Neuronal Nitric Oxide Synthase Self-inactivates by Forming a Ferrous-Nitrosyl Complex during Aerobic Catalysis*

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Neuronal NO synthase (NOS) is a flavin-containing heme protein that generates NO from L-arginine, NADPH, and O2. NO has recently been proposed to autoinhibit NOS. We have investigated whether a NOS heme-NO complex forms during aerobic steady-state catalysis. Visible and resonance Raman spectra recorded during steady-state NO synthesis by NOS showed that the majority of enzyme (70–90%) was present as its ferrous-nitrosyl complex. Ferrous-nitrosyl NOS formed only in the coincident presence of NADPH, L-arginine, and O2. Its level remained constant during NO synthesis until the NADPH was exhausted, after which the complex decayed to regenerate ferrous resting NOS.Stopped-flow measurements revealed that the buildup of the ferrous-NO complex was rapid (< 2 s) and caused a 10-fold decrease in the rate of NADPH consumption by NOS. Complex formation and decay could occur several times with no adverse effect on its subsequent formation or on NOS catalytic activity. Neither enzyme dilution nor NO scavengers (superoxide and oxyhemoglobin) diminished formation of ferrous-nitrosyl NOS or prevented the catalytic inhibition attributed to its formation. The ferrous-nitrosyl complex also formed in unfractionated cell cytosol containing neuronal NOS upon initiating NO synthesis. We conclude that a majority of neuronal NOS is converted quickly to a catalytically inactive ferrous-nitrosyl complex during NO synthesis independent of the external NO concentration. Thus, NO binding to the NOS heme may be a fundamental feature of catalysis and functions to down-regulate NO synthesis by neuronal NOS.

Nitric oxide (NO)1 is a widespread mediator of physiological and pathophysiological processes (1–5). NO is synthesized by a family of enzymes termed NO synthases (NOSs). These enzymes are homodimers that catalyze the stepwise oxidation of L-arginine to NO plus L-citrulline (3, 4, 6, 7). The NOSs contain FAD, FMN, H4biopterin, and iron protoporphyrin IX (heme) prosthetic groups. The flavins facilitate transfer of electrons from NADPH to the heme iron (8). In neuronal NOS, the flavin-to-heme electron transfer is triggered by calmodulin (CaM) binding (9, 10), which occurs in response to elevated Ca2+ concentration (3). The NOS heme iron plays a central role by binding and activating O2 at the catalytic site. This ultimately leads to oxygen incorporation into both products of the enzyme reaction (4, 7). Indeed, NO synthesis from either L-arginine or Nω-hydroxy-L-arginine is inhibited by CO, and spectral evidence demonstrates that CO inhibits the reaction by coordinating to the heme iron (11–14). In addition to binding O2 or CO, the heme iron of neuronal NOS can bind NO as a sixth ligand in both the ferrous and ferric states under an anaerobic atmosphere to generate stable NOS heme iron-NO complexes (15).

Characterization of the heme iron in neuronal NOS has recently been accomplished by employing techniques such as optical absorption (9–13), electron paramagnetic resonance (12), and resonance Raman spectroscopy (14, 15). The heme iron is axially coordinated to the protein via a cysteine thiolate, as is the case for cytochrome P450s, and is predominantly five-coordinate and high spin in ferric NOS. Substrate appears to bind directly above the heme group and can interact with ligands bound to the heme iron, such as NO or CO (14, 15). During catalysis of NO synthesis, such positioning may sterically specify substrate hydroxylation events that are catalyzed by the heme iron.

The binding of NO to neuronal NOS is particularly important in view of reports that NO can inhibit NOS activity (16–21). Studies with neuronal NOS purified from rat cerebellum indicated that NO inhibits by interacting directly with enzyme rather than with any of the soluble cofactors or substrates (19). These studies also showed that NO generated by NOS caused an inhibition of catalysis (16, 19), suggesting that NO may act as a negative feedback modulator of NOS, possibly through its interaction with enzyme-bound heme. This view was recently substantiated by optical absorption and resonance Raman spectroscopic evidence that showed neuronal NOS ends up as its ferrous heme-NO complex following NO synthesis under oxygen-limited conditions (15). However, these studies did not address whether NO binds to neuronal NOS during normal aerobic catalysis and whether such a reaction explains the catalytic inhibition attributed to NO.

Thus, our current objective was to determine whether L-arginine-derived NO would complex with the heme iron of neuronal NOS.
neuronal NOS during enzyme catalysis under aerobic, steady-state conditions. Experiments were also conducted to determine if formation of a heme-iron-NO complex was related to inhibition of NO synthesis, including stopped-flow analysis to examine the kinetic relationship between nitrosyl complex formation and inhibition of neuronal NOS by NO. Our results indicate that NO binding to the heme iron is rapid, quantitatively significant, and an integral part of the enzyme’s normal catalytic processing and functions to regulate NO synthesis by neuronal NOS.

EXPERIMENTAL PROCEDURES

Reagents—Human R293 kidney cells expressing cloned rat brain neuronal NOS were a kind gift of Drs. Solomon Snyder and David S. Bredt, Johns Hopkins School of Medicine, Baltimore, MD. Superoxide dismutase was obtained from Calbiochem and was of the ferric-manganese type. All other reagents and materials were obtained from Sigma or from sources reported previously (8, 10, 15).

Preparation of NOS—Rat neuronal NOS was purified from cultures of stably transfected kidney R293 cells by a two-column procedure as described previously (12). Protein was assayed with the Bio-Rad kit, using bovine serum albumin as a standard. Neuronal NOS concentrations were also determined spectrophotometrically at 398 nm using an estimated extinction coefficient of 72 mM$^{-1}$ cm$^{-1}$ and assuming that one heme was bound per subunit (12).

Measurement of NO Synthesis, NADPH Oxidation, L-Arginine Consumption, and Citrulline Production—The specific rate of NO synthesis by various NOS preparations was determined at 15°C using the oxyhemoglobin assay, as described previously (12, 22). This method quantitates the NO-mediated conversion of oxyhemoglobin (5–10 μM) to methemoglobin at 401 nm, using a methemoglobin minus oxyhemoglobin extinction coefficient of 38 μM$^{-1}$ cm$^{-1}$. The rate of NADPH oxidation at 15°C was measured in cuvettes as an absorbance decrease at 340 nm, using an extinction coefficient of 6.22 μM$^{-1}$ cm$^{-1}$. To measure the effect of 20 μM oxyhemoglobin on the specific rate of NADPH oxidation during NO synthesis by NOS over a range of NOS concentrations, the reactions were carried out at 27°C in triplicate in a 96-well microplate. The rate of NADPH oxidation in each 100-μl reaction was recorded at 340 nm using a Thermomax kinetic plate reader. To measure L-arginine consumption and citrulline production by neuronal NOS, 10-μl aliquots were removed from the enzyme reaction at various time points and quenched by mixing with diluted HCl. Arginine and citrulline in the samples were derivatized by reaction with orthophthalaldehyde, separated, detected using a fluorometric HPLC instrument manufacturer, and quantitated relative to amino acid standards, as described previously (23).

Optical Spectroscopy—Spectra of NOS prior to, during, and following catalysis were recorded at 15°C using a Hitachi U3010 spectrophotometer equipped with a thermostatted cuvette holder. Wavelength scans were recorded at a rate of 120 nm/min.

Resonance Raman Spectroscopy—Measurements were made on 140-μl reaction solutions sealed in a rotating Raman cell. The laser extinction wavelength used was 441.6 nm at a power of ~3 milliwatts. The scattered light was dispersed with a 1.25-μm monochromator and detected with a charged-coupled device camera. The frequencies of the Raman-shifted lines were calibrated against an indene standard. The spectra were the average of 50 scans each with a 30-s integration time.

Spectral Measurement of Ferrous-Nitrosyl Complex Formation—Reactions were carried out in cuvettes at 15°C in a final volume of 0.7 or 1 ml. Reactions were monitored either by wavelength scanning as described above or by monitoring the change in absorbance at 436 and 340 nm over time. For the standard reaction, NOS was diluted to ~1 μM in air-saturated 40 mM Bis-Tris buffer, pH 7.4, containing 0.9 mM EDTA, 1.1 mM Ca$^{2+}$, 3 μM Ca$^{2+}$, 4 μM H$_2$biopterin, 200 μM DTT, and 1 mM L-arginine. NADPH was added to initiate NO synthesis as noted in the text. In some cases, Ca$^{2+}$ was added separately to initiate NO synthesis. Inhibition of NO synthesis after NADPH had been added, or from sources reported previously (8, 10, 15).

Estimation of Ferrous-Nitrosyl NOS Formed during Steady-state Catalysis—The percentage of neuronal NOS that formed its ferrous-nitrosyl complex during reactions in which absorbance at 436 nm was monitored was estimated as follows. Prior to initiating the reaction, the concentration of NOS in the cuvette was measured by determining the absorbance difference between 395 and 700 nm and dividing this value by the extinction coefficient for ferric NOS at 398 nm (72,000; Ref. 12). After the reaction was initiated, the average absorbance value at 436 nm achieved during the steady state was noted. The difference between this value and the absorbance value obtained previously at 700 nm for ferric NOS was divided by the estimated extinction coefficient for ferrous-nitrosyl NOS at 436 nm (49,800; see text), giving an estimated steady-state concentration for ferrous-nitrosyl NOS in the experiment. The percentage of NOS present as its ferrous-nitrosyl complex during the steady state was then determined by dividing the estimated concentration of ferrous-nitrosyl NOS by the concentration of total NOS in the experiment.

RESULTS

Formation of a Neuronal NOS-NO Complex during Steady-state Catalysis—Our initial experiments utilized light absorbance spectroscopy to examine if a significant amount of neuronal NOS would form an iron-nitrosyl complex during catalysis of NO synthesis from L-arginine or during catalysis. Enzyme reactions reported here were run at 15°C$^2$ in 1-ml cuvettes in air-saturated buffer, under conditions in which NADPH was the limiting substrate and would be oxidized completely by NOS after several minutes of reaction. The upper panel of Fig. 1 contains results of a typical experiment and shows spectra that were recorded before and after initiating NO synthesis. The solid line is the spectrum of resting NOS prior to NADPH addition. The prominent Soret absorbance centered at 398 and flavin absorbance bands at 456 and 485 nm indicate that the heme iron and flavin redox centers are in their air-stable, oxidized states (9, 12). The dashed line is the spectrum recorded after

2 Reactions were run routinely at 15°C to prolong NOS catalysis at the high enzyme concentrations required to obtain spectra.

3 Based on a stoichiometry of 1.5 NADPH oxidized and NO formed from L-arginine (24), and an estimated dissolved O$_2$ concentration of ~300 μM at 15°C.
Autoinactivation of Neuronal NO Synthase

Fig. 1. Formation of a ferrous-NO complex during steady-state NO synthesis by neuronal NOS. The upper panel contains spectral scans done on a 1-ml air-saturated solution containing 40 mM Bis-Tris buffer, pH 7.4, 3 μM CaM, 0.9 mM EDTA, 4 μM H₄biopterin, 200 μM DTT, and 1 μM NOS at 15°C. The solid line is resting enzyme. The dashed line was recorded after adding 24 nmol of NADPH. The dotted line was recorded after initiating NO synthesis by adding 1 μM Ca²⁺. The dashed and dotted line was recorded after NO synthesis had stopped due to NADPH depletion by NOS. The experiment shown is representative of five. The inset contains the spectrum of NOS prior to (solid line) and during (dotted line) catalysis of NADPH oxidation in the absence of L-arginine. The reaction was started by the addition of 120 μM NADPH, and the scan was completed when the NADPH concentration had reached 25 μM. Conditions were otherwise identical to those described for the upper panel. The lower panel depicts spectral scans of an anaerobic solution containing 40 mM Bis-Tris buffer, pH 7.4, 4 μM H₄biopterin, 200 μM DTT, and 1.3 μM NOS at 15°C. The solid line is resting NOS. The dashed line was recorded after adding excess dithionite to reduce the NOS flavins and heme iron. The dotted line was recorded after the addition of NO gas to form the ferrous-nitrosyl complex of NOS. The experiment shown is representative of five.

Adding NADPH, which caused reduction of the NOS flavins, as judged by the loss of visible absorbance at 456 and 485 nm (9, 10). Note that the ferric heme absorbance was maintained at 398 nm. The dotted line is the spectrum recorded after adding Ca²⁺ to promote CaM binding to NOS, which is known to trigger heme iron reduction (9) and NO synthesis from L-arginine (3). Thus, this spectrum depicts neuronal NOS during steady-state catalysis of NO synthesis. The previously observed Soret band at 398 nm is absent, and instead absorbance peaks at 436 and ~590 nm are evident. These spectral features are identical to those of authentic ferrous-nitrosyl neuronal NOS (15). The dashed and dotted line is a spectrum that was recorded several minutes after NO production had ceased due to NADPH depletion. It is identical to the spectrum we obtained for NOS in its original ferric, oxidized state. Together, these results suggest that a significant amount of neuronal NOS was converted to its ferrous-nitrosyl complex during aerobic NO synthesis under these conditions.

The inset of the upper panel of Fig. 1 contains a spectrum of ferric resting NOS and a spectrum of the same sample undergoing steady-state catalysis of NADPH oxidation in the absence of L-arginine. In this circumstance, the NOS heme iron-catalyzed oxygen reduction still occurs but results in superoxide formation instead of NO synthesis (25). In this case, no ferrous-nitrosyl complex was observed. Instead, the spectrum is similar to that obtained previously for neuronal NOS in its reduced ferrous-deoxy state, which displays a lack of flavin absorbance and a Soret absorbance of lower extinction centered at 412 nm (8, 13–15). This indicates that formation of the ferrous-nitrosyl complex was dependent on L-arginine conversion to NO.

To quantitate the amount of ferrous-nitrosyl NOS that formed during steady-state NO synthesis, we derived an estimated extinction coefficient for ferrous-nitrosyl NOS standards that were generated as depicted in the lower panel of Fig. 1. The solid line is the spectrum of resting neuronal NOS under an anaerobic atmosphere of nitrogen. The dashed line is the spectrum recorded after adding excess dithionite, which completely reduced the NOS flavins and heme iron (13, 14). The dotted line is the spectrum that was recorded after adding NO gas to form the ferrous-nitrosyl complex of neuronal NOS. Given that the estimated extinction coefficient for ferric neuronal NOS at 398 nm is 72,000 M⁻¹ cm⁻¹ (12), we calculate an estimated extinction at 436 nm for ferrous-nitrosyl NOS of 48,200 ± 800 (n = 4). The ratio of the estimated extinction coefficients for ferric NOS and ferrous-nitrosyl NOS (1.49) is similar to that obtained for other thiolate-coordinated hemeproteins such as cytochrome P450cam (26) and cytochrome P450nor (27). Using our estimated extinction coefficient for ferrous-nitrosyl NOS, we calculate that ~90% of neuronal NOS was in its ferrous-nitrosyl form during steady-state NO synthesis in the experiment depicted in the upper panel of Fig. 1.

A replica experiment was done to characterize independently the NOS-N0 complex formed during steady-state NO synthesis in a resonance Raman cell. As depicted in Fig. 2, the resonance Raman spectrum of neuronal NOS during NO synthesis displayed a ferrous-nitrosyl stretching mode at 549 cm⁻¹. The frequencies and relative intensities of the porphyrin modes along with this ferrous-nitrosyl stretching mode are identical to those in the spectrum of ferrous-nitrosyl NOS generated with reagent NO in the presence of L-arginine (15) but differ considerably from those of ferric-nitrosyl NOS (15). These spectral features were not observed if NADPH and O₂ were omitted from the reaction. Thus, the resonance Raman data confirm independently that neuronal NOS generates its ferrous-nitrosyl complex during aerobic NO synthesis.

Influence of Ferrous-Nitrosyl Complex Formation on Neuronal NOS Catalytic Functions—Next we examined how ferrous-nitrosyl complex formation was related to NADPH oxidation and citrulline formation by NOS during steady-state catalysis. Experiments were run in cuvettes at 15°C, with NADPH (24 nmol) acting as the limiting substrate. Ferrous-nitrosyl complex formation was monitored as the gain in absorbance at 436 nm; NADPH oxidation was monitored by the loss of absorbance at 340 nm. A representative experiment is shown in the upper panel of Fig. 3. Initiation of NO synthesis was associated with commencement of NADPH oxidation and a rapid increase in absorbance at 436 nm. The absorbance at 436 nm was maintained at a relatively constant level for ~4 min, during which time a linear rate of NADPH oxidation was observed. When the NADPH was depleted, the 436 nm signal decayed to baseline within ~1 min. The dotted line in the upper panel is from an identical reaction that was run in the absence of L-arginine. In
this case, no gain in absorbance at 436 nm was observed, indicating that the 436 nm signal is dependent on L-arginine, and thus related to NO synthesis. From the absorbance value at 436 nm obtained during the steady state and our estimated extinction coefficient for ferrous-nitrosyl NOS, we estimate that approximately 76% of NOS was in its ferrous-nitrosyl form during steady-state catalysis in this particular experiment.

The bottom panel of Fig. 3 depicts the loss of L-arginine and formation of citrulline which occurred in a replica experiment. Conversion of L-arginine to citrulline was linear, consistent with the observed linear rate of NADPH oxidation, and ceased when NADPH was oxidized completely. The amount of NADPH added to the cuvette (24 nmol) enabled the conversion of ~8 nmol of L-arginine to 8 nmol of citrulline. Thus, approximately 3 NADPH were oxidized per mol of citrulline formed in this experiment, which is twice the minimum amount of NADPH required for neuronal NOS to generate 1 mol of citrulline from L-arginine (24). Together the data show that a constant degree of ferrous-nitrosyl complex formation occurs during steady-state turnover and is associated with a constant rate of NADPH oxidation and citrulline production by NOS.

Stopped-flow Analysis of Ferrous-Nitrosyl Complex Formation and Decay, and Neuronal NOS NADPH Oxidation—We utilized stopped-flow spectroscopy to examine the kinetics of complex buildup and decay and its effect on NOS NADPH oxidation. The upper panel of Fig. 4 shows the time course of ferrous-nitrosyl complex formation and NADPH oxidation at 15 °C (detected by monitoring the absorbance change at 436 and 340 nm, respectively) after rapid mixing a solution of NADPH with a solution containing CaM-bound NOS plus L-arginine, H4biopterin, and DTT. NADPH oxidation in a replica experiment that omitted L-arginine is also depicted as a dotted line and represents NADPH oxidation by NOS in the absence of NO synthesis. Formation of the ferrous-nitrosyl complex was associated with an approximate 10-fold decrease in the steady-state rate of NADPH oxidation relative to that observed in the substrate-free reaction. After NADPH was depleted the ferrous-nitrosyl signal decayed over a 40-s period. This decrease in 436 nm absorbance was best fit to a single exponential equation, giving an observed rate constant of 0.06 s⁻¹ for complex decay under the experimental conditions.

During the preequilibrium phase of the reaction, one can see that the rate of NADPH oxidation appeared to be deflected to a slower value as buildup of the ferrous-nitrosyl complex neared its steady-state value. This phase of the reaction is expanded in the lower panel of Fig. 4, which shows the absorbance changes at 436 and 340 nm that occur over the first 5 s of the reaction. The 340 nm absorbance decreased at a rate of 0.01 s⁻¹ for the first 1.4 s of the reaction, then slowed to a rate of 0.001 s⁻¹ for the duration of the reaction. This 10-fold decrease in rate took place when formation of the ferrous-nitrosyl complex neared completion, as determined by the gain in 436 nm absorbance over time. Together the kinetic data suggest that a buildup of the ferrous-nitrosyl complex causes inhibition of electron flux through neuronal NOS.

The lower panel inset of Fig. 4 shows the change in absorbance at 436 nm that takes place during the first 0.4 s of the reaction. The initial decrease in 436 nm absorbance was best fit to a single exponential equation, giving an apparent rate con-
 aliquots removed from the cuvette after each round of complex formation and decay could occur and if it would affect catalysis of citrulline production by neuronal NOS. We therefore examined if a superoxide-generating system or oxyhemoglobin would prevent the inhibition of catalysis attributed to ferrous-nitrosyl complex formation in our system.

Fig. 6 shows the effect of a xanthine oxidase/hypoxanthine superoxide-generating system on ferrous-nitrosyl NOS formation during NO synthesis. In panel A both NADPH (24 nmol) and xanthine oxidase were added to the reaction at time zero to initiate concurrent NO synthesis by NOS and superoxide production by xanthine oxidase. The conditions were such that the measured rate of superoxide production by xanthine oxidase in the reaction (30 μM/min) was approximately three times the rate of NO synthesis (9 μM/min) by NOS. Panel B is the control reaction, which received only NADPH at time zero. Cuvette buffers contained 1 mM hypoxanthine in both cases. The results show that the presence of a superoxide generator did not affect the amount of ferrous-nitrosyl complex formed nor the time required to complete the reaction.

Fig. 7 depicts the reaction time courses for identical NOS reactions that were carried out either in the presence or absence of excess oxyhemoglobin (25 μM; 10 nmol). The time course in the absence of oxyhemoglobin was followed by monitoring ferrous-nitrosyl complex formation and decay at 436 nm and is depicted as the solid line in the figure. Because it was not possible to observe directly the formation of a ferrous-nitrosyl complex at 436 nm in a reaction containing 25 μM oxyhemoglobin, we instead monitored the absorbance increase at 401 nm (dashed line in Fig. 7) which reflects the NO-mediated conversion of oxyhemoglobin to methemoglobin and can be used to quantitate both the duration and quantity of NO synthesis (22).

The linear increase in absorbance at 401 nm indicates that formation and decay and showed that identical amounts of citrulline were generated for each NADPH addition (12 ± 1 nmol). The data show that formation of the ferrous-nitrosyl complex can occur multiple times without affecting subsequent complex formation or catalysis by NOS.

Effect of NOS Scavengers—Superoxide and oxyhemoglobin react with NO at near diffusion controlled rates (28, 29) and have been used to scavenge NO or protect neuronal NOS from inhibition attributed to NO synthesis (19). We therefore examined if superoxide-generating system or oxyhemoglobin would prevent the inhibition of catalysis attributed to ferrous-nitrosyl complex formation or catalysis by NOS.

5 At this concentration, oxyhemoglobin and methemoglobin absorb strongly at 436 nm and obscure any absorbance change due to ferrous-nitrosyl NOS formation.
Oxyhemoglobin was scavenging NO as it was released into solution by neuronal NOS. A total of 3 nmol of NO was scavenged in the reaction. In spite of this, the times required to complete the reactions were approximately identical (1.8 min) in the presence or absence of oxyhemoglobin. This indicates that oxyhemoglobin scavenging of NO did not prevent the inhibition of catalysis attributed to ferrous-nitrosyl complex formation.

**Relations among NOS Concentration, Ferrous-Nitrosyl Complex Formation, and Catalytic Activity**

Because the above experiments suggested that ferrous-nitrosyl complex formation may occur independently of the solution NO concentration, we carried out experiments to determine whether the degree of complex formation or catalytic inhibition would be influenced by the concentration of neuronal NOS. Fig. 8 shows the kinetics of complex formation and decay observed for reactions that were given an identical amount of NADPH (24 nmol) but contained 1, 0.5, or 0.25 mM NOS. In all three cases there was rapid formation of the ferrous-nitrosyl complex followed by maintenance of a steady-state level and then decay. As enzyme concentrations were decreased, we observed roughly proportional decreases in the amplitude of the steady-state ferrous-nitrosyl signal, which went hand in hand with proportional increases in the duration of the reaction as determined by the time elapsed during steady state (3, 7, and 16 min). As shown in the inset, the rates of citrulline production also decreased approximately in proportion to enzyme concentration, giving 4.2, 1.8, and 0.7 nmol of citrulline/min, respectively. The average stoichiometry for the three reactions was 1.8 ± 0.2 mol of NADPH consumed per mol of citrulline formed. Together, the data show that neither the degree of ferrous-nitrosyl complex formation nor the rates of enzyme catalysis were affected by enzyme dilution within the tested concentration range.

To examine if NO-mediated catalytic inhibition would be maintained at enzyme concentrations below which direct observation of the ferrous-nitrosyl complex is possible, we compared the specific rates of NADPH oxidation during NO synthesis in reactions containing 10–200 nM neuronal NOS in the presence or absence of 20 μM oxyhemoglobin. The specific rates of NOS NADPH oxidation remained constant over the full range of dilution, and added oxyhemoglobin did not increase measurably the rate NOS NADPH oxidation in any case (data not shown). This suggests that catalytic inhibition attributed to ferrous-nitrosyl complex formation remains constant even at

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6 The 436 nm signal could not be followed reliably at NOS concentrations lower than 0.25 μM.
relatively low NOS concentrations and remains resistant to oxyhemoglobin scavenging.

**Buildup of Ferrous-Nitrosyl NOS during NO Synthesis by Cell Supernatants**—Because all of the data thus far were obtained using purified neuronal NOS, we examined if the ferrous-nitrosyl complex would also form in unfraccionated cell supernatants that normally serve as starting material to purify neuronal NOS used in these studies. In the experiment shown in Fig. 9, the cell supernatant underwent a minimal dilution such that the final concentration of NOS was estimated to be 0.4 μM, as determined by an assay that measures nitrite accumulation in cell supernatants engaged in NO synthesis (12). After the addition of a limiting amount (24 nmol) of NADPH to start NO synthesis, there was an increase in 436 nm absorbance whose duration corresponded with a linear rate of NADPH oxidation by the cell supernatant, depicted as a decrease in 340 nm absorbance. The increased 436 nm absorbance was maintained for 3.2 min and then decayed upon depletion of NADPH. As shown in the Fig. 9, an increase in 436 nm absorbance was not observed in a replica reaction that contained excess EDTA to block CaM binding to NOS and thus prevent initiation of NO synthesis when NADPH was added. Based on the absorbance increase at 436 nm which was obtained using purified neuronal NOS, we estimate that only 2 NO had been generated per heme by the time maximal complex formation occurred. Because significant complex formation can be seen even at earlier time points, it seems likely that NO generated during aerobic catalysis was surprisingly high, in that from 70 to 90% of the total enzyme was estimated to be in ferrous-nitrosyl form during the steady state under all conditions examined. These findings extend our previous work that showed that neuronal NOS is converted to its ferrous-nitrosyl complex after generating NO under oxygen-limited conditions (15).

**Discussion**

**NO Binding to NOS**

Our data and other studies establish that neuronal NOS reacts with self-generated NO during aerobic catalysis to form its six-coordinate ferrous-nitrosyl complex. In contrast, based on the position of the Soret and visible transition in the optical spectrum and the iron-NO stretching mode in the resonance Raman spectrum, there was no evidence for buildup of a ferric-nitrosyl species during catalysis. The degree of ferrous-nitrosyl complex formation during aerobic catalysis was surprisingly high, in that from 70 to 90% of the total enzyme was estimated to be in ferrous-nitrosyl form during the steady state under all conditions examined. These findings extend our previous work that showed that neuronal NOS is converted to its ferrous-nitrosyl complex after generating NO under oxygen-limited conditions (15).

**Nature of the Complex**

Stopped-flow analysis of the initial phase of NO synthesis revealed that buildup of the ferrous-nitrosyl species was rapid and was accompanied by an approximately 10-fold decrease in electron flux through neuronal NOS, as indicated by a deflection in the rate of NADPH oxidation. This catalytic inhibition remained constant during the steady state and was similar in magnitude (−90%) to the proportion of enzyme that was estimated to be in ferrous-nitrosyl form during the steady state (70–90%). Nitrosyl complex formation and the accompanying inhibition of NADPH oxidation did not occur under conditions where NOS began oxidizing NADPH without generating NO. The 10-fold decrease in NADPH oxidation rate was also unrelated to ω-arginine binding (9) because the NOS solution used here contained 1 mM ω-arginine prior to its being rapidly mixed with NADPH. Thus, a majority of NOS quickly generates its ferrous-nitrosyl complex upon initiating NO synthesis. This species appears to be catalytically inactive with regard to NADPH oxidation and, through stochastic considerations (24), to NO synthesis as well.

We attempted to reduce the catalytic inhibition attributed to ferrous-nitrosyl complex formation by adding the NO scavenger oxyhemoglobin or superoxide. These scavengers react with NO at near diffusion-controlled rates (28, 29) and are presumed to have significantly lowered the solution concentration of NO in the enzyme reactions. However, neither oxyhemoglobin nor a superoxide-generating system increased NOS catalytic activity in our system, as determined by comparing NO or citrulline formation, NADPH oxidation, or the lifetime of the ferrous-nitrosyl complex. In reactions that contained the superoxide generator we observed no decrease in the degree of ferrous-nitrosyl complex formation during the steady state. Thus, failure of the scavengers to increase neuronal NOS activity appeared to be linked to their inability to decrease ferrous-nitrosyl complex formation. This suggests that NO formed within the active site of neuronal NOS may have an opportunity to bind to the ferrous heme iron before it equilibrates with the solution as a whole. An examination of the time required for complex buildup using stopped-flow supports this contention (Fig. 4). Based on the final NOS concentration (1 μM) and the reported stoichiometry of NO synthesis (1.5 NADPH oxidized/NO formed; Ref. 24) and the amount of NADPH utilized during the time required for complex to build up to the steady-state level (3.5 μM), we estimate that only 2 NO had been generated per heme by the time maximal complex formation and the 10-fold deflection in rate of NADPH oxidation were observed. Because significant complex formation can be seen even at earlier time points, it seems likely that NO generated during catalysis can bind to the NOS heme iron before it equilibrates in solution. This suggests that NO remains in or near the distal heme pocket rather than diffusing out of the protein and may bind to the heme in a germinale fashion, similar to when NO rebinds to heme proteins following photodissociation (34–36). The NO that does escape from the heme pocket can diffuse a considerable distance from the point source in solution (37). However, our data indicate that this free NO is not involved in forming the ferrous-nitrosyl complex during steady-state catalysis.
Because formation of ferrous-nitrosyl NOS was faster than its apparent rate of decay in our system, this likely contributed to the buildup of the complex during steady-state NO synthesis. The ferrous-nitrosyl complex of neuronal NOS is an inherently stable species in the presence of l-arginine under anaerobic conditions (15). This suggests that its decay involves a reaction with dissolved O_2. After NO synthesis had ceased, decay of the ferrous-nitrosyl complex regenerated ferric NOS, which maintained its native spectral and catalytic properties even after repeated rounds of complex formation and decay. This indicates that NOS can undergo reversible complex formation with NO during catalysis. NOS is therefore similar to certain cytochrome P450s that generate stable ferrous-nitrosyl complexes (27, 30) and is dissimilar to others whose ferrous nitrosyl complexes rapidly form catalytically inactive cytochrome P420 (26). The breakdown of ferrous-nitrosyl NOS observed in our study was considerably faster than the ferrous nitrosyl complexes of hemoglobin and myoglobin under similar conditions. However, it was similar to the time required for cytochrome P450-NO complexes to regain their full catalytic activity in aerobic solution (31). This suggests that neuronal NOS and some cytochrome P450s share catalytic or structural features that promote aerobic decay of their ferrous-nitrosyl complexes.

Relation between Ferrous-Nitrosyl Complex Formation and Catalysis

A working model for neuronal NOS catalysis which incorporates our current findings is presented in Scheme I. CaM-bound NOS can undergo concurrent NO synthesis and ferrous-nitrosyl complex formation during steady-state catalysis (paths A and B) or can reduce O_2 to form superoxide in the absence of substrate (25) (path C). Upon addition of NADPH, the NOS flavins are reduced and can transfer an electron to the ferric heme iron. The ferrous heme iron then binds and activates O_2 and catalyzes either the stepwise oxidation of l-arginine to NO and citrulline (path A) or superoxide production in the absence of l-arginine (path C). When NO is formed, it can compete with O_2 for binding to the NOS ferrous heme iron before leaving the catalytic site (path B). Because the ferrous-nitrosyl complex forms faster than it decays, a rapid buildup of the complex occurs such that the major portion of NOS (70-90%) cycles through path B during the steady state, and the remainder of NOS cycles through path A. Because ferrous-nitrosyl NOS is a catalytically inactive complex, a steady but suboptimal rate of NADPH oxidation and NO synthesis is maintained which is proportional to the percentage of NOS cycling through path A. Upon cessation of NO synthesis due to depletion of NADPH, the ferrous-nitrosyl complex decays in an O_2-dependent reaction.

Decay of the Ferrous-Nitrosyl Complex

The time required for decay of the ferrous-nitrosyl complex is given a constant of 12 s\(^{-1}\). This value was inferred from the initial rate of decay of the ferrous-nitrosyl complex as measured by the rate of disappearance of the complex from the reaction mixture. The decay of the ferrous-nitrosyl complex was assumed to be negligible in the presence of arginine.

The initial concentrations of NADPH and NOS were 18 and 1 \(\mu\)M, respectively. The simulations, shown in Fig. 4, of the reactions in Scheme I qualitatively account for all of our experimental observations. Specifically, the ferrous-NO complex builds up rapidly during the initial phase of the reaction and then remains at a near constant level (~0.95 \(\mu\)M) until the reaction to yield ferric NOS.

To assess if the model presented in Scheme I is reasonable we have performed computer simulations of the reaction. The simulations were carried out by solving the simultaneous rate equations for the population of each intermediate iteratively. The rate constants used in the simulations were either inferred or derived from the measurements reported here and are listed below.

\[ k_1 = \text{The reduction of the resting enzyme by NADPH was given a bimolecular rate constant of } 2 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \text{ and } 1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}. \]

\[ k_2 = \text{The competent enzymatic pathway leading to free NO without inhibition was assigned a rate constant of } 15 \text{ s}^{-1} \text{ for the rate of formation of citrulline.} \]

\[ k_3 = \text{The rate of formation of the ferrous-NO complex was given a constant of } 12 \text{ s}^{-1} \text{ as determined in the experiments measuring its initial rate of formation.} \]

\[ k_4 = \text{The decay of the ferrous-NO complex was given a rate constant of } 0.06 \text{ s}^{-1} \text{ as measured by the rate of decay of the ferrous-NO complex after depletion of NADPH.} \]

\[ k_5 = \text{The dissociation of free NO from the ferrous NO complex was assumed to be negligible.} \]

\[ k_6 = \text{The rate of superoxide formation was assumed to be negligible in the presence of arginine.} \]

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NADPH is depleted (top panel of Fig. 10). There is an initial rapid consumption of NADPH during complex buildup which is followed by a slower rate of consumption (bottom panel of Fig. 10). After an initial burst, citrulline production continues linearly until the NADPH is depleted. These qualitative calculations based on the model in Scheme I are consistent with all of the essential features of the experimental observations. Simulations carried out in the absence of formation of the inhibitory complex (not shown) reveal that catalytic inhibition does not occur, and NADPH continues to be depleted at the initial rapid rate.

Although our model has ferrous-nitrosyl NOS acting as a catalytically inactive species whose formation is not directly involved in the generation of NO and citrulline, we cannot rigorously exclude the alternative possibility that ferrous-nitrosyl NOS is an intermediate in the pathway of NO synthesis. However, this seems unlikely, given the stability of most ferrous nitrosyl complexes (32) and that the rate of ferrous-nitrosyl complex decay in our experiments (0.06 s\(^{-1}\)) is too slow to account for the rate of NO synthesis during the steady state.

As depicted in Scheme I, the degree of ferrous-nitrosyl NOS formation during the steady state likely depends on several factors, including the rate of NOS heme iron reduction, the concentration of \(O_2\), the affinity of the ferrous heme iron toward NO versus \(O_2\), and the rate of ferrous-nitrosyl complex breakdown. Regarding heme iron reduction, our spectral data suggest that very little neuronal NOS exists in ferric form during steady-state NO synthesis. A considerable buildup of ferrous-deoxy NOS (\(\sim 70\%\)) also occurs during its catalysis of NADPH oxidation in the absence of substrate (see Fig. 1, inset), which involves \(O_2\) binding to ferrous heme and electron transfer to form superoxide (8, 25). This suggests that the slow step may involve \(O_2\) access and binding. Given that ferrous heme proteins generally display a greater affinity toward binding NO than their ferric forms (32, 33), the propensity of neuronal NOS to generate ferrous heme iron during catalysis may increase its susceptibility to NO.

Although the maximal solution NO concentrations which could be achieved in our studies (0–15 \(\mu M\)) were less than the dissolved oxygen concentration (\(\sim 300 \mu M\) initially), we observed significant ferrous-nitrosyl NOS formation during NO synthesis in all cases. This is consistent with ferrous iron having a greater affinity toward NO than \(O_2\) (32, 33). Also, \(O_2\) must diffuse into the active site from solution, whereas NO is not subject to this constraint. Ligand binding to the NOS heme iron may also be influenced by substrate. L-Arginine decreases the rate of CO binding to ferrous NOS by a factor of 12 (10). This is consistent with resonance Raman studies showing that substrate binds directly above the heme iron and can affect ligands coordinated to the heme iron such as CO and NO (14, 15). L-Arginine was also found to stabilize ferrous-nitrosyl NOS (15). How these and other factors interact to control NO and \(O_2\) binding to NOS during catalysis will require continued investigation.

In earlier studies describing NO inhibition of neuronal NOS, the inhibition was found to occur gradually, was enhanced by superoxide removal, diminished by the NO scavenger oxyhemoglobin, and became irreversible under some circumstances (16–19). In our current study the ferrous-nitrosyl complex formed immediately after NO synthesis was initiated, reaching a steady state within 2 s. The complex was maintained at a constant level while NOS continued to catalyze NADPH oxidation and NO synthesis. Neither the degree of complex formation nor the enzyme specific activity was affected by enzyme dilution or by NO scavengers such as oxyhemoglobin and superoxide. Multiple rounds of complex formation and decay were not accompanied by a gradual loss of enzyme activity. Thus, the characteristics of inhibition which we attribute to ferrous-nitrosyl complex formation in our current system do not match those observed previously for NO-mediated inhibition of NOS.

The discrepancy between our experiments showing no effect of superoxide or oxyhemoglobin on the rate of NO synthesis during the steady state in comparison with prior reports showing strong effects is particularly intriguing. At present we can only postulate possible origins for these differences. The NOS concentrations used in most of our measurements were high (see Figs. 6 and 7) in comparison with the earlier studies. Thus, in our studies of the influence of NO scavengers on the reaction, the NADPH was depleted within 2–3 min. In contrast, in the earlier studies the NOS concentration was much lower, and the NADPH concentration was higher so the reaction progressed for many tens of minutes. In addition, the temperature in our measurements was controlled at 15 °C, whereas in the earlier experiments it was 37 °C. These time and temperature differences could have a significant effect, considering that two phases of NO inhibition have been reported for cytochrome P450 (31). At early times NO reversibly inhibited P450 by coordinating to the ferrous iron of the heme just as occurs in neuronal NOS. However, a second phase was characterized at longer incubation times in which there was an irreversible loss of catalytic activity compared with enzyme never exposed to NO. It was proposed that the irreversible inhibition resulted from oxidation of certain amino acids in P450 by reactive oxides of nitrogen. Thus, neuronal NOS inhibition by NO could also display two phases: one phase in which NO coordinates to the ferrous heme iron prior to leaving the active site and is thereby not affected by the solution conditions; and a slower phase in which the NO escapes from the heme pocket to eventually react with the protein component of the enzyme. Additional experiments are needed to test these ideas.

To help determine if ferrous-nitrosyl complex formation is a general property of neuronal NOS catalysis, we examined whether the inhibition of NADPH oxidation which is associated with buildup of the ferrous-nitrosyl complex would be maintained at lower enzyme concentrations and if complex formation would still occur in an unfraccionated cell supernatant that contained neuronal NOS. We found that the specific rate of NADPH oxidation remained unchanged even at the lowest enzyme concentration tested (10 nM), and this rate was not increased in the presence of oxyhemoglobin. This suggests that the degree of complex formation is constant and independent of solution NO over a wide range of enzyme concentrations. Ferrous-nitrosyl NOS also formed during NO synthesis in an unfraccionated cell supernatant that was estimated to contain 0.4 \(\mu M\) neuronal NOS. Thus, cytosolic constituents potentially capable of scavenging NO (thiols, ascorbate, metalloproteins, superoxide) were unable to prevent ferrous-nitrosyl complex formation in the supernatant, implying that its formation is not restricted to purified systems and may also occur in intact cells that express neuronal NOS. We have carried out similar studies with inducible macrophage NOS and found that this isoform also generates a nitrosyl complex during aerobic NO synthesis. However, in contrast to neuronal NOS, catalytic inhibition related to nitrosyl complex formation is prevented by added oxyhemoglobin. Thus, fundamental differences appear to exist between neuronal and macrophage NOS regarding their autoinhibition due to nitrosyl complex formation.

To conclude, we report that a majority of neuronal NOS quickly converts to its inactive ferrous-nitrosyl complex after initiating catalysis, causing the enzyme to operate at only a fraction of its maximum possible activity. Complex formation and associated catalytic inhibition are unaffected by enzyme...
dilution, NO scavengers, and cellular constituents and seem to arise from a reaction between NO and the ferrous heme iron within the enzyme's active site. That an enzyme would evolve to function largely autoinhibited is remarkable and suggests that there are biological roles for neuronal NOS which remain to be considered. These are under current investigation in our laboratories.

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