Nitric Oxide Complexes of Inducible Nitric Oxide Synthase: Spectral Characterization and Effect on Catalytic Activity†

Amy R. Hurshman‡ and Michael A. Marletta*,‡,§

Interdepartmental Program in Medicinal Chemistry, College of Pharmacy, and Department of Biological Chemistry, School of Medicine, University of Michigan, Ann Arbor, Michigan 48109-1065

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ABSTRACT: Nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine to citrulline and nitric oxide (•NO). NOS is a hemoprotein containing a cytochrome P-450-type heme that has been shown to be involved in catalysis. It has been suggested that •NO is able to bind tightly to the heme of NOS and may in this way serve to regulate enzymatic activity. We report here the formation of both ferric and ferrous heme nitrosyl complexes with the inducible NOS from murine macrophages. The ferric nitrosyl complex is characterized by a Soret peak at 443 nm and two distinct peaks in the α/β region at 549 and 585 nm. The ferrous nitrosyl complex has absorbance maxima at 436 and 566 nm. A transient spectral intermediate is observed under conditions of NOS turnover. This intermediate appears to be a mixture of ferric and ferrous nitrosyl complexes and is unstable in the presence of oxygen. Binding of L-arginine decreases the affinity of •NO for the ferric heme but does not appear to decrease the affinity of •NO for the ferrous heme. Addition of either oxyhemoglobin or methemoglobin to NOS assays results in a nearly 2-fold increase in enzymatic activity. This result is attributed to the ability of both forms of hemoglobin to decrease the concentration of •NO in solution and is consistent with •NO inhibition of NOS under assay conditions. Our results show that NOS nitrosyl complexes form under certain conditions but suggest that the relevance of such complexes to activity *in vivo* may be limited by their instability in an aerobic environment.

Nitric oxide synthase (NOS; EC 1.14.13.39)[1] catalyzes the conversion of the amino acid L-arginine to citrulline and nitric oxide (•NO) (Marletta, 1993; Nathan, 1992). The reaction requires O_2 and NADPH as cosubstrates (Scheme 1). Three distinct isoforms have been characterized: (i) a particulate, constitutive type from vascular endothelium, (ii) a soluble, constitutive type from neuronal cells typified by that isolated from the cerebellum, and (iii) an inducible isoform, the best characterized example being that from murine macrophages (Marletta, 1994). The isoforms are different gene products and differ in their molecular weight and mode of regulation. The constitutive isoforms are regulated by calcium and calmodulin, the binding of calmodulin being required for enzymatic activity (Bredt & Snyder, 1990; Schmidt & Murad, 1991). The inducible NOSs are active in the absence of exogenous calmodulin; however, it has recently been shown that the inducible isoform purified from murine macrophages contains calmodulin as a tightly bound subunit (Cho et al., 1992; Stevens-Truss and Marletta, unpublished observations). This observation suggests that binding of calmodulin may be required for activity in all the NOS isoforms.

The enzyme shows homology to NADPH−cytochrome P-450 reductase (Bredt et al., 1991) and, as the sequence of the reductase would predict, has been found to have 1 equiv each of FAD and FMN (Hevel et al., 1991; Stuehr et al., 1991a; Mayer et al., 1991). In addition, NOS contains an iron protoporphyrin IX heme prosthetic group. This heme moiety has been characterized spectrally as a cytochrome P-450-type heme on the basis of its ability to form a reduced CO complex with an absorbance maximum at ~446 nm (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; McMillan et al., 1992). N^G-Hydroxy-L-arginine, formed by the N-hydroxylation of one of the equivalent guanidino nitrogens of L-arginine, has been identified as an intermediate in the NOS reaction (Stuehr et al., 1991b; Pufahl et al., 1992; Klatt et al., 1993). CO inhibition studies with L-arginine or N^G-hydroxy-L-arginine as the substrate suggest a catalytic role for the heme in both steps of the reaction (White & Marletta, 1992; Pufahl & Marletta, 1993). The enzyme also contains a tightly bound reduced pterin (Hevel & Marletta, 1992; Schmidt et al., 1992). The function of the pterin is still not fully understood and a direct role for this cofactor, such as in the hydroxylation of L-arginine, has not been ruled out.

The well-known affinity of hemoproteins for •NO has led to the speculation that NOS might be inhibited by •NO during enzymatic turnover. In fact, it has been reported that exogenously added •NO or •NO-releasing compounds inhibit citrulline formation by crude preparations of NOS from

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* To whom correspondence should be addressed at the College of Pharmacy, 428 Church St., University of Michigan, Ann Arbor, MI 48109-1065.

‡ Interdepartmental Program in Medicinal Chemistry.

§ Department of Biological Chemistry.

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[1] Abbreviations: NOS, nitric oxide synthase; H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hb, hemoglobin; BSA, bovine serum albumin; SOD, superoxide dismutase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; PCA, protocatechuic acid; PCD, protocatechuate 3,4-dioxygenase; DTT, dithiothreitol; TCA, trichloroacetic acid.

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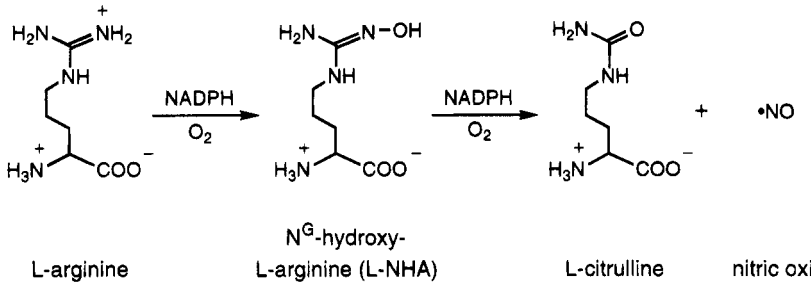
Scheme 1: Reaction Catalyzed by Nitric Oxide Synthase



bovine cerebellum (Rogers & Ignarro, 1992; Rengasamy & Johns, 1993) and rat alveolar macrophages (Griscavage et al., 1993). This inhibition has more recently been observed with purified rat cerebellar NOS (Griscavage et al., 1994). •NO has also been reported to inhibit cytochrome P-450 catalyzed reactions (Wink et al., 1993; Stadler et al., 1994). Although in principle •NO might be expected to inhibit NOS by forming a tight complex with the heme, previous investigations of the ferric and ferrous nitrosyl P-450 complexes show them to be stable only under anaerobic conditions (O'Keeffe et al., 1978). Furthermore, •NO inhibition might arise from several alternative mechanisms. Since O_2 reacts avidly with •NO, the addition of relatively high micromolar concentrations of •NO may result in inhibition of the NOS reaction by making the solution partially anaerobic. The reported ability of oxyHb to prevent the inhibition by •NO could be due to the inherent reactivity of oxyHb with •NO (Doyle & Hoekstra, 1981) but could also be due, at least in part, to oxyHb serving as an O_2 reservoir. The nitrosylating agents N_2O_3 and N_2O_4 are formed during the solution decomposition of •NO to NO_2^- and NO_3^- and could also contribute to NOS inhibition at high •NO concentrations.

To investigate the role that enzyme-generated •NO might play in turnover-dependent inhibition of the enzyme, we have formed both the ferric and ferrous nitrosyl complexes of the inducible murine macrophage NOS and have examined their spectral properties. Although unstable in the presence of oxygen, these heme nitrosyl complexes can be observed spectrally under turnover conditions at high NOS concentrations. Furthermore, we show that the rate of reaction of NOS is enhanced by the addition of either oxyHb or metHb to enzyme assays. Our results indicate that the effect of Hb on the NOS reaction rate is complex and involves a series of reactions.

METHODS

Materials and General Methods. OxyHb (human A_0, ferrous), Hb (equine), globin (human), BSA, SOD (bovine erythrocyte), catalase (bovine liver), HEPES, DTT, NADPH, $NADP^+$, glycerol (molecular biology grade), L-arginine, potassium ferricyanide, Dulbecco's modified Eagle's medium, LPS (*Escherichia coli*), penicillin, and streptomycin were purchased from Sigma Chemical Co. Macrophage RAW 264.7 cells were purchased from American Type Culture Collection (ATCC TIB 71). Calf serum was obtained from HyClone Laboratories and was heat-inactivated at 56 °C for 30 min before use. H_4B was purchased from Dr. B. Schircks Laboratory (Jona, Switzerland) and prepared in 15 mM HEPES (pH 7.5) containing 100 mM DTT. 2′,5′-ADP Sepharose 4B was purchased from Pharmacia-LKB Biotechnology Inc. DEAE-Bio-Gel-A, AG 50W-X8 cation-exchange resin, and Bradford protein dye reagent were purchased from Bio-Rad. L-[U-^{14}C]Arginine (specific activity = 319 mCi/mmol) was purchased from Amersham Corp. Ecolume scintillation cocktail was obtained from ICN-Flow. Sodium dithionite was purchased from Aldrich, made up anaerobically, and standardized against ferricyanide before use (Di Iorio, 1981). NADPH concentrations were determined using an extinction coefficient of 6200 M^{-1} cm^{-1} at 339 nm (pH 7.5). SOD and catalase activities were defined as those given by the supplier: one unit of SOD inhibits the rate of reduction of cytochrome *c* by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 and 25 °C in a 3.0-mL reaction volume; one unit of catalase decomposes 1 μmol of hydrogen peroxide/min at pH 7.0 and 25 °C while the peroxide concentration falls from 10.3 to 9.2 mM. Reactivials and silicone/Teflon septa (Tuf-Bond) were obtained from Pierce (Rockford, IL). PCA and PCD were gifts from Dr. David Ballou (University of Michigan). Argon (99.998%) was further purified by passage through two oxygen traps (G5301-4 from Baxter Diagnostics, Romulus, MI) followed by a third, indicating oxygen trap (Oxisorb I inline system from Messer-Griesheim GmbH Industriegase, Düsseldorf, Germany). •NO gas (99%) was purchased from Matheson (Chicago, IL) and was purified of other nitrogen oxides by bubbling through a saturated solution of KOH.

Purification of NOS. Cell culture procedures, induction of murine macrophage NOS activity, and preparation of the 100000*g* supernatant were carried out as previously described (Tayeh & Marletta, 1989), with the following modifications: 10−12 plates (150 × 25 mm) of confluent RAW 264.7 cells were activated for 18−20 h with 1 L of Dulbecco's modified Eagle's medium containing 10% heat-inactivated calf serum, 4 mM L-glutamine, penicillin (50 units/mL), streptomycin (50 μg/mL), *E. coli* LPS (75 ng/mL), and recombinant murine IFN-γ (3.5 units/mL). NOS was purified as previously described (Hevel & Marletta, 1992). H_4B (7 μM) was included in all steps throughout the purification in order to obtain enzyme that contained nearly 1 pterin per subunit and that showed only a slight rate enhancement (12 ± 6%) upon addition of exogenous H_4B. Protein concentration was determined by the Bradford microassay with BSA as the standard. NOS purified by this method was greater than 98% pure as judged by SDS−PAGE stained with Coomassie Blue R-250. One unit of NOS is defined as the amount of enzyme required to form 1 nmol of product/min at 37 °C.

NOS Activity Assays. Enzymatic activity was measured by one of the following assays: (A) •NO formation was measured by the oxidation of oxyHb to metHb as previously

described (Hevel & Marletta, 1994), using an extinction coefficient of 60 000 M^{-1} cm^{-1} for the increase in absorbance at 401 nm. (B) Citrulline formation was measured as previously described (Hevel & Marletta, 1992) with the following modifications. Assays (total volume 200 μL) contained L-[U-^{14}C]arginine (100 μM, specific activity = 3.3 μCi/μmol), NADPH (200 μM), H_4B (12 μM), DTT (100−150 μM), and NOS (5−40 nM) in 100 mM HEPES (pH 7.5) and were initiated with the addition of enzyme. Reactions were terminated after an incubation of 0−15 min at 37 °C by the addition of TCA (120 mM final concentration) and were placed immediately on ice. The samples were neutralized by the addition of 1.5 M HEPES (100 μL, pH 7.5). Unreacted arginine was removed from the reaction mixtures by applying the samples to AG 50W-X8 cation-exchange columns (500 μL of resin, sodium form) and washing with water (3 × 0.5 mL) directly into Ecolume scintillant (15 mL). Control experiments demonstrated that citrulline recovery under these conditions was greater than 97%.

UV−Visible Absorbance Spectroscopy and Anaerobic Procedures. UV−visible spectra were obtained on a Cary 3E spectrophotometer equipped with a Neslab RTE-111 circulating water bath. Unless otherwise indicated, all spectral experiments were carried out at 4 °C and under anaerobic conditions. A specially designed anaerobic cuvette sealed with a silicone/Teflon septum was used in all these experiments. The cuvette's side arm, equipped with a stopcock, allowed equilibration of the sample with argon. The total volume of the anaerobic cuvette was 15.0 mL, with a typical sample volume of 400 μL. NOS samples were deoxygenated by 10 cycles of alternate evacuation and purging with argon, using a gas train constructed in our laboratory. PCA (250 μM) and PCD (0.32 unit; 1 unit is the amount of enzyme that catalyzes the disappearance of 1 μmol of substrate/min at 25 °C in 100 mM HEPES, pH 7.5) were added to consume any residual oxygen. All other anaerobic solutions were prepared by extensive purging with prepurified argon in reacti-vials sealed with silicone/Teflon septa. PCA (250 μM) and PCD (0.8 unit/mL) were also included in these solutions. Additions and transfers were made with gas-tight syringes (Hamilton Co., Reno, NV). All spectra were normalized to 0 at 800 nm and were corrected for dilution.

Formation of Heme Nitrosyl Complexes. NOS (1−2.5 μM) in 100 mM HEPES (pH 7.5) with 10% glycerol was made anaerobic as described above. The ferric nitrosyl complex was formed by adding •NO gas in a gas-tight syringe to the anaerobic enzyme. The ferrous nitrosyl complex was formed by reducing anaerobic NOS prior to the addition of •NO gas. The amount of •NO added to the samples was typically that required to give 0.1% •NO in the gas phase, which corresponds to about 3 μM in solution at 4 °C (Young, 1981). A slight excess of either NADPH or sodium dithionite (both anaerobic, final concentration 5−8 μM) was necessary to obtain complete reduction, judged by the decrease in heme extinction and shift of the Soret peak to ~409 nm. The ferrous nitrosyl complex was also formed by direct reduction of the ferric nitrosyl complex.

Complex Formation during Turnover. Enzymatic turnover was initiated by the addition of NADPH (20−100 μM) to a cuvette containing NOS (1−2.5 μM), L-arginine (500 μM), H_4B (5 μM), and DTT (40−60 μM) in 100 mM HEPES (pH 7.5) with 10% glycerol. Spectra were recorded every minute until the reaction was complete, as evidenced by the depletion of NADPH. Since NOS requires oxygen for turnover, these experiments were carried out aerobically at 25 °C.

Arginine Titrations. An anaerobic solution of L-arginine (50 mM) was added to a cuvette containing either ferric nitrosyl NOS or ferrous nitrosyl NOS (1−2.5 μM, formed as described above). Spectra were recorded after each addition of arginine and were corrected for dilution. The final concentration of arginine at the end of the titrations was 10 mM. These titrations allowed the determination of the apparent dissociation constant (K_d) for arginine with the ferric nitrosyl complex of NOS. This K_d was determined by plotting the absorbance changes as a function of arginine concentration and fitting the data by a nonlinear least squares fit to the saturation binding equation: $y = (\Delta Abs_{max} \times [arginine])/(K_d + [arginine])$ (KaleidaGraph 3.0.4, Abelbeck Software).

Effect of Hb on NOS Activity. Hemoglobin solutions were made up in 100 mM HEPES buffer (pH 7.5). MetHb was prepared by oxidation of hemoglobin with a 1.2-fold excess of potassium ferricyanide for 1 h at room temperature, followed by dialysis against 100 mM HEPES (Di Iorio, 1981; Benesch et al., 1973), and was used within 24 h. The concentration of oxyHb and metHb used in assays was 10 μM; all concentrations of hemoglobin species reported here refer to the heme (*i.e.*, hemoglobin monomer) concentration. NOS assays which contained globin (250 μg/mL) were carried out in 100 μM HEPES (pH 7.5) due to the insolubility of this protein at higher buffer concentrations. Control assays (*i.e.*, without globin) were also carried out in 100 μM HEPES, since NOS activity is significantly lowered under these conditions. The effect of oxygen on enzyme activity was also examined by carrying out assays in oxygen-saturated buffer (100 mM HEPES, bubbled with oxygen for 10 min at 4 °C before use) and under an atmosphere of oxygen. These assays were carried out in borosilicate test tubes (10 × 50 mm) sealed with rubber septa and were initiated with NOS added with a gas-tight syringe. Some control assays contained BSA (250 μg/mL), SOD (50 or 100 units), catalase (48 or 96 units), or H_4B (25, 50, or 100 μM).

Redox State of Hemoglobin during NOS Turnover. NOS (5−10 nM) was added to a cuvette containing 100 μM L-arginine, 200 μM NADPH, 12 μM H_4B, 100−150 μM DTT, and 10 μM either oxyHb or metHb in aerobic 100 mM HEPES (pH 7.5). Spectra were recorded at 37 °C every minute for 20 min. Concentrations of oxy-, deoxy-, and metHb were determined at each time point using the following equations, developed from published extinctions (pH 7.5; Van Assendelft & Zijlstra, 1975) of the three species at 560, 576, and 630 nm:

$$[\text{oxyHb}]\ (\mu M) = (1.055A_{576} - 0.4275A_{630} - 0.7630A_{560}) \times 10^2$$

$$[\text{deoxyHb}]\ (\mu M) = (1.444A_{560} - 0.7853A_{576} - 0.7847A_{630}) \times 10^2$$

$$[\text{metHb}]\ (\mu M) = (3.090A_{630} + 0.2026A_{576} - 0.4191A_{560}) \times 10^2$$

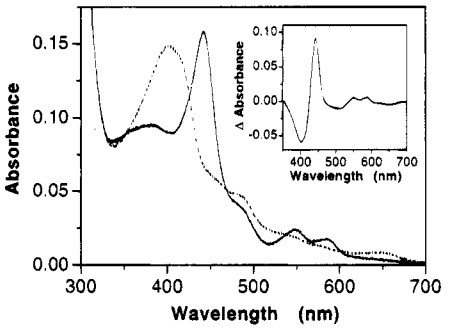
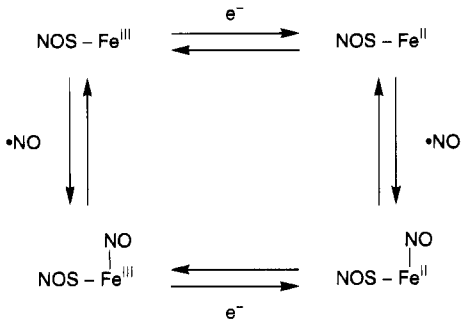


FIGURE 1: Spectrum of the ferric nitrosyl complex of NOS. Shown are the spectral changes induced by addition of •NO (3.7 μM final concentration) to anaerobic ferric NOS (2.4 μM) at 4 °C. The Soret peak for native ferric NOS (dashed line) is centered at 400 nm. The maximum for the ferric nitrosyl complex (solid line) is at 443 nm, with additional peaks at 549 and 585 nm. The inset shows the calculated difference spectrum for binding of •NO to ferric NOS. The difference spectrum shows absorbance maxima at 443, 552, and 588 nm.

Scheme 2: Formation of Heme Nitrosyl Complexes of NOS



RESULTS

Nitrosyl Complexes of NOS. Addition of •NO gas to a solution of ferric NOS results in the formation of a spectrally distinct species. This species is characterized by an absorbance maximum in the Soret region at 443 nm and distinct α/β bands at 549 and 585 nm (Figure 1). This spectrum, particularly the splitting in the α/β region, is similar to those of ferric heme nitrosyl complexes observed with other cytochrome P-450 enzymes (O'Keeffe et al., 1978; White & Coon, 1982). The ferrous heme nitrosyl complex of NOS can be similarly formed by the addition of •NO to ferrous NOS or alternatively by the direct reduction of the ferric nitrosyl complex (Scheme 2). The identical spectrum is obtained in either case and is characterized by a Soret maximum at 436 nm and a broad single α/β peak centered at 566 nm (Figure 2). These spectral shifts are consistent with the formation of a ferrous nitrosyl complex (O'Keeffe et al., 1978). Complete conversion to the ferrous nitrosyl spectrum described was obtained only when sodium dithionite was the reductant. When NADPH was the source of reducing equivalents, the resulting spectrum was that of a mixture of 10−15% ferric and 85−90% ferrous heme nitrosyl complexes (data not shown). This observation may reflect the difference in reduction potential of the two reductants; alternatively, the 10−15% ferric nitrosyl complex may arise from some portion of NOS that has lost a flavin during the purification procedure. NOS with one or no bound flavin would not be reduced by NADPH, a two-electron donor which can reduce the heme of NOS only via the flavins.

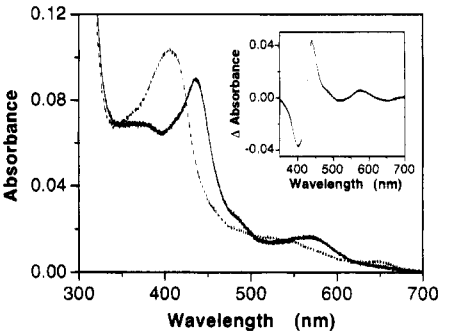


FIGURE 2: Spectrum of the ferrous nitrosyl complex of NOS. The spectrum of ferrous NOS (1.6 μM) at 4 °C before and after the addition of •NO (3.0 μM final concentration) is shown. The spectrum of ferrous NOS (dashed line) was obtained by reduction of the ferric enzyme with NADPH (5.2 μM final concentration) and displays a Soret peak at 408 nm. Following addition of •NO, 10−15% of the nitrosyl NOS was in the ferric state; sodium dithionite (3.5 μM final concentration) was added to obtain full reduction. The absorbance maxima for the ferrous nitrosyl complex (solid line) are centered at 436 and 566 nm. The inset shows the calculated difference spectrum for •NO binding to ferrous NOS, with λ_{max} at 440 and 577 nm.

Table 1: Absorbance Maxima (λ_{max}, nm) of Nitrosyl Complexes of Cytochrome P-450s

	ferric nitrosyl		ferrous nitrosyl		
protein[a]	Soret	α/β	Soret	α/β	
P-450$_{cam}$	432	540, 571	440	586	*b*
P-450$_{LM}$	436	543, 574	444	585	*b*
P-450$_{LM2}$	433	543, 575			*c*
P-450$_{LM4}$	433	542, 571			*c*
bNOS	440	549, 580	436	567	*d*
mNOS	443	549, 585	436	566	*e*

[a] P-450$_{cam}$, camphor hydroxylating P-450 from *Pseudomonas putida*; P-450$_{LM}$, P-450 from rat liver microsomes; P-450$_{LM2}$, phenobarbital-induced P-450 from rabbit liver microsomes; P-450$_{LM4}$, 5,6-benzoflavone-induced P-450 from rabbit liver microsomes; bNOS, constitutive NOS from rat brain; mNOS, inducible NOS from murine macrophages. [b] From difference spectra (O'Keeffe et al., 1978). [c] From absolute spectra (White & Coon, 1982). [d] From absolute spectra (Wang et al., 1994). [e] From absolute spectra; this work.

The two heme nitrosyl complexes can be distinguished not only in the position of the Soret peak but also in the α/β bands. As with the nitrosyl complexes of other P-450s characterized to date, the ferric NO species has two resolved peaks in the α/β region whereas the ferrous NO complex exhibits a single, broad peak in this region (Table 1). Additionally, the extinction of the Soret peak of the ferric nitrosyl complex is significantly larger than that of the ferrous nitrosyl. Both nitrosyl complexes of NOS are stable only under anaerobic conditions (data not shown). The ferrous nitrosyl complex was particularly unstable in the presence of oxygen. This complex was readily oxidized to ferric NOS, and in the presence of excess •NO, a spectrum identical to that of ferric nitrosyl was obtained even at low oxygen tension (data not shown).

Complex Formation during Turnover. Addition of low concentrations of NADPH to a cuvette containing NOS, L-arginine, H_4B, and DTT in the presence of oxygen results in the formation of a transient spectral intermediate. This intermediate is characterized by absorbance maxima at 442, 552, and 584 nm. The concentration of this species is highest in the first minute of the reaction and subsequently decreases as the reaction progresses and NADPH is depleted (Figure

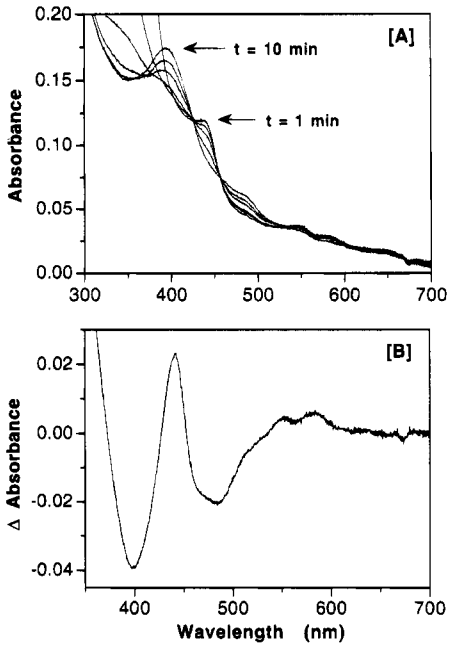


FIGURE 3: NOS spectra during turnover. (A) Spectra were recorded every minute following the addition of NADPH (100 μM final concentration) to a cuvette containing NOS (1.5 μM), L-arginine (500 μM), and H_4B (5 μM) at 25 °C. For clarity, only spectra at 1, 4, 5, 6, 7, and 10 min are shown. The transiently formed complex disappears with time, regenerating high-spin ferric NOS (λ_{max} 393 nm). (B) This panel shows the difference spectrum calculated by subtracting the $t = 0$ spectrum (not shown) from that obtained at $t = 5$ min. Absorbance maxima are observed at 442, 552, and 584 nm.

3A). The spectrum of the intermediate suggests that it is a mixture of both ferric and ferrous nitrosyl complexes. The difference spectrum shown in Figure 3B clearly illustrates this mixture of oxidation states. The splitting in the α/β region is characteristic of the ferric nitrosyl complex, whereas the position and extinction of the Soret indicate the presence of the ferrous nitrosyl complex.

Arginine Titrations. Arginine was added to both the ferric and ferrous nitrosyl complexes of NOS. Upon addition of arginine to the ferric nitrosyl complex, the Soret maximum shifts to 393 nm, characteristic of high-spin ferric NOS. This increase in absorbance at 393 nm occurs concomitantly with a decrease at 443 nm, the absorbance maximum of the ferric nitrosyl, with an isosbestic point at 420 nm (Figure 4). The apparent K_d for arginine binding to ferric nitrosyl NOS was determined by nonlinear least squares fit of the data to a saturation binding equation (see Figure 4 inset). This value was in the range of 60−200 μM ($n = 4$) but was found to vary depending on the concentration of •NO initially added to form the ferric nitrosyl complex. No spectral changes were observed upon addition of arginine to the ferrous nitrosyl complex (data not shown).

Effect of Hb on NOS Activity. To investigate whether •NO formed by NOS has an inhibitory effect on enzyme activity, assays were carried out in the presence or absence of oxyHb. •NO reacts rapidly with oxyHb to form NO_3^- and ferric Hb (Doyle & Hoekstra, 1981); therefore, oxyHb acts as an •NO scavenger. The addition of 10 μM oxyHb to NOS reaction mixtures results in a nearly 2-fold increase in activity (177 ± 16% of control, $n = 13$). Citrulline formation under the assay conditions was linear up to 15 min in both the presence and absence of oxyHb (Figure 5). An increase of similar

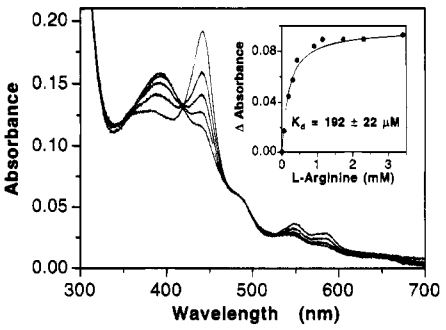


FIGURE 4: Titration of the ferric nitrosyl complex with arginine. The spectra show the absorbance changes upon addition of arginine to a preformed ferric nitrosyl complex (2.4 μM, formed in the presence of 6 μM •NO). The initial spectrum has an absorbance maximum at 443 nm, and the final spectrum has a maximum at 393 nm. The inset shows the titration data, which for this experiment gave a K_d of 192 ± 22 μM. The ordinate is the difference in absorbance calculated by subtracting the decrease at 443 nm from the increase at 393 nm ($\Delta A_{393} - \Delta A_{443}$).

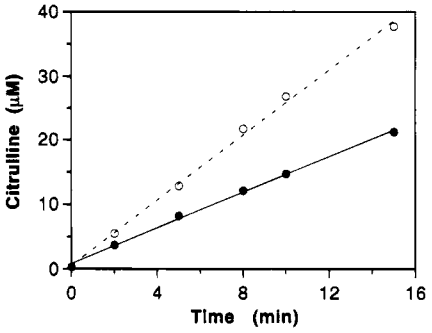


FIGURE 5: Effect of Hb on NOS activity. Assays contained 100 μM L-arginine, 200 μM NADPH, 12 μM H_4B, 125 μM DTT, and 14.4 nM NOS in 100 mM HEPES (pH 7.5). The closed circles (●) are NOS assays to which no Hb was added; the open circles (○) are assays carried out in the presence 10 μM oxyHb. In this representative experiment, the addition of 10 μM oxyHb resulted in an 84% increase in activity.

magnitude (163 ± 17% of control, $n = 4$) was also observed in assays containing 10 μM metHb (data not shown). Addition of globin (250 μg/mL, $n = 1$), BSA (250 μg/mL, $n = 1$), SOD (50 and 100 units, $n = 1$) or catalase (48 and 96 units, $n = 1$) had no effect on the rate of citrulline formation (data not shown), suggesting that the observed activity increase is specific to the heme moiety of Hb. The dependence of the catalytic rate on oxygen concentration was also examined. Assays carried out under an atmosphere of oxygen showed a slight increase in activity (120% of control, $n = 1$); however, the rate in the presence of oxyHb was unchanged by the addition of oxygen (data not shown). High concentrations of H_4B (25, 50, or 100 μM) did not increase the reaction rate (data not shown, $n = 2$), whereas similar concentrations of H_4B have been reported to nearly double the rate of citrulline formation by purified rat cerebellar NOS (Griscavage et al., 1994).

Redox State of Hb during NOS Turnover. Spectral studies were carried out under the same conditions as the assays described above. These allowed the determination of the concentrations of several Hb species (met, oxy, and deoxy) at time points throughout the NOS reaction. With the addition of either oxyHb or metHb, an equilibrium mixture consisting of ~80% metHb and 10% each of oxyHb and deoxyHb was reached within 15 min (Figure 6).

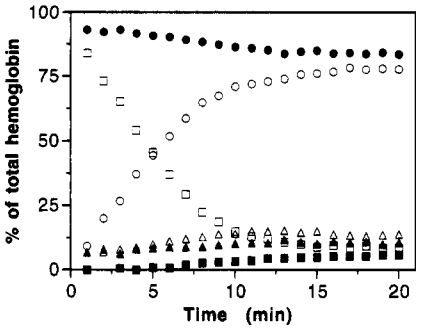


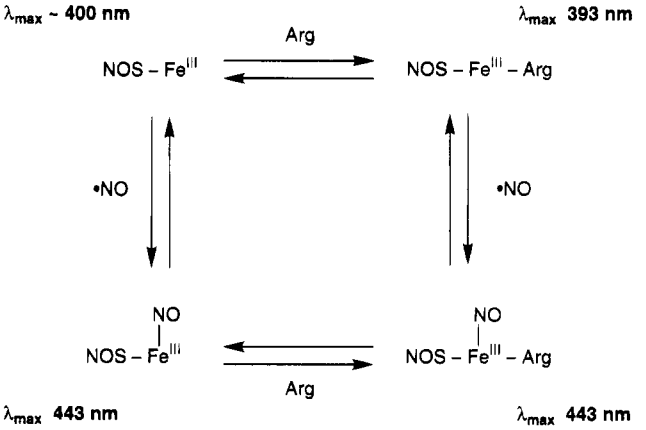
FIGURE 6: Redox cycling of Hb during NOS turnover. Squares (■, □) denote oxyHb, triangles (▲, △) deoxyHb, and circles (●, ○) metHb. The concentrations of the three species are plotted as the percentage of total Hb and are the average of triplicate data points. Open symbols represent data points for the experiment carried out in the presence of 10 μM oxyHb, while closed symbols are the values for the metHb experiment. The same equilibrium concentrations of the three Hb species were reached in both cases.

DISCUSSION

In order to investigate the possibility that end-product inhibition by •NO may be a physiological regulatory mechanism for NOS, we have initiated spectral studies to characterize the complexes of •NO with NOS. •NO has often been used to probe the nature of the heme environment of hemoproteins, and the nitrosyl complexes of several cytochrome P-450 isoforms have been previously characterized (O'Keeffe et al., 1978; White & Coon, 1982). We report here that both ferric (Figure 1) and ferrous nitrosyl (Figure 2) complexes can be formed with the murine macrophage inducible NOS. The spectra obtained upon •NO binding to NOS in either oxidation state are similar to those reported for other P-450s (O'Keeffe et al., 1978; White & Coon, 1982). Both nitrosyl complexes of NOS are stable for several hours under anaerobic conditions. Stuehr and co-workers recently reported the formation of •NO complexes with the constitutive neuronal NOS (Wang et al., 1994). The spectra we obtain for the inducible isoform are similar to those of the constitutive isoform (Table 1). In contrast to that report, however, we are able to observe formation of the ferrous nitrosyl complex in the absence of substrate.

The observation that NOS nitrosyl complexes could be formed upon the addition of high concentrations of exogenous •NO led us to determine whether the same nitrosyl complexes could be obtained in the presence of NOS-generated •NO. The conditions used in this turnover experiment are not typical assay conditions, since enzyme is present at a concentration of 1−2 μM, which is several orders of magnitude higher than that used in activity assays. A spectral intermediate is transiently formed under these conditions (Figure 3). This intermediate, which appears to be a mixture of ferric and ferrous nitrosyl complexes, forms within the first minute of the reaction but is stable only during the time of the enzymatic reaction. As the reaction progresses and NADPH is depleted, the complex is converted back to high-spin ferric NOS, as evidenced by the increase in absorbance at 393 nm. These results suggest that nitrosyl complexes of NOS can form during turnover but that the steady-state concentration of these complexes is limited by their instability in an aerobic environment. The high enzyme concentration in this experiment likely results in •NO concentrations far greater than those normally attained under assay conditions. This would not only increase the amount of NOS nitrosyl complex formed but may also contribute to its stability by making the reaction solution partially anaerobic.

Scheme 3: Model for •NO and Arginine Binding to Ferric NOS



The addition of arginine to the ferric nitrosyl complex caused a shift in the Soret maximum from 443 to 393 nm, consistent with the regeneration of unliganded ferric high-spin heme. This result can be explained by analogy to the binding of arginine to native ferric enzyme. Arginine binding induces a change in the spin state of the native ferric NOS heme from low spin to high spin. The change in spin state is visualized by a shift in the Soret maximum from ~400 to 393 nm and is thought to be accompanied by the displacement of a water molecule from the sixth coordination position of the heme (McMillan & Masters, 1993; White and Marletta, unpublished observations). Given that the binding of arginine to the ferric nitrosyl form of NOS causes a similar shift in Soret maximum, we conclude that the binding of arginine leads to a shift from ferric nitrosyl heme to an unliganded high-spin ferric heme. Thus, the binding of substrate appears to weaken the affinity of •NO for the ferric heme in the same way as it weakens the affinity of water for the ferric heme.

Even in the presence of saturating concentrations of arginine (10 mM), however, a shoulder remained at 443 nm, indicating that not all of the ferric nitrosyl was converted to the unliganded high-spin form (Figure 4). Because this shoulder persisted in the presence of saturating concentrations of arginine, we conclude that the species contributing this absorbance is arginine-bound ferric nitrosyl NOS. Two results support this conclusion: (i) in the presence of higher •NO concentrations, the shoulder at 443 nm was more prominent; and (ii) the calculated dissociation constant for the binding of arginine to the ferric nitrosyl complex increased with increasing concentration of •NO. The simplest model, therefore, which can account for these results is one in which •NO can bind to either the substrate-free or substrate-bound ferric enzyme (Scheme 3). At high arginine concentrations, the only relevant species in this scheme are those with bound arginine: the unliganded high-spin (λ_{max} 393 nm) and the arginine-bound ferric nitrosyl NOS (λ_{max} 443 nm), and the equilibrium between these two species is dependent on the concentration of •NO. These results indicate that the substrate-bound ferric nitrosyl is spectrally indistinguishable from the substrate-free ferric nitrosyl.

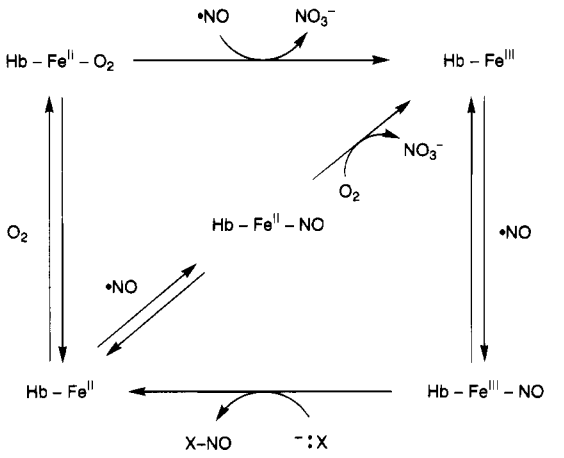
Similar arginine binding experiments were also carried out with the ferrous nitrosyl complex. In this case, no spectral shifts were observed. It thus appears that substrate-bound ferrous nitrosyl NOS and substrate-free ferrous nitrosyl NOS

are spectrally identical, similar to what is observed with the ferric nitrosyl complex. Furthermore, arginine does not decrease the affinity of •NO for the ferrous heme. These observations are not surprising, since the ferrous nitrosyl complex is expected to be structurally similar to the ferrous dioxygen complex, which is formed following substrate binding during the normal P-450 catalytic cycle. This ferrous dioxygen complex is also expected to form in the NOS reaction. If subsequent hydroxylation is to occur, arginine and oxygen must be able to bind simultaneously to the ferrous NOS heme. Ferrous nitrosyl complexes of other P-450s have been reported to be spectrally identical in the presence and absence of substrate (O'Keeffe et al., 1978); in some cases, the stability of these complexes is increased upon substrate binding (O'Keeffe et al., 1978; Tsubaki et al., 1987). Similarly, the ferrous nitrosyl complex of constitutive NOS is apparently stable only in the presence of arginine (Wang et al., 1994). The effect of arginine binding on the stability of either the ferric or ferrous nitrosyl complex of inducible NOS was not investigated in this work. An alternative explanation for the results discussed here is that arginine does not bind to the ferrous nitrosyl complex. This seems unlikely, given that the concentration of arginine used here (10 mM) is 3 orders of magnitude greater than the K_m for arginine (8−15 μM).

In parallel to these spectral experiments, we carried out assays to assess the effect of •NO and possible nitrosyl complex formation on the activity of NOS. Since oxyHb is an effective scavenger of •NO, the addition of oxyHb to NOS assays should result in lowered •NO concentrations in solution and perhaps in higher enzymatic activity. A significant increase in rate is indeed observed with the addition of 10 μM oxyHb to NOS assay mixtures, as previously observed for constitutive NOS from rat brain (Griscavage et al., 1994) and crude preparations of NOS from several sources (Rogers & Ignarro, 1992; Rengasamy & Johns, 1993; Griscavage et al., 1993). In the experiments reported here, the rate increase was found to require the heme moiety of Hb, since the addition of globin or BSA had no effect on enzyme activity. Similarly, neither catalase nor SOD affected the rate of citrulline formation, suggesting that neither peroxide nor superoxide plays an important role in the observed inhibition or in the decomposition of NOS-generated •NO under these conditions. The rate was slightly enhanced in the presence of oxygen. This rate enhancement can be rationalized in the same way as the oxyHb data, since the rate of decomposition of •NO and probably that of the NOS heme nitrosyl complexes is faster at higher oxygen concentrations. That product formation is linear for up to 15 min in both the presence and absence of Hb indicates that the steady-state concentrations of •NO in solution and of the NOS heme nitrosyl complexes must be reached within the first 2 min of the reaction.

The role of Hb in these reaction mixtures was examined more carefully on the basis of the following observations: (i) The addition of 10 μM metHb caused an increase in product formation by NOS of nearly the same magnitude as that observed with oxyHb. The rate of reaction of •NO with metHb relative to that with oxyHb would predict that the ferric form should be less efficient as a scavenger of •NO and that a smaller rate increase should be observed (Sharma et al., 1987; Traylor & Sharma, 1992; Kobayashi et al., 1982). (ii) The addition of either oxyHb or metHb (10 μM heme) resulted in the linear formation of up to 40 μM citrulline. These two observations together support a catalytic rather than stoichiometric reaction of Hb with •NO.

Scheme 4: Potential Reactions Involving •NO and Hb[a]



[a] The results presented here support an •NO scavenging role not only for oxyHb but also for metHb. This scheme summarizes some of the reactions of Hb with •NO.

Spectrophotometric determination of the concentrations of oxy-, deoxy-, and metHb under NOS assay conditions showed that the same equilibrium concentrations of these three species were reached regardless of the initial oxidation state of the Hb. The equations used to calculate the concentrations of Hb species were determined for a three-component mixture of only oxy-, deoxy-, and metHb, and the presence of nitrosyl complexes of Hb was not taken into account. Nonetheless, the equations remain valid for these calculations because the accumulation of Hb nitrosyl complexes is limited by their reactivity. The ferric nitrosyl complex of Hb is unstable and has been shown to be rapidly converted in the presence of excess •NO and water or another nucleophile to the ferrous nitrosyl complex (Chien, 1969; Sharma et al., 1987; Wade & Castro, 1990). The contribution of the ferrous complex is more significant and, based on its absorbance in the α/β region (Ohdan et al., 1994), may result in a slight overestimation of the concentration of oxy- and deoxyHb. Like the ferric nitrosyl complex, however, the ferrous nitrosyl complex does not accumulate to a large extent; instead, it reacts with oxygen to form NO_3^- and metHb (Chiodi & Mohler, 1985; Maeda et al., 1987). Thus, the data in Figure 6 clearly demonstrate that Hb undergoes complex redox chemistry with •NO. The reactions shown in Scheme 4 are consistent with our observations.

It has been suggested that •NO inhibits the reaction of NOS by its ability to form nitrosyl complexes with the heme moiety of NOS (Rogers & Ignarro, 1992; Rengasamy & Johns, 1993; Griscavage et al., 1993, 1994). The formation of such nitrosyl complexes might result in inhibition of enzyme activity by several mechanisms. The heme prosthetic group of NOS, like the thiolate-ligated hemes of cytochrome P-450s, is presumed to bind and activate molecular oxygen for substrate oxidation. Heme ligands, such as •NO, compete with oxygen for binding and thus interfere with the catalytic role of this cofactor. In addition, our results demonstrate that the K_d for arginine with the ferric enzyme is increased in the presence of •NO. This modulation of the affinity of arginine for NOS might also contribute to inhibition of NOS by •NO. Our activity data in the presence

or absence of Hb are consistent with •NO inhibition of NOS. This inhibition is probably due to the formation of nitrosyl complexes of NOS; however, the formation of such complexes cannot be directly observed under the assay conditions used here.

In summary, •NO can bind to the heme iron of NOS in either the ferric or ferrous oxidation state to form heme nitrosyl complexes with unique spectral properties. Such complexes can also be detected during turnover, under conditions described here. Clearly, the physiological importance of feedback-inhibition of NOS by •NO and of NOS nitrosyl complex formation will depend on the concentration of •NO in solution and on the stability of the complexes once formed. As demonstrated here, two of the factors influencing this stability are the concentrations of oxygen and the substrate L-arginine. It remains to be shown whether •NO inhibition of NOS has any relevance to the regulation of this enzyme *in vivo*.

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