes.

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