Mechanism of Free-Radical Generation by Nitric Oxide Synthase

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Contents

I. Introduction

In 1986, it was reported that nitric oxide (NO•) mimicked the physiologic activity[1] of an uncharacterized endothelium-secreted factor that had previously been shown to relax blood vessels.[2] Verification soon followed.[3] We now know that NO• is a novel transient cell messenger that augments intercellular communications and governs many intracellular events.[4] Additionally, NO• plays an essential role in host immune response, particularly effective against a number of intracellular pathogens.[5]

Nitric oxide synthases (NOSs; EC 1.14.13.39) are a class of hemeproteins that specifically catalyze the oxidation of L-arginine (**1**) to N^G-hydroxy-L-arginine (**2**) and eventually to L-citrulline (**3**) and NO• (Scheme 1).[6] Considering the breath of physiologic actions purported for NO•, it is not surprising that there are multiple isoforms of this enzyme. These include a constitutive neuronal NOS (nNOS or NOS I),[6a,7] an endotoxin- and cytokine-inducible NOS (iNOS or NOS II),[8] and a constitutive endothelial NOS (eNOS or NOS III).[9] These isozymes are encoded by three distinct genes.[10]

Nitric oxide synthases are members of a superfamily of heme-containing monooxygenases that include the well-characterized enzyme, cytochrome P-450.[11] Despite the common ancestry, NOSs have unique properties that support as well as regulate the formation of NO• from L-arginine (**1**). These enzymes are composed of a flavin-containing C-terminal domain with binding sites for FAD, FMN, and NADPH and a catalytic N-terminal oxygenase domain with binding sites for L-arginine (**1**) and 6-R-tetrahydrobiopterin (H_4B). The domains are connected by a highly conserved Ca^{2+}/calmodulin binding region.[12] To be activated, all isozymes of NOS must become dimeric, as electrons pass from the reductase domain of one monomer to the oxidase domain of the other.[13] As noted above, there are significant differences among the various isoforms of NOS; however, the mechanism by which these enzymes metabolize L-arginine (**1**) to L-citrulline (**3**) and NO• is probably identical and independent of the isozyme.

II. Enzymology of Nitric Oxide Synthase Derived Nitric Oxide

1. Formation of N^G-Hydroxy-L-arginine from L-Arginine

To generate NO• from L-arginine (**1**), NOS must cycle twice, generating N^G-hydroxy-L-arginine (**2**), as an intermediate.[14] It is proposed that during the initial oxidation sequence electrons are shifted from NADPH, as a hydride, to FAD, forming $FADH_2$. Disproportionation with FMN would lead to FADH•/FMNH• (Figure 1).[11,15] As is the case for cytochrome P-450,[16] electron flow from FMNH• to Fe^{3+} gives the reduced heme, Fe^{2+}, and FADH•/FMN ↔ FAD/FMNH• (Figure 1).[17] Binding of O_2 in the sixth ligand position affords the ferric heme−oxygen intermediate $Fe^{3+}-O_2^{\bullet}$, which is further reduced by FAD/FMNH• to $Fe^{3+}-O_2H$.[18] Recent data point to a role for H_4B in advancing the formation of $Fe^{3+}-O_2H$, either through donating the second electron or assisting in electron flow from NADPH through the flavins.[19] Thereafter, an electron-rich thiolate, presumably a cysteine residue at the fifth ligand position,[19d,20] promotes formation of a high oxidation state iron−oxygen species, proposed to be $[Fe^{5+}{=}O]^{3+}$, concomitant with the release of H_2O. [21]

It has been suggested that the binding of the guanidino nitrogen in an ordered position near the perferryl complex[19d,20,22] expedite hydrogen atom abstraction. Within this cage, rapid transfer of "HO•" to the 2-position would facilitate formation of N^G-hydroxy-L-arginine (**2**). This intermediate has been observed under specific experimental conditions.

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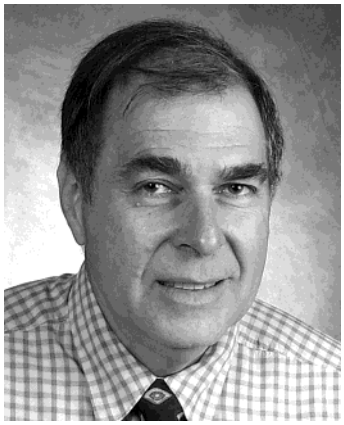
Pei Tsai was educated at the University of Oregon, where she received her Ph.D. in organic chemistry. She is a postdoctoral fellow with Gerald Rosen, developing spin traps for the in vivo in situ detection of free radicals, including nitric oxide, in animal models.

However, N^G-hydroxy-L-arginine (**2**) is not released by NOS.[14,23] Further oxidation to L-citrulline (**3**) and NO• is kinetically favored (Scheme 2).[14,24] Equally as fascinating is the specificity of NOS toward the L-isomer of arginine.[14,25]

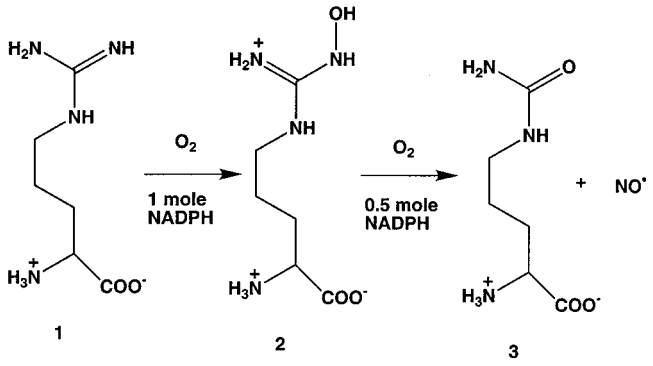
2. Generation of L-Citrulline and NO• from N^G-Hydroxy-L-arginine

While there is a general consensus on the mechanism by which NOS converts L-arginine (**1**) to N^G-hydroxy-L-arginine (**2**), there is much debate as to the pathway by which this enzyme metabolizes N^G-hydroxy-L-arginine (**2**) to L-citrulline (**3**) and NO•. The source of the controversy centers on the requirement of only 0.5 mol of NADPH − one-electron.[14] Since the actual oxidation requires two electrons, what may be the source of the second electron? One theory envisions a hydrolytic reaction, giving L-citrulline (**3**) and *N*-hydroxylamine. Oxidation of *N*-hydroxylamine by catalase, for instance, produces NO•.[26] Two independent observations cast doubts on this mechanism. First, crude neuroblastoma cell cytosolic fractions containing NOS generates NO• from L-arginine (**1**), whereas this same preparation does not oxidize



Gerald Rosen, after receiving his Ph.D. in organic chemistry from Clarkson University and spending three years of postdoctoral training in pharmacology, joined the Department of Pharmacology, Duke University, where he spent most of his academic career. In 1988, he became Chairman of the Department of Pharmacology at the University of Maryland School of Pharmacy, until 1993, when he returned to full-time research, conducting experiments on the in vivo identification of biologically generated free radicals such as nitric oxide using spin trapping/EPR spectroscopy. He is currently Isaac E. Emerson Professor of pharmacology.

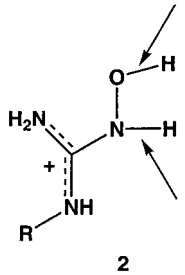
Scheme 1



N-hydroxylamine to NO• in the absence of catalase.[27] Second, the oxygen atom introduced into L-citrulline (**3**) and NO• has its origin in O_2 and not from H_2O.[28]

Another theory suggests that an oxazirine, produced during the metabolism of N^G-hydroxy-L-arginine (**2**), rearranged, yielding L-citrulline (**3**) and HNO. Loss of an electron afforded NO•.[29] Again, there is little corroborative evidence to support such a proposition.

An additional hypothesis envisions that N^G-hydroxy-L-arginine (**2**) is the source of the second electron. These models propose that this oxidation occurred at either N−OH or N^G N−H of (**2**). A review of these theories follows.



2

R = $(CH_2)_3CH(NH_3^+)COO^-$

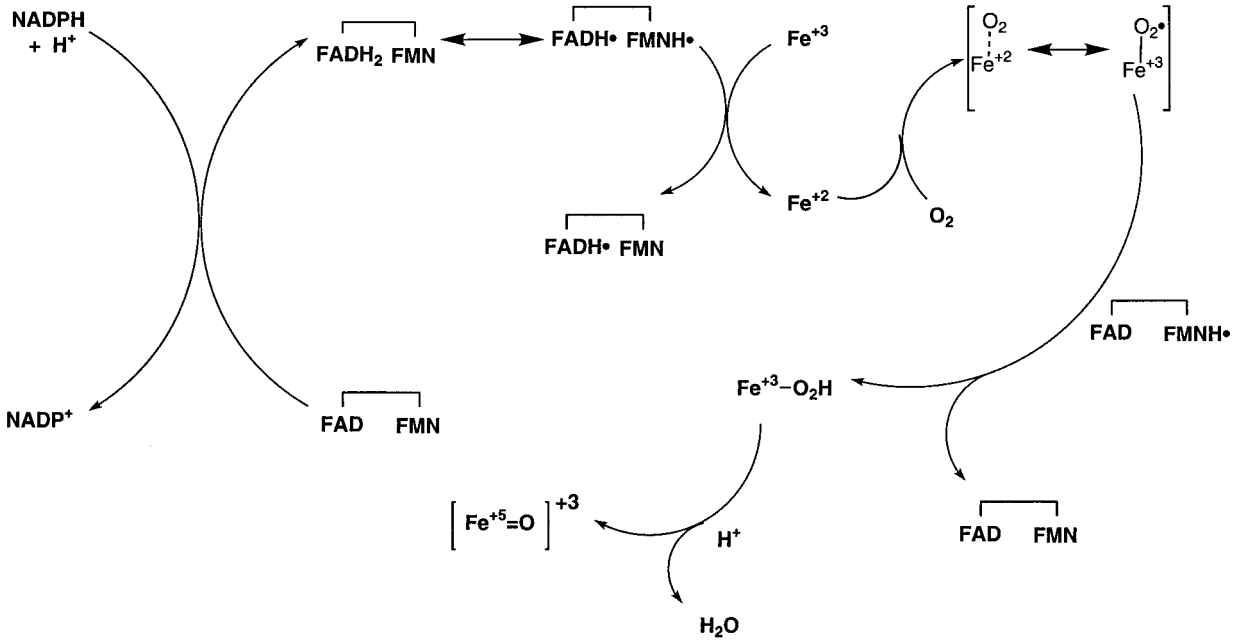
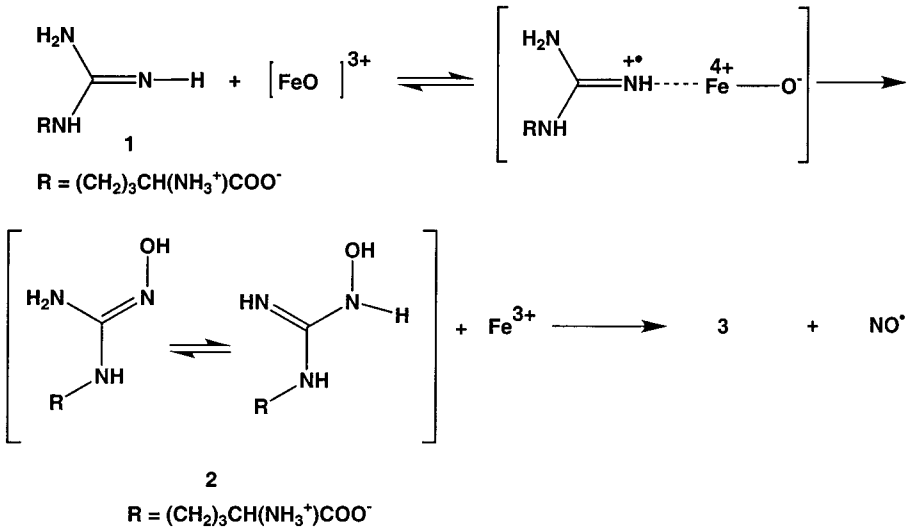


Figure 1. Proposed model for electron transport through NOS, culminating in the formation of the perferryl complex, the proposed reactive species responsible for the catalytic oxidation of L-arginine (**1**) to L-citrulline (**3**) and NO•. Electrons flow from NADPH to the flavins in the reductase domain to the heme-iron in the oxidase domain. These domains are connected by a highly conserved Ca^{2+}/calmodulin binding region. See text for details.

Scheme 2



All but one mechanism proposes a one-electron oxidation of the *N*-hydroxylamine (**2**) to the corresponding nitroxide. One of the earliest reports, however, suggests that $Fe^{3+}O_2^{\bullet}$ abstracted a hydrogen atom from the HO of N^G-hydroxy-L-arginine (**2**).[12a] Attack by $Fe^{3+}-O_2^-$ with release of a proton followed by rearrangement leads to L-citrulline (**3**) and NO• (Scheme 3).

A more recent theory, whose central theme is similar to that described,[12a] proposes a hydrogen atom donation followed by ferri-peroxyl addition to the α-carbon (Scheme 4). After rearrangement, L-citrulline (**3**) and NO• are generated.[30]

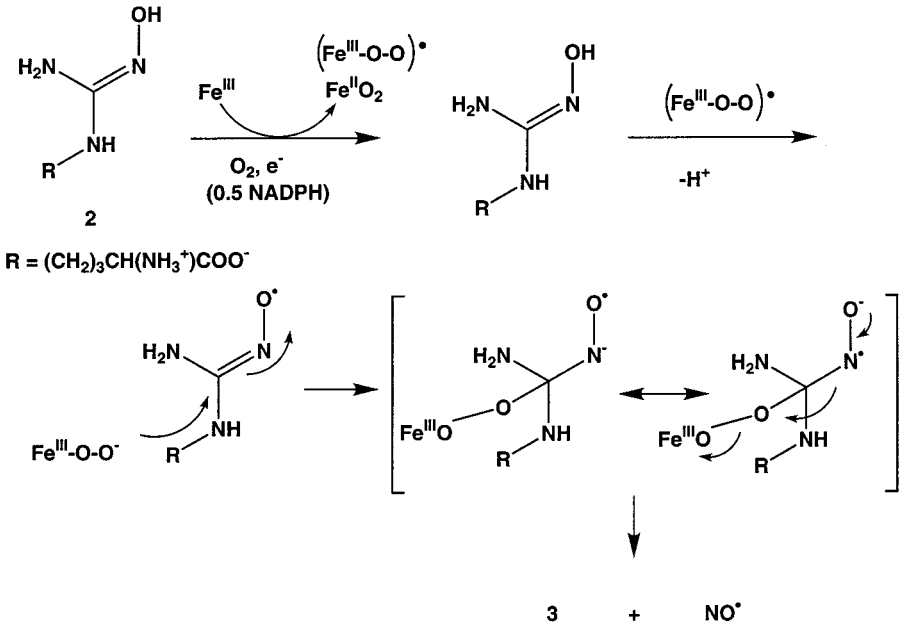
Another mechanism[31] hints that upon hydrogen atom abstraction from N^G-hydroxy-L-arginine (**2**) $[Fe^{5+}=O]^{3+}$ and H_2O are generated. The perferryl complex subsequently removes a hydrogen atom from the secondary amino group. After addition of HO• to the α-carbon followed by rearrangement, L-citrulline (**3**) and NO• are thereafter produced (Scheme 5).

A further theory[32] proposes that heme-Fe^{3+} is reduced by N^G-hydroxy-L-arginine (**2**). To the ensuing heme-Fe^{2+}, O_2 adds, which is followed by a rapid addition to the resultant α-carbon radical. After rearrangement, L-citrulline (**3**) and NO• are afforded (Scheme 6).

In two similar pathways, either a poorly defined oxidant[33a] or $Fe^{3+}-O_2^{\bullet}$[33b] abstracts an electron from the *N*-hydroxylamine. Addition of the resultant peroxide to the carbon−nitrogen double bond results in an intermediate that decomposes to L-citrulline (**3**) and NO• (Schemes 7 and 8).

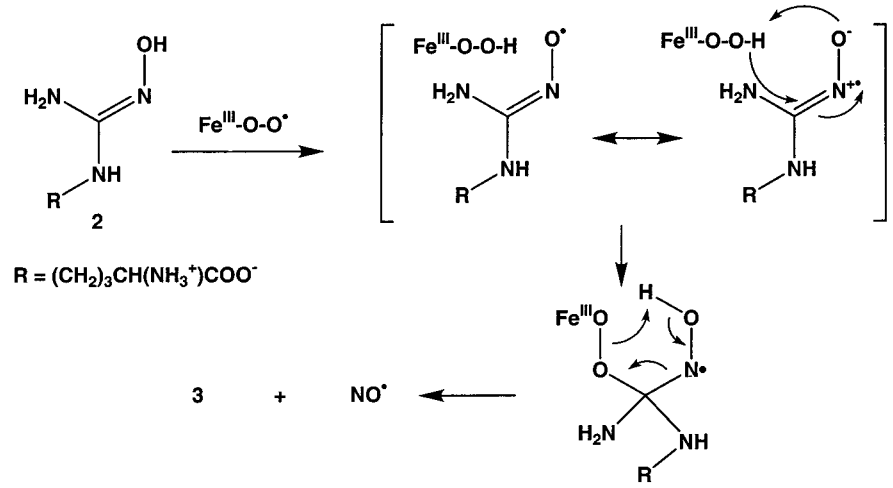
One of the most novel ideas proffers that the site of hydrogen atom abstraction is at the N^G N−H,

Scheme 3[a]



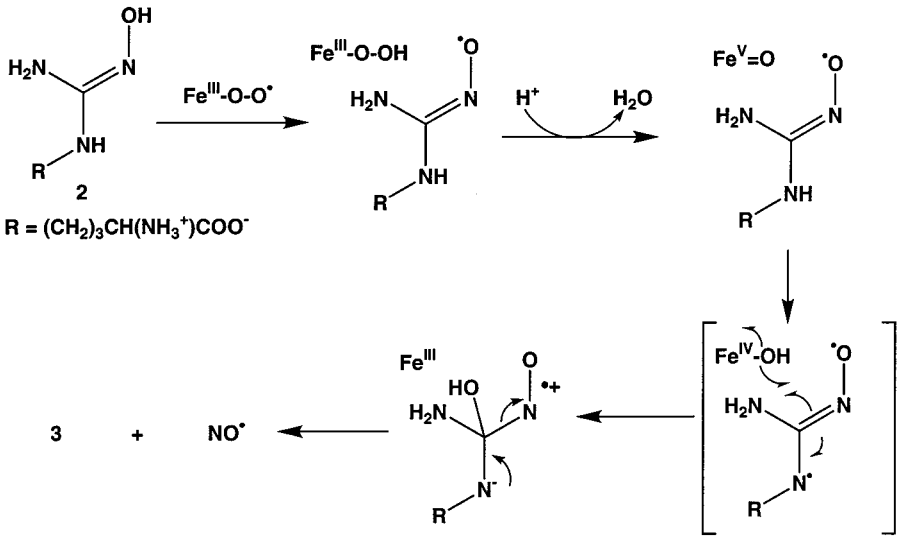
[a] Adapted from refs 12a and 34.

Scheme 4[a]



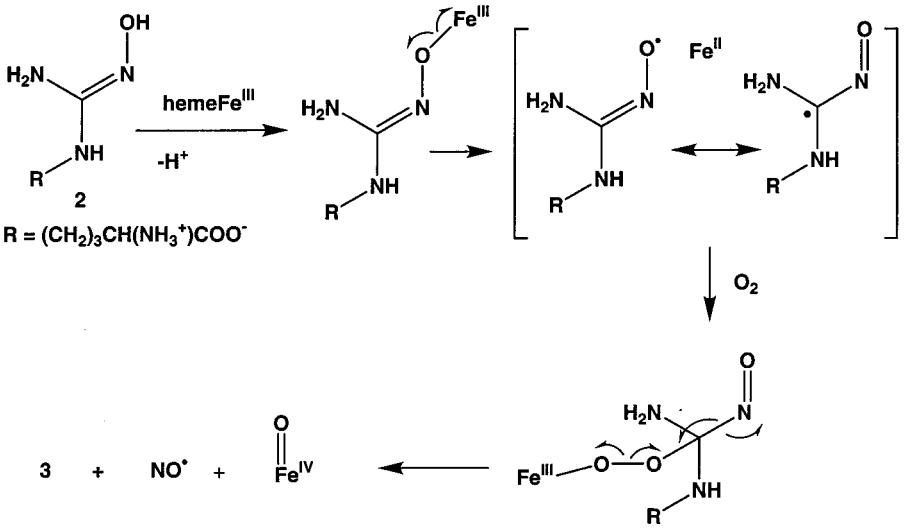
[a] Adapted from refs 30a and 34.

Scheme 5[a]



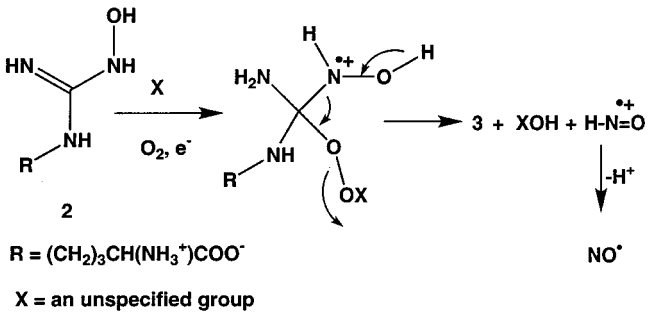
[a] Adapted from refs 31 and 34.

Scheme 6[a]



[a] Adapted from refs 32 and 34.

Scheme 7[a]



[a] Adapted frpm refs 33a and 34.

instead of N−OH on N^G-hydroxy-L-arginine (**2**).[34] Evidence in support of this theory comes from two separate series of experiments. First, ENDOR spectroscopic analyses suggest that the N−H of N^G-hydroxy-L-arginine (**2**) when bound to NOS is oriented toward the heme.[35] Second, in the case of NOS II, crystallographic findings demonstrate that N−OH is oriented away from the heme, whereas N^G N−H is correctly positioned for hydrogen atom abstraction.[36] Density functional theory calculations of N^G-hydroxy-L-arginine (**2**) in which the protonated form of N^G-hydroxy-L-arginine (**2**) is bound to the enzyme further supports this hypothesis.[37] With this model, the N-radical is a reasonable alternative to the O-radical (Scheme 9).

Recently, this theory was tested by synthesizing a readily removable *O-tert*-butyl- and *O*-(3-methyl-2-butenyl)- attached to L-arginine (**4** and **5** of Scheme 10).[34] Upon exposure to NOS, NO• was generated. Research described in ref 34 and Scheme 10 support the ability of NOS to abstract a hydrogen atom from the N^G N−H of (**2**) or electron transfer from that nitrogen atom. These studies, however, cannot differentiate between these pathways. Further, these experiments[34] do not discount the possibility that NOS may oxidize the N−OH of N^G-hydroxy-L-arginine (**2**) to the corresponding nitroxide. Future experiments will undoubtedly address these issues.

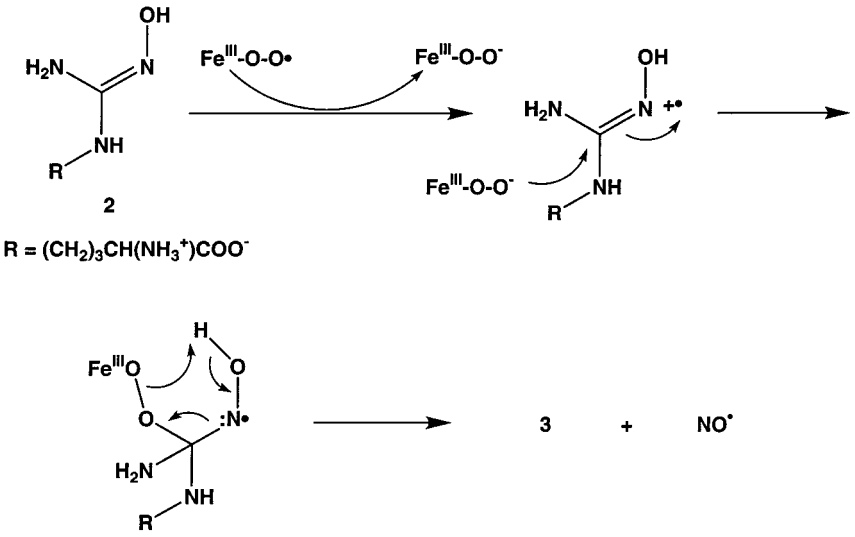
III. Superoxide Generation by Nitric Oxide Synthase

The ease by which NOS can reduce ferricytochrome *c*[38] and the inability of SOD to inhibit this reaction led to the initial hypothesis that during the aerobic cycling of this enzyme in the absence of L-arginine (**1**), O_2 was directly reduced to H_2O_2.[39] These data suggest that NOS is unique among heme monooxygenases in that in the absence of substrate a two-electron pathway is the sole route for O_2 reduction. However, spin trapping/EPR spectroscopic studies revealed that NOS, independent of the isozyme, generates $O_2^{\bullet-}$, which subsequently dismutates to H_2O_2.[38,40] This latter reaction may account for the perceived production of H_2O_2 from O_2, although several recent reports have suggested that H_4B may promote the direct formation of H_2O_2 at the expense of $O_2^{\bullet-}$ (Scheme 11).[17b,19]

Nitric oxide synthase has three redox active centers, FAD/FMN in the reductase domain and H_4B and heme in the oxidase domain, all of which could conceivably reduce O_2 to $O_2^{\bullet-}$ and/or H_2O_2. The flavins in the reductase domain are similar to a family of flavoproteins that are sterically hindered toward O_2 binding, thereby limiting the generation of H_2O_2.[41] While $FMNH_2$ and $FADH_2$ are not immune to reducing O_2 to $O_2^{\bullet-}$,[42] this one-electron transfer does not appear to be a relevant pathway for NOS. Typical NOS flavin autoxidation rates range from 0.01 to 0.03 s^{-1},[43] which is about a thousand-times slower than free $FADH_2$ in aerated solutions.[44] Further, there appears to be control elements in the reductase domain that protect the flavins from autoxidation.[43b]

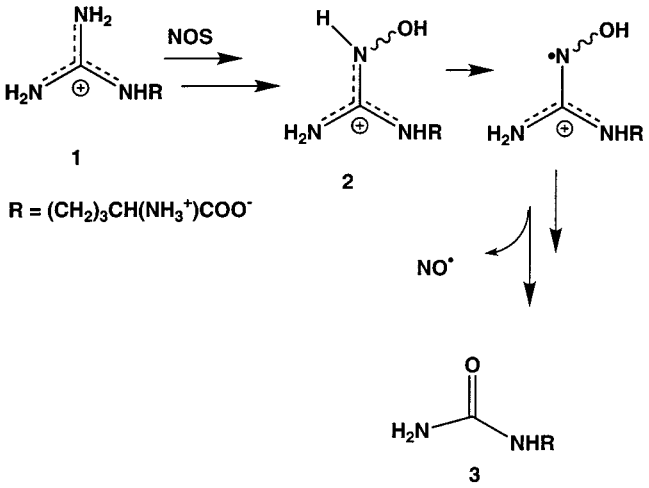
In aqueous aerobic solutions, H_4B is oxidized, affording $O_2^{\bullet-}$; however, the rate is considerably slower than that for reduced flavins.[45] For NOS, H_4B binds next to the heme and near the dimer interface,[19d,46] and as such, it is stabilized against autoxidation. On the other hand, heme in NOS is attached at the bottom of a solvent-exposed substrate channel[19d,46] and can bind O_2 rapidly whether L-arginine is bound or not.[47] As electrons flow from

Scheme 8[a]



[a] Adapted from refs 33b and 34.

Scheme 9[a]



[a] Adapted from ref 37.

NADPH to FAD/FMN and eventually to heme, O_2 attaches to the fifth ligand position. Decay of the heme−O_2 species can lead to $O_2^{\bullet-}$ or H_2O_2, depending on the reductive state of the enzyme. It appears that the release of either $O_2^{\bullet-}$ or H_2O_2 is considerably faster in NOS than it is in cytochrome P-450.[19c,47a,48] Interestingly, turnover rate of the enzyme, resulting in production of $O_2^{\bullet-}$ and/or H_2O_2 from decomposition of NOS ferrous heme−O_2 species, is increased when H_4B is bound, even in the absence of substrate.[19a,c,46a] Only at saturating levels of L-arginine (**1**) are the levels of $O_2^{\bullet-}$ minimal.[40d] Finally, since the conversion of L-arginine (**1**) to L-citrulline (**3**) and NO$^\bullet$ requires O_2, and since this catalytic reaction takes place at the heme, it is fitting that the primary site of O_2 activation should, likewise, be at the heme.

Despite the tight control of electron transport through NOS, there are xenobiotics that can uncouple this enzyme.[49] One such compound is paraquat.[49c] When NOS is incubated with this herbicide, electrons are shunted from the flavins of the reductase domain to this bipyridylium salt (Scheme 12). Under anaerobic conditions, paraquat free radical can be observed by EPR spectroscopy.[49c] However, in aerobic solutions paraquat free radical can reduce O_2, affording $O_2^{\bullet-}$. Despite this, NOS is not fully uncoupled, as the addition of L-arginine (**1**) results in formation of NO$^\bullet$ at the expense of $O_2^{\bullet-}$.[49c] Other one-electron acceptors, such as *o*-quinones and adriamycin, behave similarly.[49a,b]

IV. Secondary Free-Radical Formation by Nitric Oxide Synthase

The consensus is that NOS is capable of metabolizing only L-arginine (**1**) to L-citrulline (**3**) and NO$^\bullet$, with N^G-hydroxy-L-arginine (**2**) as an intermediate, despite a few exceptions.[50] In fact, D-arginine is not a substrate for this enzyme.[25] Recent studies, however, have found that when L-arginine is bound to NOS, this enzyme can oxidize other compounds, leading to other free-radical intermediates.[25]

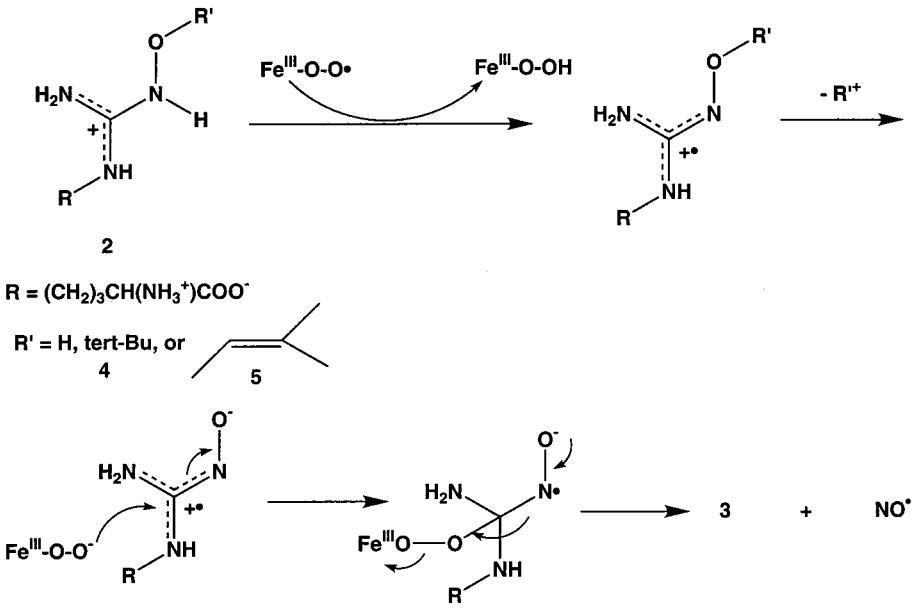
During enzymatic cycling of NOS, NO$^\bullet$ and $O_2^{\bullet-}$ are produced, and the ratio of each is dependent upon the concentration of L-arginine.[40d] These free radicals react at diffusion-controlled rates to produce $ONOO^-$,[51] which has subsequently been shown to decompose to give small amounts of HO$^\bullet$ (Scheme 13).[52]

Further, NOS produces H_2O_2 either directly or from the dismutation of $O_2^{\bullet-}$. In the presence of ferric salts, HO$^\bullet$ will be generated by means of a metal ion-catalyzed Haber−Weiss reaction (Scheme 14).[53]

Therefore, HO$^\bullet$ can be produced by one of two different pathways. During the course of exploring these NOS-catalyzed free radical reactions, it became apparent that other free radicals were generated through a pathway that required the presence of L-arginine.[54] As this is a novel observation with enormous ramification for drug metabolism, the mechanism responsible for this unanticipated finding is described.

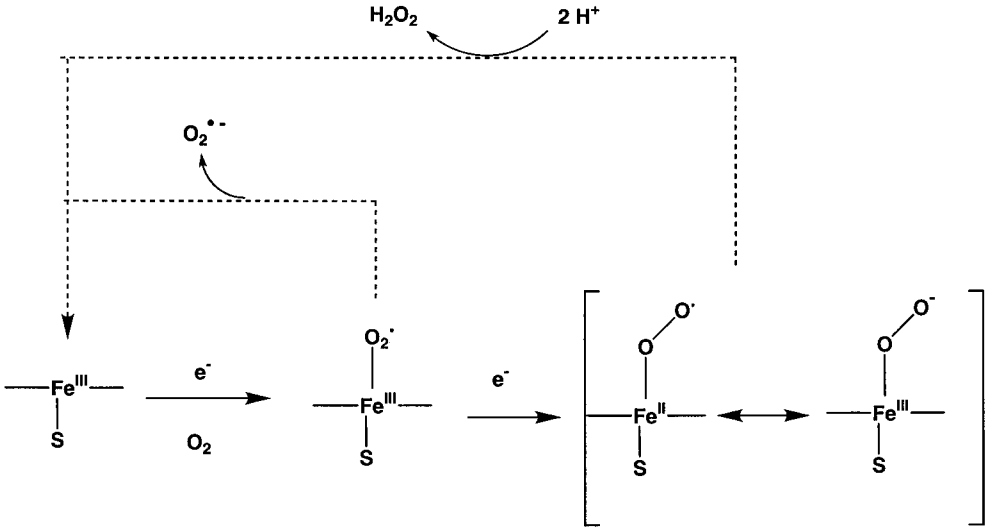
As stated earlier, once L-arginine (**1**) binds to NOS, this interaction promotes the formation of hypothetical perferryl complex NOS−$[Fe^{5+}{=}O]^{3+}$. This reactive intermediate is believed to be the proximal oxidant that metabolizes L-arginine (**1**) to N^G-hydroxy-L-arginine (**2**) and eventually to L-citrulline (**3**) and NO$^\bullet$

Scheme 10[a]

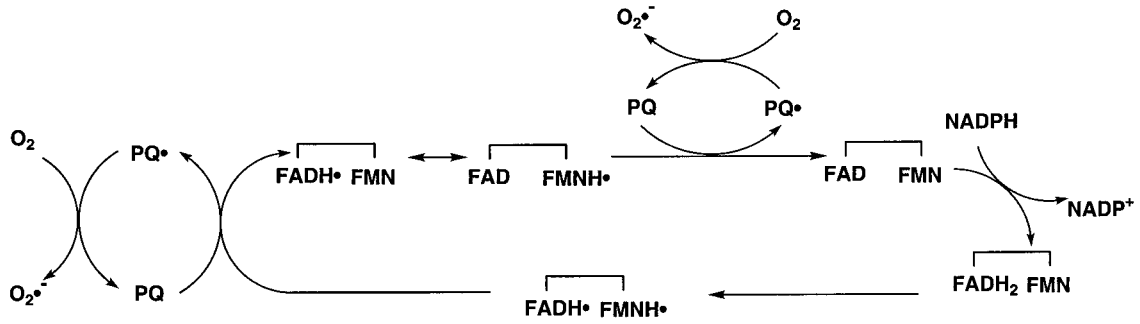


[a] Adapted from ref 34.

Scheme 11

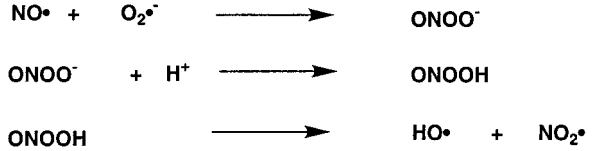


Scheme 12[a]

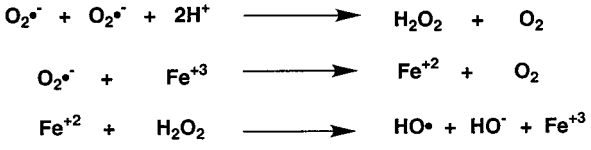


[a] Adapted from ref 49c.

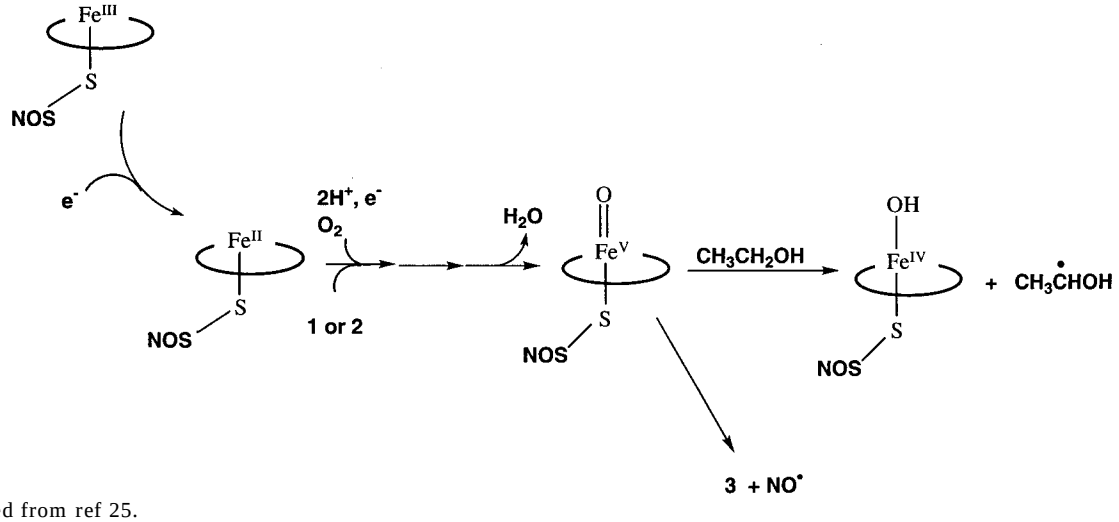
Scheme 13



Scheme 14



Scheme 15[a]



[a] Adapted from ref 25.

and, when ethanol is included in the reaction mixture, to $CH_3{}^{\bullet}CHOH$ as well (Scheme 15).[25] With other primary and secondary, but not tertiary alcohols, the corresponding α-hydroxyalkyl radical is likewise produced.

It appears that the perferryl complex is the catalytic intermediate that mediates these one-electron oxidations as long as the alcohol contains at least one hydrogen atom at the α-position. It has been proposed[25] that formation of the corresponding α-hydroxyalkyl radical is dependent upon electron transfer from the alcohol to the perferryl complex.[55] We envision that the perferryl complex abstracts a hydrogen atom from the carbon α to the oxygen of each alcohol, directly affording α-hydroxyalkyl radical (Scheme 15).

V. Conclusion

Nitric oxide synthases have some unique features that distinguish them from other hemeprotein monooxygenases such as cytochrome P-450s.[11] One of the singular characteristics of NOS is that N^G-hydroxy-L-arginine (**2**) participates in its own metabolism by donating an electron to the enzyme despite the fact that there is much discussion as to the exact mechanism. In the absence of L-arginine, electron flow through NOS leads to the generation of $O_2{}^{\bullet-}$ and/or H_2O_2. The distribution of O_2 reduction products is dependent upon the availability of H_4B. These findings, although different from that noted for cytochrome P-450, parallel earlier observations with FAD-containing monooxygenase.[56]

Superoxide and NO$^{\bullet}$ were detected by spin trapping/EPR spectroscopic experiments with NOS I.[40d] This is remarkable, considering these free radicals combine at near diffusion-controlled rates, producing $ONOO^-$ (Scheme 13).[51] These findings suggest that $O_2{}^{\bullet-}$ and NO$^{\bullet}$ are generated sequentially at the same site, the heme iron.[40d] After $O_2{}^{\bullet-}$ is produced, NOS I must cycle twice before NO$^{\bullet}$ is released. This is apparently sufficiently long to allow $O_2{}^{\bullet-}$ to diffuse from the enzyme to the surrounding milieu, where it reacts with its physiologic target or the spin trap.[40d]

For cytochrome P-450, with its breadth of xenobiotics that undergo metabolsim, it is not usual to find substrates that act as competitive inhibitors of each other. However, until recently, there was no evidence that NOS would behave similarly. In light of a new report,[25] our assessment of this enzyme cannot be so limited. Rather, we must now view NOS as an enzyme that may participate in secondary metabolism. Considering the number of cell types that contain or in the case of NOS II that can be induced to synthesize NOS, the importance of this enzyme in secondary metabolism cannot be minimized.

VI. Acknowledgment

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VII. References

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