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Heme coordination of NO in NO synthase

(cytochrome P-450/Raman scattering/flavoprotein/L-arginine)

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ABSTRACT **A current question in nitric oxide (NO) biology is whether NO can act as a feedback inhibitor of NO synthase (NOS). We have approached this problem by examining the interaction of NO with neuronal NOS by optical absorption and resonance Raman scattering spectroscopies. Under an inert atmosphere NO coordinated to the heme iron in both the oxidized and reduced forms of NOS. The Soret and visible optical absorption transitions are detected at 436 and at 567 nm, respectively, in the Fe^{2+}–NO heme complex and at 440 nm and at 549 and 580 nm, respectively, in the Fe^{3+}–NO heme complex. In the resonance Raman spectrum of the ferrous complex the Fe–NO stretching mode is located at 549 cm^{-1} in the presence of L-arginine and at 536 cm^{-1} in the absence of L-arginine, whereas in the ferric enzyme the mode is located at 540 cm^{-1} (in the absence of L-arginine). The interaction between bound L-arginine and the NO indicates that L-arginine binds directly over the heme just as do the substrates in cytochrome P-450s. In the absence of L-arginine, NO readily oxidized the ferrous heme iron. The oxidation was prevented by the presence of bound L-arginine and enabled NOS to form a stable ferrous NO complex. Under oxygen-limited conditions, NO generated by neuronal NOS coordinated to its heme iron and formed a spectrally detectable ferrous–NO complex. Taken together, our results show that NO can bind to both ferric and ferrous NOS and may inhibit NO synthesis through its binding to the heme iron during catalysis.**

Nitric oxide (NO) has been shown to be involved in several important physiological processes acting as a neurotransmitter, a vasodilator, and a cytotoxic agent (1–5). NO is formed by a homodimeric family of enzymes termed NO synthases (NOSs), which oxidize L-arginine to citrulline and NO. The synthases bind several prosthetic groups, including calcium-calmodulin, tetrahydrobiopterin (H_4BPT), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and heme, the putative site at which the oxidation of L-arginine occurs (1, 2, 5, 6). The NOSs of different tissues vary with regard to their primary sequence, mode of expression, and regulation by calcium. Both constitutive and inducible isoforms of the enzyme have been characterized. The rat brain NOS used in the experiments reported here is a soluble isoform that is expressed constitutively in distinct populations of neurons (7).

The properties of the heme in NOS have recently been characterized by optical absorption (8–11), electron paramagnetic resonance (9), and resonance Raman spectroscopy (11). These techniques have shown that the heme iron is coordinated to the protein by thiolate just as in the cytochrome P-450s. Like these enzymes, the heme pocket of NOS is envisioned to form the catalytic site where the stepwise oxidation of L-arginine takes place (Fig. 1) (12, 13). The NOS heme iron is thought to participate in both steps by binding and activating O_2 (5). Indeed, NO synthesis from either L-arginine or N^{ω}-hydroxy-L-arginine is inhibited by CO (9, 14), and various spectral data suggest that CO inhibits the reaction by coordinating directly to the heme iron (8–11, 15), thus preventing O_2 binding and activation.

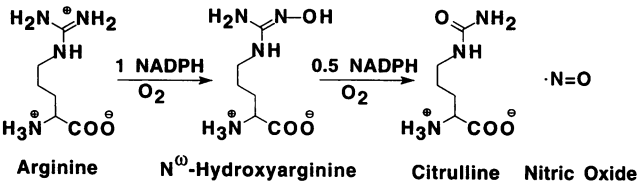

FIG. 1. Reaction catalyzed by NOS. The reaction is carried out in steps and involves formation of the intermediate N^{ω}-hydroxy-L-arginine (12, 13). A total of 1.5 NADPH is consumed per NO formed, distributed in the manner shown (12).

Recent evidence suggests that the reaction product, NO, acts as a feedback inhibitor of NOS (16–19). Although NO binds as an axial ligand to the iron of many heme proteins (20–22), its binding to the heme iron in NOS has not been established, and the mechanism by which NO inhibits NOS is still unclear. In this report, we have utilized optical absorption and resonance Raman spectroscopies to characterize the interaction of NO with the ferrous and ferric forms of NOS. Optical absorption is valuable because the electronic transitions of NO-bound heme proteins are well characterized in both oxidation states (21). However, the optical spectra probe only the transitions of the porphyrin macrocycle and thereby do not give unequivocal proof that NO has bound to the central heme iron rather than inducing some other change in the coordination structure of the heme. Resonance Raman scattering probes the vibrational spectra of the porphyrin macrocycle, so yields information that is complementary to the absorption spectrum (23). The resonance Raman spectrum also gives direct information concerning the coordination of ligands to the heme group in those cases in which modes involving the ligand are present in the spectra. The technique has been especially useful in this regard in the identification of modes involving bound oxygen intermediates in various enzymes (24, 25) and other bound ligands such as CO and NO, which serve as useful probes to study the properties of the physiological binding site (26–30). We now demonstrate that NOS binds NO as a sixth ligand to the heme iron in both its ferric and ferrous forms and generates a ferrous NOS–NO complex by reacting with self-generated NO during limited catalysis.

METHODS AND MATERIALS

The cloned and expressed Ca^{2+}-sensitive NOS from rat brain (31) was purified from cultures of stably transfected kidney cells as described (9). The enzyme preparations were con-

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Abbreviations: NOS, nitric oxide synthase; H_4BPT, tetrahydrobiopterin.

centrated to ≈50 μM in 40 mM Tris buffer (pH 7.8) containing 3 mM dithiothreitol and 4 μM H_4BPT. Experimental solutions did not contain added FAD, FMN, Ca^{2+}, or calmodulin. When added, the final L-arginine concentration was 2 mM. To reduce the enzyme, sodium dithionite solutions were used. $^{14}N^{16}O$ was purchased from Matheson (>99% purity). Immediately prior to exposure of the enzyme to NO, it was scrubbed by passage through a saturated solution of KOH and then a solution of phosphate buffer. $^{15}N^{18}O$ was purchased from ICON Services (Summit, NJ) and used without further purification.

Samples for the resonance Raman measurements were prepared by sealing aliquots (140 μl) of about 30 μM NOS in a rotating cell. The laser excitation wavelength used to generate the heme resonance Raman spectra, at a power of about 3 mW, was 441.6 nm. This excitation wavelength was selected to optimize the quality of the spectra from the NO-bound form of the enzyme. The scattered light was dispersed by a 1.25-m monochromator and detected with a charged-coupled device camera. Typically, several 1-min spectra were obtained and averaged to improve the signal-to-noise ratio. The data reported here have been baseline corrected but are unsmoothed. The frequencies of the Raman shifted lines were calibrated against an indene standard. Absorption spectra of the samples [SLM Aminco (Urbana, IL) model DW-2000] were obtained in the sealed Raman cell (pathlength, 2 mm) before and after measuring the Raman spectra to ensure that the low-power laser excitation did not modify the enzyme.

NOS catalysis under oxygen-limited conditions was carried out in 1.5-ml cuvettes equipped with a rubber stopper. Cuvette solutions (1 ml) contained 1.3 μM neuronal NOS, 4 μM H_4BPT, 2 μM calmodulin, and 0.9 mM EDTA in 40 mM bis-Tris propane (pH 7.4). All solutions were bubbled with N_2 to reduce their oxygen concentration prior to transfer into the cuvette. Subsequent additions of L-arginine (2 mM), 7 μM NADPH, and 2 mM Ca^{2+} were made such that the volume of the cuvette solution changed <3%. In some cases, CO gas was introduced into the sample by bubbling as a final addition. Spectra were recorded on a Hitachi U3100 spectrophotometer at 15°C following each addition as described in the text.

RESULTS

NO coordinates to many heme proteins in both the ferrous and ferric oxidation states of the heme iron atom, yielding characteristic spectra (21). To determine if NO is able to bind to the heme of NOS we have exposed both the fully oxidized and the fully reduced forms of the enzyme to NO. Changes in the spectrum are detected that are consistent with the coordination of NO to the heme of NOS (see Fig. 2) as discussed below. For the reduced enzyme the Soret maximum is located at 436 nm and the visible transition is at 567 nm. For the oxidized enzyme the corresponding absorption bands are found at 440 nm and at 549 and 580 nm, respectively.

The spectrum of the NO-bound oxidized enzyme, in Fig. 2, was obtained by the direct addition of gaseous NO to the resting enzyme containing a ferric heme. However, as is evident in Fig. 3A, the same spectrum is obtained by the addition of excess NO to the reduced enzyme. Spectrum a in Fig. 3A is that of the resting enzyme, which is converted to the reduced ligand-free form by the addition of sodium dithionite (spectrum b). Upon exposure of the reduced enzyme to an atmosphere of NO gas, spectrum c, that of the NO-bound oxidized enzyme is obtained. Thus, NO appeared to oxidize the ferrous NOS upon binding. Removal of most of the NO by flushing with nitrogen gas regenerates the spectrum of the ligand-free oxidized enzyme with no evidence for any ligand-bound contribution (spectrum d). Rereduction of the enzyme with dithionite yields spectrum e, which is a mixture of the ligand-free reduced enzyme (Soret at ≈410 nm) and the NO-bound reduced enzyme (Soret at ≈433 nm) in spite of the fact that no additional NO was added to the sample. This indicates that NO binds more strongly to the reduced heme than to the oxidized heme. By allowing the sample to incubate for ≈1 hr, the conversion of the enzyme to the NO-bound reduced form continued (spectrum f). To determine if the intensity of the small peak at 433 nm, presumed to originate from the NO-bound reduced enzyme, could be increased, more NO was introduced to the sample

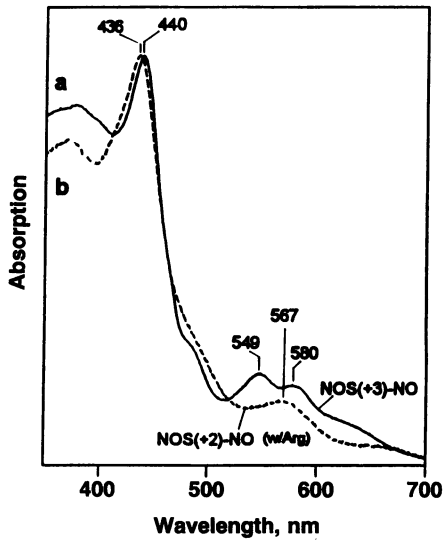

FIG. 2. Absorption spectra of NOS with NO coordinated to the heme in the ferric (solid line) and ferrous (dashed line) oxidation states. For the ferrous sample L-arginine was present.

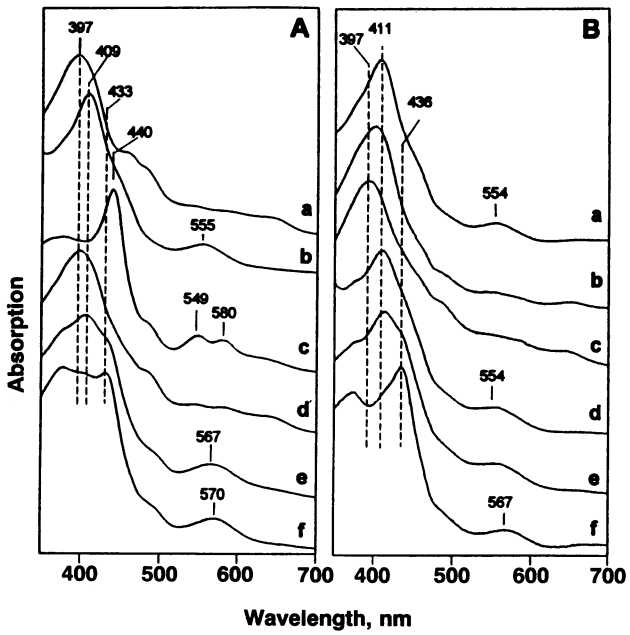

FIG. 3. Absorption spectra of NOS showing the effect of the addition of reductant, NO, and L-arginine. (A) Spectra: a, resting enzyme; b, dithionite reduced (10-fold excess); c, flush with NO gas; d, flush with nitrogen gas to remove the NO; e, reduce again with 10-fold excess dithionite; f, incubate for 40 min. (B) Spectra: a, dithionite-reduced enzyme; b, add 7-fold excess of NO; c, add 2 mM L-arginine; d, reduce again with 10-fold excess dithionite; e, add 7 equivalents of NO; f, add 8 equivalents of NO.

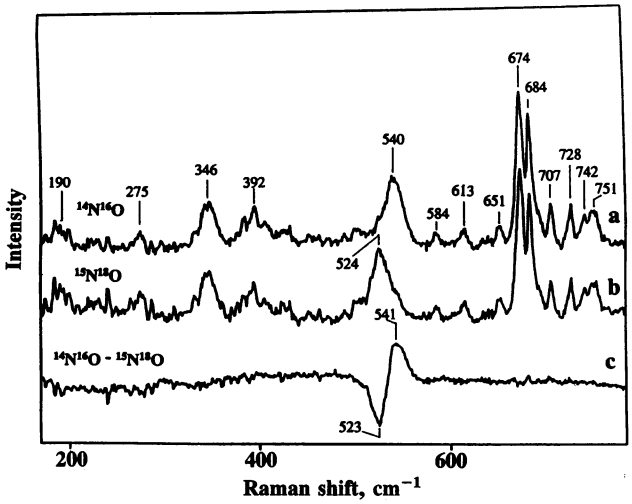

FIG. 4. Low-frequency resonance Raman spectra of NOS in which NO is coordinated to the ferric heme. Spectra: a, $^{14}N^{16}O$ adduct; b, $^{15}N^{18}O$ adduct; c, difference spectrum of spectrum a minus spectrum b.

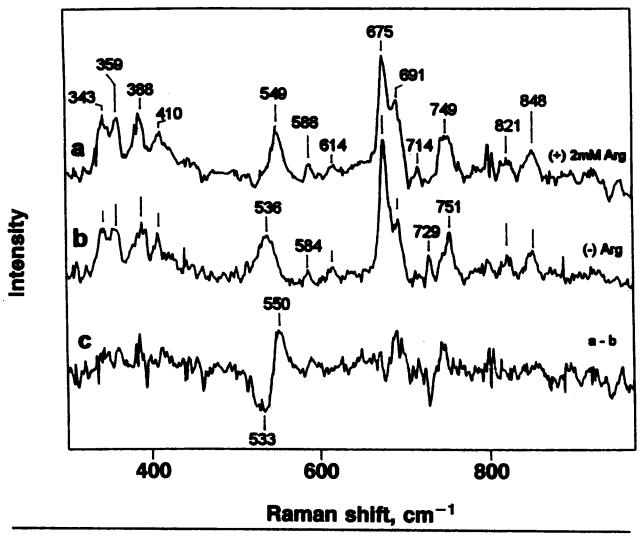

FIG. 5. Low-frequency resonance Raman spectra of NOS in which NO is bound to the ferrous heme. The spectra were obtained from samples in the presence (spectrum a) and absence (spectrum b) of L-arginine (2 mM). Spectrum c is the difference spectrum of spectrum a minus spectrum b.

(≈0.5 equivalent). However, instead of increasing the intensity of this presumed NO band, the sample became partially oxidized. The addition of more NO and rereduction with dithionite regenerated a spectrum similar to spectrum f. We found that this form of the enzyme was not very stable.

Confirmation that NO oxidizes the heme in NOS is illustrated in the spectra from a different sample shown in Fig. 3*B*. In this series, the first spectrum (a) is that of the ligand-free dithionite-reduced enzyme. Seven molar equivalents (per heme) of NO were added to the reduced sample. Instead of forming an NO-bound adduct, the enzyme progressively became oxidized as is evident in spectra b and c. L-Arginine (2 mM) was added and the sample was rereduced with dithionite, resulting in spectrum d with a shoulder in the 430- to 440-nm region, which results from NO-bound enzyme due to the presence of some residual NO in the solution. The addition of 7 more equivalents of NO increased the intensity of the shoulder (spectrum e) and the addition of 8 more equivalents yielded spectrum f. This NO–adduct formed in the presence of L-arginine was quite stable.

The resonance Raman spectra of the NO-bound form of NOS in the ferric state is shown in Fig. 4. Spectrum a is from a sample that was exposed to $^{14}N^{16}O$ and spectrum b is from one that was exposed to $^{15}N^{18}O$. The difference spectrum (spectrum c) identifies the strong broad line at 540 cm^{-1} for $^{14}N^{16}O$ as a mode that involves motion of the NO since it shifts by 16 cm^{-1} upon the isotope substitution. As summarized in Table 1, this frequency is very similar to the Fe—NO stretching mode of chloroperoxidase (30) and is somewhat higher than that of cytochrome P-450 in the absence of substrate (27, 28). In analogy to the other proteins in which thiolate is coordinated to the iron and the strongest NO isotope-sensitive line present in the spectrum is the Fe—NO stretching mode, we tentatively assign the mode at 540 cm^{-1} in NOS as the Fe—NO stretching mode. No other modes are present in the isotope difference spectrum of NOS that could be identified as a possible Fe—N—O bending mode. However, the stretching and bending modes of the Fe–NO complexes have been shown to be close in frequency and thereby have mixed character (27, 28, 30). Additional isotope measurements are needed to firmly establish the normal coordinate composition of the mode we detect.

The resonance Raman spectra of NO coordinated to the reduced enzyme in the presence and absence of L-arginine are shown in Fig. 5. Spectrum a was obtained from a sample containing L-arginine and spectrum b was obtained from a sample in the absence of L-arginine. The difference spectrum is shown in spectrum c. We assign the lines at 536 and 549 cm^{-1} in the absence and presence of L-arginine, respectively, as Fe—NO stretching modes from the reduced enzyme. As may be seen in Table 1, these frequencies are very consistent with the frequency of the Fe—NO stretching mode of other ferrous NO complexes, although, as evident in Table 1, again

Table 1. Frequencies (cm^{-1}) of the Fe—NO modes in selected heme proteins

Protein	Fe^{3+}		Fe^{2+}		Source or ref(s).
	ν_{Fe-NO}	δ_{Fe-N-O}	ν_{Fe-NO}	δ_{Fe-N-O}	
Mb	595	573		554	32
HbA	594				32
HRP	604				32
CcO			545		33
P-450 (−)	528		547	444	27, 28
P-450 (+)	520–524	542–546	545–554	441–446	27, 28
CPO	538	558	542		30
NOS (−)	540		536		This work
NOS (+)			549		This work

Mb, myoglobin; HbA, human adult hemoglobin; HRP, horseradish peroxidase; CcO, cytochrome *c* oxidase; P-450 (+ or −), cytochrome P-450 in the presence or absence of substrate; CPO, chloroperoxidase; NOS (+ or −), NOS in presence or absence of arginine. ν_{Fe-NO} and δ_{Fe-N-O} refer to the Fe—NO stretching mode and Fe—N—O bending modes, respectively.

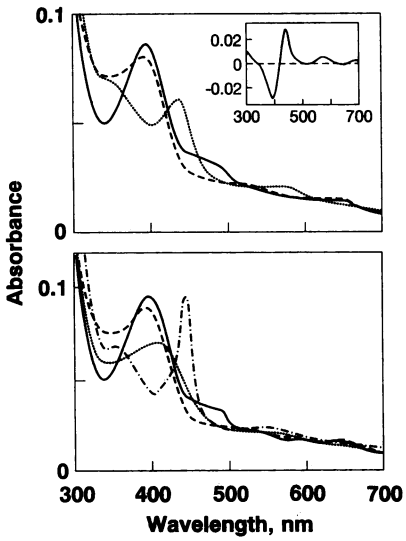

FIG. 6. Formation of the ferrous NOS–NO complex during NO synthesis. Experiments were performed in the presence (*Upper*) or absence (*Lower*) of L-arginine under oxygen-limited conditions. The individual spectra were recorded in the following sequence: 1.3 μM resting ferric NOS (———); following addition of 7 μM NADPH (- - - -); following addition of Ca^{2+} to trigger calmodulin binding and heme iron reduction (· · · ·); and following addition of CO gas (- · - · -, *Lower* only). Experiment shown is representative of two.

the assignment is complicated by the presence of the nearby bending mode.

Given that reagent NO coordinated to the iron of the ferrous and ferric NOS heme, we sought to determine if a NOS–NO complex would form under catalytic conditions—i.e., when NOS was synthesizing NO. Experiments were carried out under O_2-limited conditions in order to maximize the possible buildup of a NOS–NO complex by minimizing its reaction with dissolved O_2. As shown in Fig. 6 *Upper*, addition of excess NADPH to the calcium-free, L-arginine-bound neuronal NOS led to exclusive reduction of the NOS flavins, as indicated by the loss of absorbance 456 and 485 nm. Addition of Ca^{2+} triggered calmodulin binding and caused electrons to transfer to the NOS heme iron (34). Under the current conditions, this enabled NOS to synthesize a small amount of NO until the oxygen concentration became limiting. The resultant spectrum shows that a ferrous–NO complex had formed, with characteristic absorption bands at 436 and 537 nm (compared with Fig. 2). To estimate the amount of NO synthesis, nitrite determination on two separate reactions was carried out, showing that 2.2 nmol of nitrite had been generated from L-arginine by NOS, under the O_2-limited conditions. This gave a nitrite concentration of 2.2 μM in the cuvette. A control experiment was run in an identical manner, except that L-arginine was omitted (Fig. 6 *Lower*). In this case NOS would be expected to generate superoxide and H_2O_2 instead of NO in response to Ca^{2+}-triggered calmodulin binding (35, 36) and would stabilize simply as deoxy-ferrous NOS when the oxygen concentration became limiting. As shown in Fig. 6 *Lower*, calmodulin-triggered heme reduction in the absence of L-arginine resulted in the buildup of a distinct species that was identified to be deoxy-ferrous NOS based on its Soret absorbance at 412 nm (11) and its absorbance at 444 nm following CO binding (9, 34).

DISCUSSION

The optical absorption and the resonance Raman spectra of NOS in the presence of NO clearly demonstrate that NO can bind directly to the heme iron of NOS. Several specific features of the interactions of NO with the heme iron in NOS give additional insights into the complexity of these interactions (Fig. 7). (*i*) NO binds to the heme of the reduced form of NOS more strongly than it does to the oxidized form of the enzyme. However, in the reduced form the complex is unstable, resulting in oxidation of the heme. (*ii*) In the presence of L-arginine the coordination of NO to the heme of the reduced enzyme is stabilized. (*iii*) The presence of L-arginine influences the Fe—NO stretching mode in the reduced complex, causing frequency shifts in the Fe—NO stretching mode such as those observed in CO-bound, O_2-bound, and NO-bound cytochrome P-450s upon substrate addition (24, 27–29).

The occurrence of different Fe—NO stretching frequencies in the reduced form of the NO-bound enzyme, depending on the presence or absence of L-arginine, is very similar to the behavior observed in cytochrome P-450s (27, 28). In that case the frequency of the Fe—NO stretching mode is dependent on the specific substrate that is bound to the complex. In general, the presence of substrate causes an increase in the Fe—NO stretching frequency of the NO-bound ferrous heme. This sensitivity is a consequence of the direct interaction between the substrate and the bound ligand through either hydrogen bonding, polar forces, or steric forces (37). In the substrate-bound NOS reported here the increase in the frequency of the Fe—NO stretching mode indicates that the substrate (L-arginine) binds directly over the NO-bound heme group so as to interact directly with the NO, as illustrated schematically in Fig. 7. Thus, in the catalytic process when O_2 is bound to the heme, L-arginine is located over the iron-bound oxygen to sterically specify the hydroxylation event. This is the same structure/function motif that occurs in cytochrome P-450-type enzymes, which also carry out oxygenation reactions (38). Additional studies with substrate analogs should yield further information concerning the specific orientation of the substrate in the catalytic pocket.

The binding of L-arginine over the Fe-coordinated NO allows for interpretation of the reduced enzyme's differential susceptibility to NO-mediated oxidation. Two different factors could play a role in this L-arginine-induced difference. First, in other enzymes such as cytochrome *c* oxidase, it has been shown that binding of two molecules of NO in the heme pocket leads to oxidation of the heme as well as the nearby copper atom (33). This process involves the uptake of two protons and the generation of N_2O and H_2O. Such a process could also occur in NOS: in this case one electron could come from the heme and another could come from distinct redox centers such as H_4BPT. In this scenario, the lack of oxidation in the presence of L-arginine could be explained by a steric effect that allows only one NO molecule to fit within the heme pocket when L-arginine is present, thereby preventing the two-molecule oxidation of the heme iron. A second factor that could influence the oxidation of the heme involves a redox potential modification by L-arginine. Bound L-arginine

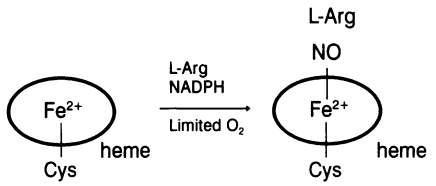

FIG. 7. Model for the formation of a NOS–NO complex during catalysis. Addition of L-arginine (L-Arg) and excess NADPH to ferric NOS under O_2-limited conditions led to a small amount of NO synthesis and resulted in the buildup of a substrate-bound, ferrous NOS–NO complex.

might stabilize the ferrous NO species by increasing the reduction potential of the NOS heme iron, thus preventing its oxidation by bound NO. Our related work with NOS has shown that bound L-arginine does stimulate the buildup of electron density on the heme iron during electron titration experiments (39), consistent with substrate binding functioning in NOS to increase the reduction potential of the heme iron as occurs in cytochrome P-450s (40).

The central issue of whether NOS–NO complexes can form during NO synthesis has now been settled by our results. A ferrous NOS–NO complex was generated when NOS initiated catalysis under O_2-limited conditions. Thus, enzyme-generated NO was able to coordinate to the NOS heme iron in an identical manner as that for reagent NO. Because the solution concentration of enzymatically generated NO was very low, it is likely that NOS bound NO that was generated at or near its own active site.

Although we detected a ferrous NOS–NO complex under our conditions, its possible role as an obligate catalytic intermediate is an open question. However, the fact that an NOS–NO complex did form during NO synthesis strongly suggests that NO binding to the heme iron may contribute to the feedback inhibition of NOS observed in several settings (16–19) and thereby offers a structural basis for the inhibition. The molecular mechanism of the inhibitory process appears to be complex inasmuch as our results demonstrate that NO coordinates to the heme iron in both oxidation states and is sensitive to the presence of L-arginine. Furthermore, it has been shown that the inhibitory process is also sensitive to several other factors, including the presence of oxidants or reductants and the presence of H_4BPT (19). Additional studies of the properties of NO coordination to the ferric and ferrous hemes of NOS regarding the influence of the dissolved oxygen concentration are necessary to formulate a more complete mechanism for the interaction of NO with NOS under physiological conditions.

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