

# Nitric oxide synthase: models and mechanisms

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The overproduction or underproduction of nitric oxide has been implicated in pathological symptoms such as endotoxic shock, diabetes, allograft rejection, and myocardial ischemia/reperfusion injury. A thorough understanding of the biosynthesis of nitric oxide is necessary to probe and manipulate these signaling events. There is also considerable pharmacological interest in developing selective inhibitors of the several isoforms of nitric oxide synthase. The recently determined crystal structures of complexes between nitric oxide synthase and substrate, the mechanisms of the enzymatic reaction that generate nitric oxide and chemical precedents and models for these reactions are now coming into focus, but there are still numerous fascinating and unanswered questions regarding nitric oxide biosynthesis.

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## Abbreviations

<b>CaM</b>	calmodulin
<b>Co(salen)</b>	[bis(salicylaldehyde)ethylenediimnato]cobalt(II)
<b>ENDOR</b>	electron-nuclear double resonance
<b>eNOS</b>	endothelial NOS
<b>EPR</b>	electron paramagnetic resonance
<b>FAD</b>	flavin adenine dinucleotide
<b>FMN</b>	flavin mononucleotide
<b>H4B</b>	tetrahydrobiopterin
<b>iNOS</b>	inducible NOS
<b>NADH</b>	nicotinamide adenine dinucleotide, reduced form
<b>NADPH</b>	nicotinamide adenine dinucleotide phosphate, reduced form
<b>NHA</b>	<i>N</i> -hydroxy-L-arginine
<b>NHE</b>	normal hydrogen electrode
<b>nNOS</b>	neuronal NOS
<b>NO</b>	nitric oxide
<b>NOS</b>	nitric oxide synthase
<b>Sk</b>	skatole
<b>SOD</b>	superoxide dismutase

## Introduction

Nitric oxide (NO) is a small, reactive molecule that is an extraordinarily important bioregulator. Although NO was first characterized more than 200 years ago by Joseph Priestly, its functions as a biomessenger were not recognized until the mid-1980s. NO is now recognized as a ubiquitous biomessenger, existing in a wide variety of organisms. In mammals, NO is used for a number of inter-cellular and intra-cellular signaling functions including blood-vessel dilation, neuronal signal transmission, cytotoxicity against pathogens and tumors, coordination of heart rhythm and the regulation of cellular respiration activity.

NO is a unique biological messenger molecule. Endogenous NO is produced by the heme-containing metalloenzyme

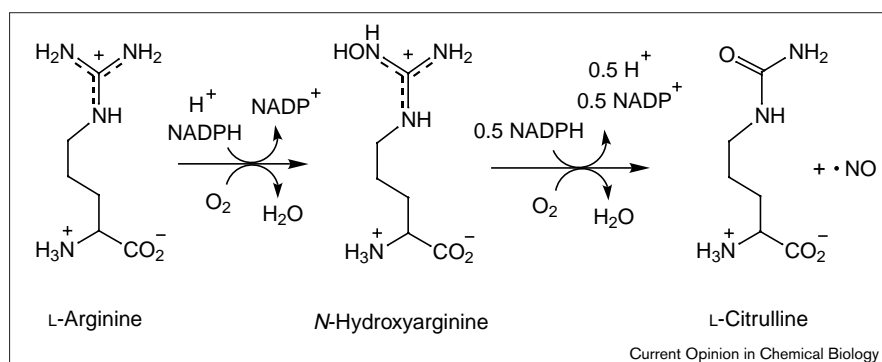
nitric oxide synthase (NOS; EC 1.14.13.39) [1–3]. There are several NOS isoforms and these are homodimers with each monomer containing binding sites for NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), calmodulin (CaM), tetrahydrobiopterin (H4B), and a heme group. Catalytic activity assays reveal that H4B is an especially important NOS cofactor serving both allosteric and redox functions. The X-ray crystal structures of substrate-bound NOS show that both the substrate and H4B are bound at the heme site with a substantial network of hydrogen bonds. A cysteinyl thiolate-ligated heme group reminiscent of cytochrome P450 is the redox active center that mediates the NOS reactions. NOS catalyzes the two-step, five-electron oxidation of L-arginine (L-Arg) via *N*-hydroxy-L-arginine (NHA) to citrulline and NO [4,5]. The first step of the NOS reaction recruits two NADPH-derived reducing equivalents to furnish the two-electron oxidation of L-Arg to NHA by O<sub>2</sub>• (Figure 1; [6,7]). The second step of the NOS reaction is unusual because it furnishes the three-electron aerobic oxidation of NHA to NO and citrulline by consuming only one NADPH-derived reducing equivalent [8,9]. A nucleophilic hydroperoxo-Fe(III)heme, the oxenoid (oxo-Fe(IV)(Por<sup>•+</sup>)) species known in P450 oxygenase reactions, and a radical-type autoxidation mechanism have been proposed as the oxidizing intermediates for the oxidation of NHA. Our current understanding of these processes is discussed in light of the characteristics of the NOS structure, biochemical studies, enzymatic precedents and model chemical systems. The principal focus is on results reported in 1999 and 2000.

## Nitric oxide synthase

The catalytically active NOS is a homodimer of hemoprotein. The molecular weight of the monomer ranges from 110 kDa to 160 kDa depending on the isoform [4,10,11]. Each NOS monomer contains an oxidase domain at its amino-terminal end and a reductase domain at its carboxy-terminal end. The NOS reductase domain contains binding sites for the redox cofactors NADPH, FMN, FAD, and CaM. Two reducing equivalents are transferred from NADPH, via FMN and FAD, to the oxidase domain under the control of the Ca<sup>2+</sup>/CaM complex. The oxidase domain tightly binds one equivalent each of H4B and a cysteinyl thiolate-ligated heme group, which is the reactive center of the oxidation reaction (Figure 2).

NOS is particularly interesting and complicated because of the variety of redox cofactors in the catalytic assembly. CaM activates NOS function mainly by influencing the reductase domain [12]. NADPH is the reductant of the NOS reactions. This redox active cofactor has a reduction potential of –0.324 V (Table 1). Upon binding to NOS, NADPH is oxidized by hydride loss to generate NADP<sup>+</sup>. Notably, although the structure of NADH is only slightly

Figure 1



different from NADPH, NADH cannot support the NOS reactions at all.

During the catalytic cycle of the NOS reaction, electrons are transferred one-by-one to the heme group. The flavin cofactors are located in the NOS reductase domain downstream from the NADPH-binding site. The electrons from NADPH are transferred to the oxidized forms of FMN or FAD and are stored here. It has been shown that in the absence of NADPH, the half-reduced flavin cofactors, flavin semiquinone free radicals (FSQ), support the oxidation of L-Arg to NHA and small amounts of citrulline [13]. Intriguingly, half-reduced FMN and FAD do not support the oxidation of NHA to citrulline and NO in the absence of NADPH [14].

Among the various cofactors of NOS, H4B is functionally the most versatile and mysterious. H4B supports the NOS reactions in two ways. One is an allosteric action stabilizing the structure of functioning NOS, the other is as a redox cofactor for the NOS reaction. The allosteric effects of H4B include stabilizing the dimeric structure of NOS, stabilizing the conformation of high-spin heme iron, and

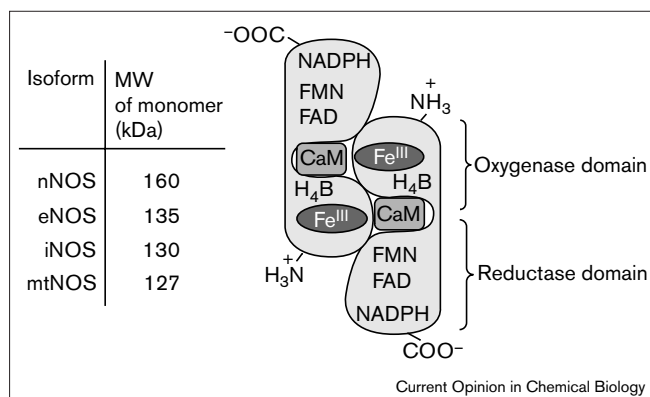
supporting the binding of substrate L-Arg [15]. Recent data support a redox function of H4B in the NOS reaction. It was found that L-Arg was converted to NHA by the oxygenase domain of eNOS (endothelial NOS) alone [16]. And recently, the radical species  $H_3B^\bullet$  was directly observed during the production of NHA from arginine [17<sup>\*\*</sup>]. When a pterin analog of H4B was mixed with H4B-bound nNOS (neuronal NOS) under catalytic conditions, only 50% of recovered pterin was H4B, whereas the other 50% pterin was a derivative different from H2B and H4B [18]. This result supports the notion that H4B functions in the NOS reaction by serving as a direct electron donor to the heme group [19,20].

### The structure of nitric oxide synthase

Crystal structures of substrate-bound iNOS (inducible NOS), eNOS and nNOS show that both the substrates and H4B bind close to the heme by a network of hydrogen bonds. L-Arg is located immediately above the heme group. The central guanidine-N of L-Arg is 3.8 Å away from the heme iron [21–23]. The other two guanidine-N of L-Arg are bound to the NOS active site by two hydrogen bonds to a Glu371 and a hydrogen bond to the carbonyl group of Trp366 (Figure 3). In addition, the amino group of L-Arg is hydrogen-bonded with the propionate group of heme pyrrole ring D. The carboxylate group of the L-Arg group is hydrogen-bonded with Tyr367, a water molecule, and the carboxylate group of Asp376.

The crystal structure of NHA-bound iNOS is very similar to that of L-Arg-bound iNOS, except that an additional hydrogen bond exists between the NHA-hydroxy oxygen and the amido hydrogen of Gly365 [24]. The structure indicated that NHA does not ligate Fe(III) heme because the distance between Fe(III) and *N*-hydroxy oxygen ( $\approx 4.3$  Å) is too long for a Fe–O bond hydrogen-bonded to the propionate group of heme pyrrole ring D (Figure 3; [16,21,24,25]). Overall, the arrangement is similar to one of the structures of NHA-bound nNOS determined by the ENDOR (electron-nuclear double resonance) spectroscopic technique, which showed the hydroxylated NHA nitrogen nearest the iron and, therefore, protonated [26,27<sup>\*</sup>].

Figure 2



Schematic structure of nitric oxide synthase. mtNOS, mitochondrial NOS; MW, molecular weight.

Table 1

**Redox potentials of NOS cofactors, NHA derivatives and various iron porphyrin oxidation states.**

Redox couples	Redox potentials*	Reference
OxoFe(IV)(P <sup>+</sup> )/oxoFe(IV)P	≈1.4–1.6 V	[67]
OxoFe(IV)P/Fe(OH)P	≈1.0–1.3 V	[67]
NHA/NHA-derived iminoxy radical	≈710 mV (pH 7.5)	[8]
H4B/qH2B	150–180 mV	[68]
SuperoxoFe(III)/peroxoFe(III)heme	10 mV	[69]
NHA <sup>-</sup> /NHA <sup>•</sup>	≈-200 mV	[57]
NOS Fe(III)/Fe(II)heme	-248~-263 mV	[70]
FAD/FAD <sup>+</sup>	-270~-290 mV	[71]
NADPH	-324 mV	[72]

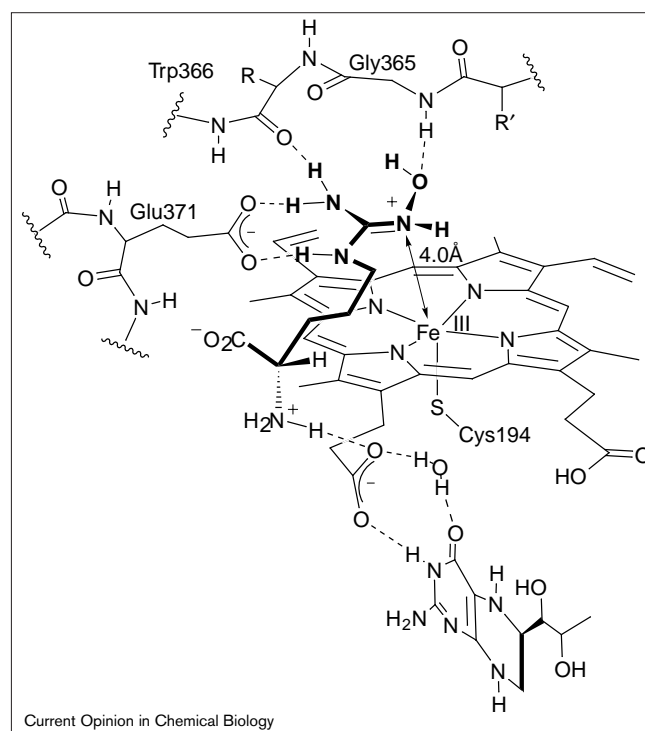
\*E<sub>1/2</sub> versus NHE, at pH 7 aqueous solution except where otherwise cited. †Estimated from the redox potential of the amidoximate/amidoxyl iminoxy radical couple. NHA<sup>-</sup>, oximate derived from the N-hydroxy group of NHA; NHA<sup>•</sup>, the iminoxy radical derived from NHA; qH2B, quinonoid dihydrobiopterin.

These structures inform the discussion of the mechanisms of NOS in a number of interesting ways. The hydrogen-bonding network seems to orient arginine and NHA rigidly with respect to the heme as shown in Figure 3. It is immediately apparent that NHA is bound as the *anti* stereoisomer, a fact that has not been previously discussed. This is noteworthy because the hydroxylimine oxygen and the guanidinium carbon of the NHA are both distant from the heme iron (4.3 Å and 4.4 Å, respectively). This arrangement presents significant difficulties for some of the proposed mechanisms of NOS discussed below.

**Characteristics of the NOS reactions**

The NOS reaction generates citrulline by oxidizing L-Arg through the intermediacy of NHA. However, the identity of the nitrogen oxide product, NO, or the nitroxyl anion, NO<sup>-</sup>, was only determined recently. Several reports suggested that the nitroxyl anion, NO<sup>-</sup>, was generated by NOS and converted to NO under the catalysis of superoxide dismutase (SOD) [28,29]. Recently, however, NO from the NOS reaction was directly detected in the absence of SOD [30]. There has been a debate regarding the stoichiometry of NADPH and the amount of citrulline generated in the NOS reaction. The controversy arises because several NADPH-consuming reactions occur along with the NOS reaction. These side reactions include the generation of superoxide ion (O<sub>2</sub><sup>-</sup>) by the NOS heme [31<sup>•</sup>,32] and the oxidation of NADPH by peroxynitrite generated from NO and O<sub>2</sub><sup>-</sup> [28]. Hevel and Marletta [9] have correctly determined the NADPH/citrulline stoichiometry by conducting the reaction with H4B-saturated iNOS in the presence of SOD. Another important characteristic of the NOS reaction products is the oxygen source for NO and the urea-oxygen of citrulline. Using <sup>16</sup>O-labeled NHA under <sup>18</sup>O<sub>2</sub> conditions, the urea oxygen of citrulline was found to contain exclusively <sup>18</sup>O,

Figure 3



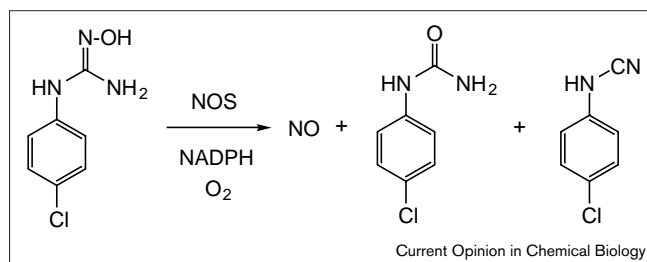
Schematic structure of the active site of NHA-bound murine iNOS. NHA is shown in bold.

whereas the NO product originated completely from the N-hydroxy group of NHA [33,34].

The N-hydroxylation of L-Arg consumes one equivalent each of NADPH and O<sub>2</sub>, and is typical of a P450 oxygenase reaction. The generally accepted P450 reaction mechanism can account for the stoichiometry and the product formation [35]. Marletta *et al.* [36] have proposed a non-P450-type mechanism to account for the oxidation of L-Arg in which O<sub>2</sub> activation by an H4B and a non-heme metal ion was proposed to furnish the intermediate oxidizing L-Arg to generate NHA. This mechanism is analogous to that of the pterin-dependent aromatic amino acid hydroxylases [37]. However, this notion has been challenged by two recent discoveries. First, the 5-methyl H4B analog supports the NOS reaction, but it does not support O<sub>2</sub> activation [38]. Secondly, catalytic activity analysis of a mutated NOS, in which the histidine residues near the heme group were substituted, and metal ion analysis both suggest that there is no catalytic role for a non-heme redox-active metal in NOS [39].

In the second step of the NOS reaction, the heme group activates O<sub>2</sub> by recruiting only one reducing equivalent from NADPH and, apparently, one from NHA. A key question that needs to be answered is the order of the redox events involved in the oxidation of NHA. Particularly, it is unclear whether the NOS Fe(III)-heme is reduced by NHA or the

Figure 4



The NOS-catalyzed oxidation of *N*-aryl-*N'*-hydroxyguanidines.

NADPH-derived reducing equivalent to initiate the second step. Several groups have attempted to address this question. Pufahl and Marletta [40] have shown that the incubation of NHA with NOS under CO does not give the  $\lambda = 450$  nm difference spectrum that is characteristic of an iron(II)-heme thiolate species, whereas the NADPH-containing solution of NOS does. Nonetheless, CO inhibits the NOS reaction by competing with  $O_2$  to bind to Fe(II)-heme, and the inhibition occurs regardless of the presence of either L-Arg or NHA. These observations were interpreted as evidence indicating that NHA cannot reduce the Fe(III)-heme of NOS. 'Single turnover' oxidation of NHA by NOS also supports this view. [41–43] Notably, unnatural substrates have been found that bind and react at the NOS active site. For example, the NOS-catalyzed oxidation of *N*-aryl-*N'*-hydroxyguanidines, with a  $K_m$  30-fold higher and  $V_{max}$  fivefold lower than those of NHA, consumes NADPH to generate the corresponding urea and cyanamide along with nitrite (Figure 4; [44]).

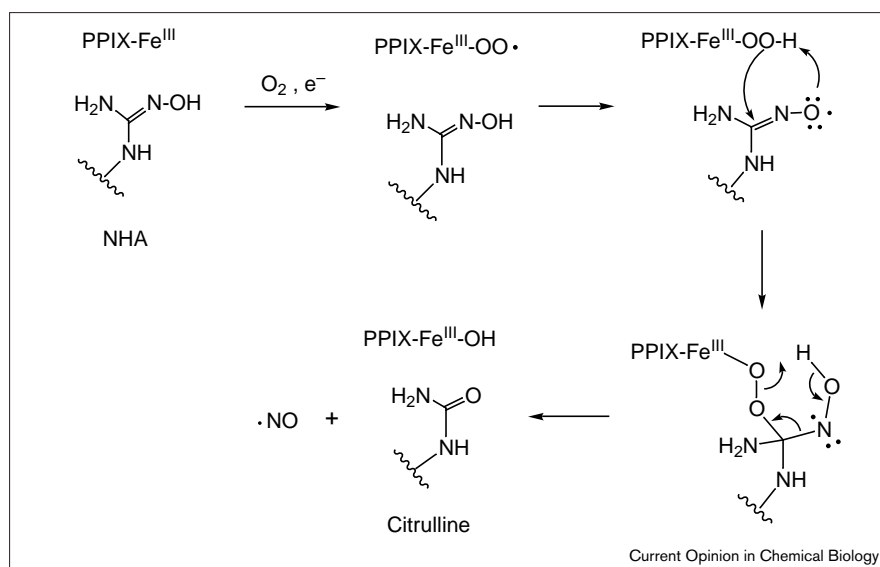
### Proposed mechanisms and analogous reactions

Several proposals have been advanced to account for the unusual redox chemistry of the NOS-catalyzed oxidation

of NHA. These proposals include an oxenoid species ((Por<sup>+</sup>)Fe<sup>IV</sup>(O)) analogous to the cytochrome P450 oxygenase, [45,46] and versions of a nucleophilic peroxyFe(III)P (for P, see Figure 6) species analogous to that proposed for the aromatase reaction [8,36,47,48]. Recently, we presented a radical-type autoxidation mechanism based on model systems that mimic many of the important properties of the NOS-catalyzed oxidation of NHA [49\*]. A comparison of the proposed mechanisms based on chemical and enzymatic precedents will now be discussed.

Marletta [47] has advanced a mechanism involving a nucleophilic peroxyFe(III)heme species to account for the NOS-catalyzed oxidation of the *N*-hydroxyguanidine group. Later, this mechanism was modified by Ingold and co-workers [8] on the basis of thermodynamic considerations (Figure 5). A superoxoiron(III)heme intermediate resulting from NOS Fe(II)heme and  $O_2$  was proposed to abstract the hydrogen atom of the NHA hydroxy group to furnish a peroxyiron(III)heme and the NHA-derived iminoxy radical. A nucleophilic attack of the hydroperoxyiron(III)heme on the carbon of the iminoxy radical is followed by a pericyclic reaction on the peroxy intermediate to furnish the generation of NO and citrulline. Citrulline and N<sup>δ</sup>-cyanoornithine were generated along with nitrite and nitrate in the  $H_2O_2$ -supported oxidation of NHA under the catalysis of nNOS and iNOS [50,51]. Significantly, however, the NOS–NHA crystal structure shows that the NHA hydroxyl is held 4.3 Å away from the iron and is inaccessible to the proposed Fe(III)-superoxo intermediate. Further, Stuehr and co-workers [52] have reported Raman data for Fe– $O_2$ –nNOSoxy indicating that the bound oxygen and arginine do not interact. This is difficult to accommodate with the X-ray structures unless the oxygen is bent in a direction away from the substrate. Accordingly, mechanisms involving interactions of Fe– $O_2$  with NHA would require

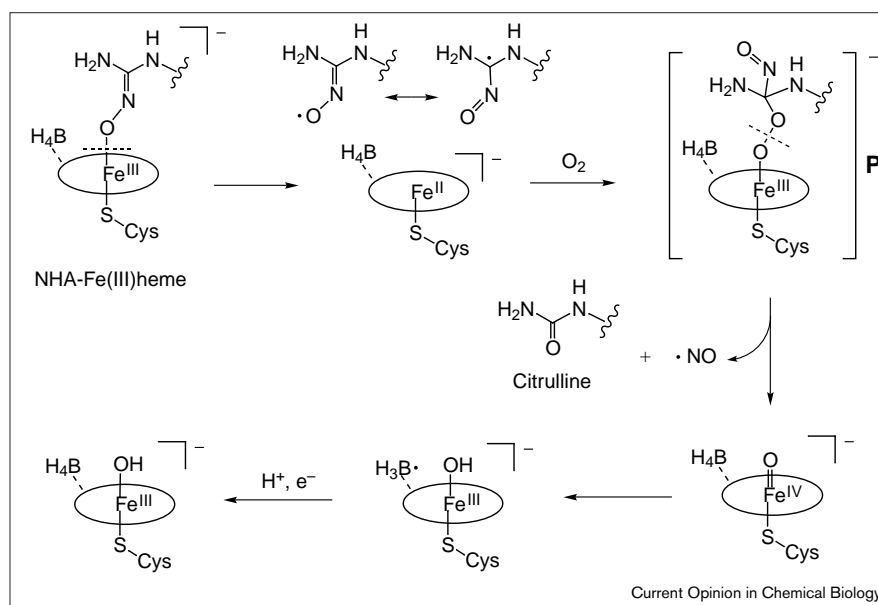
Figure 5



Proposed nucleophilic hydroperoxyFe(III)heme mechanism for the NOS-catalyzed oxidation of *N*-hydroxyarginine. PPIX, protoporphyrin IX.

Figure 6

Proposed radical-type autoxidation mechanism of the NOS-catalyzed oxidation of NHA. Ellipses denote the heme group. P, peroxyFe(III)heme intermediate.



considerable rearrangement of the reactants at the NOS active site.

### Radical-type autoxidation mechanism

We have proposed that the NOS-catalyzed aerobic oxidation of NHA occurs via a radical-type autoxidation process on the basis of the observation that Fe(III) porphyrins can oxidize oximes to an iminoxyl radical intermediate [49<sup>•</sup>,53]. Specifically, we propose that the hydrogen-bonded NHA in NOS can be oxidized by the Fe(III)heme to generate Fe(II)heme and the NHA-derived iminoxyl radical, which would tautomerize to the  $\alpha$ -nitroso radical. The insertion of a dioxygen molecule between the  $\alpha$ -nitroso radical and Fe(II)heme would form an energetic  $\alpha$ -nitroso peroxyFe(III)heme intermediate as in Figure 6. This peroxyFe(III)heme intermediate (P in Figure 6) would decompose via the homolysis of the energetic O-O bond, as other energetic peroxyFe(III)P species do in nonpolar environments [54,55] to generate NO, citrulline and oxoFe(IV)P.

OxoFe(IV)P can be immediately reduced back to the resting heme Fe(III) by harvesting one electron from the adjacent H<sub>4</sub>B cofactor. The resulting H<sub>3</sub>B<sup>•</sup> radical could be recycled back to H<sub>4</sub>B by the NADPH-derived reducing equivalent, and return the system to the resting state. Thermochemical considerations indicate that the NHA-derived oximate is capable of reducing the NOS Fe(III)heme (Table 1). Although the reduction potential of NOS Fe(III)heme (-248 to about -263 mV versus NHE [normal hydrogen electrode] at pH 7.0) [56] is much lower than the oxidation potential of NHA (580 mV versus NHE) [8]; it is about the same as that of the NHA-derived oximate should be lower than that of acetamidoxime-derived oximate (-200 mV versus NHE) [57,58]. Thus, the autoreduction of the Fe(III) in the NHA-Fe(III)heme adduct should be energetically accessible ( $\Delta G^\ddagger < 1.1 \sim 1.5$  kcal/mol, calculated from  $\Delta G^\ddagger = -23.06$  kcal/mol $\cdot\Delta E^\circ$ , where  $\Delta E^\circ = 0.5 \sim 0.6$  V). Direct ligation of the NHA to the Fe(III) heme appears to be precluded by the X-ray crystallographic data, however.

Figure 7

The reaction of an iminoxyl radical and O<sub>2</sub>, which is proposed to generate an  $\alpha$ -nitroso oxy radical species, which then loses NO to generate a ketone. R, alkyl.

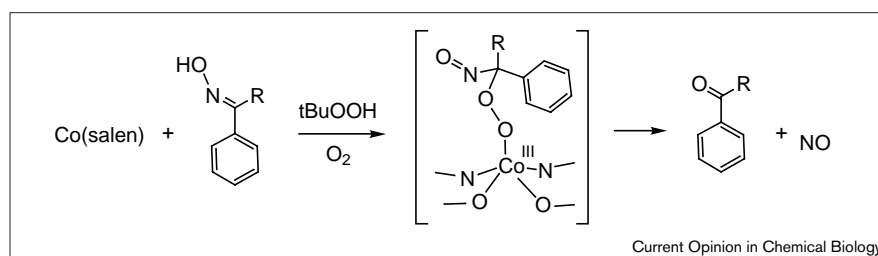
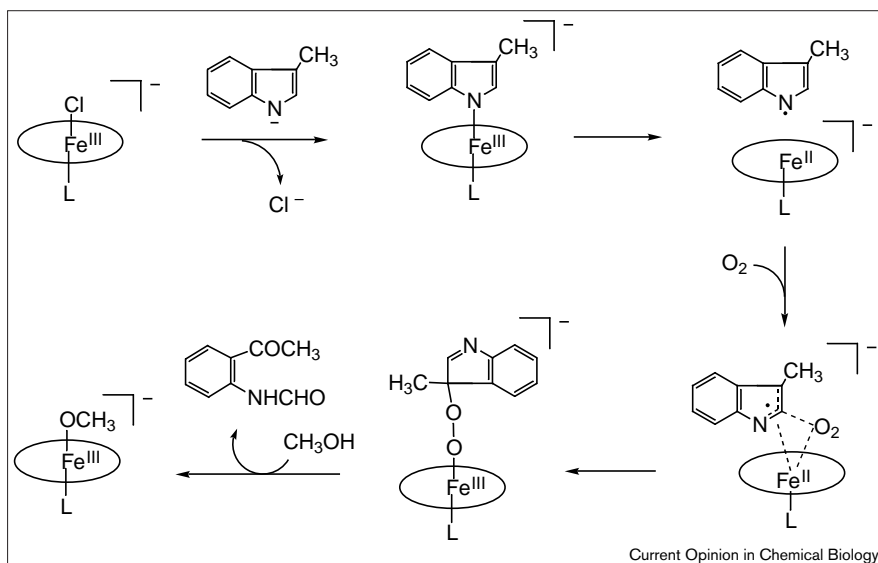


Figure 8



## Chemical and enzymatic precedents

### Co(II)(salen)-catalyzed oxime oxidation

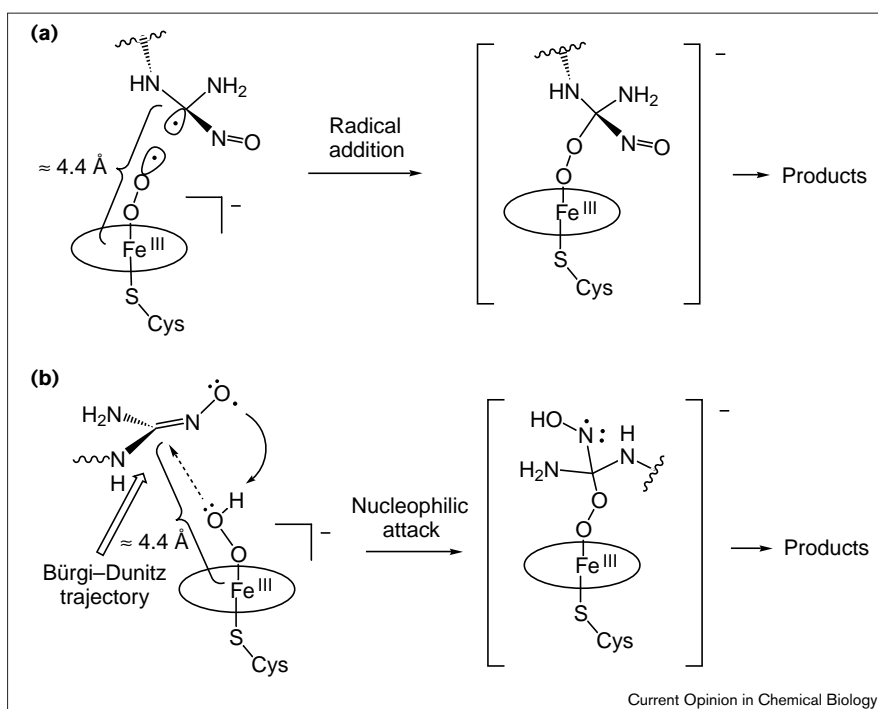
It was demonstrated some time ago that simple Co(II)(salen) complexes can mediate the oxidation of acetophenone oxime with *t*-butyl hydroperoxide [59]. The reaction generates ketones and Co(III)(ONO)(salen). The proposed mechanism of this reaction involves the one-electron oxidation of an oxime to an iminoxy radical by a *t*-butoxyl radical resulting from the decomposition of

Co(III)(*t*-BuOO)(salen). The reaction of the iminoxy radical and O<sub>2</sub> was proposed to generate an  $\alpha$ -nitroso oxy radical species, which could lose NO to generate the ketone (Figure 7).

### Tryptophan dioxygenase

A substrate-derived peroxyFe(III)heme species has been implicated in the tryptophan dioxygenase reaction. The enzyme is assumed to generate a ternary complex containing

Figure 9



an indole-derived radical, O<sub>2</sub>, and heme-Fe(III) as the reactive intermediate [60]. Models of the ternary complex have been prepared from the toluene/methanol solution of Fe(III)P, O<sub>2</sub>, and 3-methylindole (skatole; Sk) [61,62]. Electron paramagnetic resonance (EPR) spectroscopic analysis reveals the signals of the nitrogen and carbon-centered Sk neutral radical, the Sk-peroxide radical, and two low-spin peroxyFe(III) complexes, which were assigned as [Fe(III)P(OCH<sub>3</sub>)(OOSk)]<sup>-</sup> and [Fe(III)P(OOSk)<sub>2</sub>]<sup>-</sup> (Figure 8). The two Sk-peroxide-containing Fe(III)-heme species decomposed upon warming to generate the dioxygenated indole derivatives. Interestingly, the reaction rates of the Sk dioxygenation are proportional to the reduction potentials of the Fe(III)P catalysts [61]. These results suggest that Sk is oxidized by Fe(III)P, and then reacts with O<sub>2</sub> and Fe(II)P to form the Sk-peroxide-containing Fe(III)P intermediate.

### The oxygenation of the NHA-derived radical

The X-ray crystal structures have shown that the NOS heme iron and the guanidinium carbon of the NHA-derived iminoxy radical would be 4.4 Å apart and that the substrate restricts oxygen binding [22,24]. Further, bound O<sub>2</sub> seems to point away from the substrate according to the Raman data [52]. Accordingly, the distal oxygen of bound O<sub>2</sub> would have to be very far away from the NHA hydroxyl group and both the orientation and the distance between Fe(III)heme and guanidinium carbon are awkward for the proposed peroxo nucleophilic attack. This orientation also deviates significantly from the ideal Bürgi–Dunitz trajectory [63,64] for the nucleophilic addition [65]. By contrast, the spatial arrangement of the NHA-derived iminoxy radical and superoxoFe(III)-heme could be well poised for a radical addition reaction. A front-side approach of the heme-superoxoFe(III) radical to the guanidinium carbon radical could induce the iminoxy radical to tautomerize to the α-nitroso radical, which could combine with superoxoFe(III)heme to form the peroxyFe(III)heme intermediate (P in Figure 6) in a manner analogous to the reaction between a carbon radical and an alkene ([66]; Figure 9).

### Conclusions

The NOS reactions provide an array of unprecedented chemistry yet to be unraveled. Many puzzles remain unsolved, particularly regarding the function of the tetrahydrobiopterin, the possible allosteric action of NADPH, and the possibility of an NHA-effected reduction of Fe(III)heme. Carefully designed model systems along with structural and biochemical studies of the enzyme have begun to reveal the essence of these reactions. The various proposed mechanisms for the NOS reactions discussed above explain many of the observations but none of them are fully consistent with the known facts at this time. A fuller understanding of NOS reaction mechanisms, which is essential for the rational development of mechanism-based inhibitors, must await the outcome of experiments currently underway in a number of laboratories.

### Acknowledgements

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  - The conversion of *N*-hydroxy-L-arginine (NOHA) or L-arginine to citrulline and NO was examined under single-turnover conditions using the oxygenase domain of neuronal nitric oxide synthase (nNOSox) and rapid scanning stopped-flow spectroscopy. When anaerobic nNOSox saturated with H4B and NOHA was provided with 0.5 or 1 electron per heme and then exposed to air, it formed 0.5 or 1 mol of citrulline/mol of heme, respectively, indicating that NOHA conversion had 1:1 stoichiometry with respect to electrons added. Transient spectral intermediates were investigated. For NOHA, four species were observed in the following sequence: starting ferrous nNOSox; a transient ferrous-dioxygen complex; a transient ferric-NO complex; and ferric nNOSox. The results establish that the ferrous-dioxy enzyme reacts quantitatively with NOHA but not with arginine and that its reaction with NOHA forms one NO-heme, which immediately binds to form a ferric-heme-NO complex.
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