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Optical Difference Spectrophotometry as a Probe of Rat Brain Nitric Oxide Synthase Heme–Substrate Interaction†

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ABSTRACT: NO synthase (NOS) is a family of enzymes that catalyzes the NADPH-dependent formation of NO and citrulline from L-arginine and molecular oxygen. The reaction involves an initial hydroxylation of L-arginine to form the isolable intermediate N^G-hydroxy-L-arginine (NOHArg). The subsequent incorporation of a second atom of oxygen during the metabolism of NOHArg is required to yield the final products NO and citrulline. NOS contains heme iron, FAD, FMN, and tetrahydrobiopterin prosthetic groups. To examine the interaction of substrates with the heme prosthetic group, substrate perturbation difference spectrophotometry was employed. By analogy with substrate binding interactions with cytochromes P450, NOS exhibits "type I" substrate perturbation difference spectra with the substrates L-arginine and NOHArg and the inhibitor N^G-methyl-L-arginine (NMA). These spectral perturbations are characterized by the appearance in the difference spectrum of a peak at ~380 nm, a trough with an absorbance minimum at ~420 nm, and an isosbestic point at ~405 nm. The spectral binding constants, K_s, for L-arginine and NMA were determined to be ~2.5 μM. These values are in agreement with the reported kinetic constants for these compounds. The "apparent K_s" values for NOHArg were 0.4 μM (2.0 μM NOS) and 0.8 μM (3.5 μM NOS), respectively. Furthermore, NOS exhibits "type II" difference spectra upon titration with imidazole, characterized by the appearance of a peak at ~430 nm and a trough at ~395 nm, with a spectral binding constant of ~160 μM. The conversion of NOS (~75% total protein at 1 mM imidazole) to a "low-spin" species suggests the displacement of an unknown bound ligand that interacts with the heme prosthetic group. The high-spin-state species is restored upon addition of L-arginine, independent of the presence of imidazole. D-Arginine, which is neither a substrate nor an inhibitor of NOS, does not exhibit a titratable interaction with the heme. Thus, the substrate L-arginine and the reaction intermediate NOHArg exhibit a specific and titratable interaction with the heme prosthetic group, implicating the heme as the reaction center for the initial binding of substrate and the subsequent incorporation of oxygen to form both products, NO and citrulline.

Rat brain NO[1] synthase (constitutive) catalyzes the NADPH-dependent, calcium/calmodulin-dependent formation of NO and citrulline from L-arginine (Bredt & Snyder, 1990) and molecular oxygen (Kwon *et al.*, 1990). This reaction involves two oxygenation steps during the metabolism of the substrate L-arginine. N^G-Hydroxy-L-arginine (NOHArg) has been identified as an isolable, oxygenated reaction intermediate, which is capable of serving as a substrate for NOS (Stuehr *et al.*, 1991a). Brain NOS is a 160-kDa enzyme that contains heme iron (McMillan *et al.*, 1992; Stuehr & Ikeda-Saito, 1992), FAD, FMN (Stuehr *et al.*, 1991b), and tetrahydrobiopterin (Mayer *et al.*, 1991) prosthetic groups.

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[1] Abbreviations: DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NO, nitric oxide; NOHArg, N^G-hydroxy-L-arginine; NMA, N^G-methyl-L-arginine; NOS, nitric oxide synthase.

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The C-terminal 641 amino acids display consensus sequences for flavin and pyridine nucleotide binding and 58% sequence similarity to the rat NADPH–cytochrome P450 reductase (Bredt *et al.*, 1991) and presumably serve as the oxidoreductase domain. Carbon monoxide inhibits NO formation by ~80% and results in a reduced, CO difference spectrum with an absorbance wavelength maximum at about 445 nm, suggesting the heme prosthetic group serves as an oxygenation reaction center (McMillan *et al.*, 1992). White and Marletta (1992) also have reported CO inhibition of rat brain NOS in a partially purified preparation and documented the presence of stoichiometric amounts of heme in macrophage NOS. It has been suggested, by analogy with the NADPH–cytochrome P450 reductase/cytochrome P450 system (Porter & Coon, 1991), that electrons are shuttled from NADPH by the flavin-containing oxidoreductase domain of NO synthase to the heme prosthetic group, which serves as an oxygenation reaction center.

In attempting to determine the role of the heme domain in rat brain NO synthase, our laboratory has utilized substrate perturbation difference spectrophotometry (Schenkman *et al.*, 1967), applied to the detection of substrate interactions and determination of binding constants with the various cytochromes P450. The appearance of difference spectra due to heme perturbation by the putative substrates or inhibitors constitutes direct evidence that the heme prosthetic group of rat brain NO synthase binds initially with the substrate, presumably prior to serving as the monooxygenase reaction center in this enzyme.

Wolff *et al.* (1993) have reported that imidazole inhibits citrulline formation, in a noncompetitive manner, by bovine brain NOS. The mechanism of inhibition for imidazole is likely the formation of a distal heme axial ligand, thus precluding substrate oxygenation. Cytochromes P450 exhibit "type II" difference spectra upon perturbation with antifungal imidazole agents and nitrogenous base compounds, such as aniline. Type II ligands are characterized by the appearance of a peak at about 425–430 nm and a trough at about 380–390 nm in the difference spectrum. The formation of a type II difference spectrum upon perturbation of NOS by imidazole provides additional evidence in support of this mechanism of inhibition.

MATERIALS AND METHODS

Human kidney 293 cells stably transfected with rat brain NO synthase cDNA were the gift of Dr. Solomon Snyder, The John Hopkins University, Baltimore, MD. N^G-Hydroxy-L-arginine dihydrochloride was the gift of Dr. Owen Griffith, Medical College of Wisconsin, Milwaukee, WI. N^G-Methyl-L-arginine monoacetate was obtained from Calbiochem (San Diego, CA). (6*R*)-5,6,7,8-Tetrahydrobiopterin was obtained from Research Biochemicals (Natick, MA). L-Arginine, D-arginine hydrochloride, and all other biochemicals were from Sigma Chemical Co. (St. Louis, MO).

Enzyme Purification. NO synthase was purified from stably transfected human kidney 293 cells by 2′,5′-ADP–Sepharose 4B and Sephacryl HR200 chromatography as previously described (McMillan *et al.*, 1992). The final product was obtained in 50 mM Tris-HCl, 10% glycerol, 0.1 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT, pH 7.5, buffer and was stored frozen at −80 °C. The purified enzyme was determined to be >98% homogeneous by SDS–polyacrylamide gel electrophoresis (7.5% gel). The optical spectral properties were consistent for all preparations, displaying a Soret band wavelength maximum at ~397 nm and a broad shoulder in the region of 470 nm, representing flavin absorbance.

Measurement of NO Formation. Nitric oxide formation was measured by the hemoglobin–NO capture assay (Kelm, 1988; Stuehr *et al.*, 1991a). The 1.0-mL reaction mixture contained 8.0 μM reduced hemoglobin, 100 μM L-arginine, 0.1 mM DTT, 10 μM calcium chloride, 1 μg/mL calmodulin, 5 μM tetrahydrobiopterin, and 100 μM NADPH in 50 mM Hepes, pH 7.5. The hemoglobin was prepared by sodium dithionite reduction in 50 mM Hepes, pH 7.5, buffer. The reduced hemoglobin was desalted using Sephadex G25 and stored at −20 °C. Reactions were performed at 25 °C and started by the addition of NADPH. Initial velocity rates were determined by monitoring the $\Delta A_{401}-A_{411}$ per minute using an extinction coefficient of 0.038 μM^{-1}. A Beckman DU 7400 diode array spectrophotometer, with a Peltier temperature control accessory, was used for the kinetics measurements. The specific activities of NO production by purified NOS ranged from 150 to 300 nmol min^{-1} mg^{-1}, representing turnover numbers of 24–48 min^{-1}, based upon heme content.

Protein Determination. Protein concentration was determined by the Bradford dye binding micromethod (Bradford, 1976) using human serum albumin as the standard. All determinations were performed in triplicate.

Difference Spectrophotometry. Substrate perturbation difference spectrophotometry measurements of NOS were conducted in the absence of calcium/calmodulin and added tetrahydrobiopterin. Optical spectra were recorded using a Shimadzu Model 2101 UV/visible dual-beam spectrophotometer, with a Peltier temperature control accessory, and 1.0-mL masked quartz cuvettes. Titration experiments were performed at 10 or 15 °C. All substrates were dissolved in 50 mM Tris-HCl, 10% glycerol, pH 7.5, buffer. Additions of substrate solution were made using a 10-μL Hamilton syringe, and the final sample volume changes were <2%. Samples of 0.5 mL of NOS were placed in the cuvettes, the absorbance difference was adjusted to zero, and a baseline was recorded. Difference spectra were recorded after each addition at 0.2-nm intervals, from 350 to 500 nm, using a 2-nm slit width. The spectral binding constants, K_s, were determined from the x-intercept of a double-reciprocal plot of the difference in the respective peak to trough absorbances versus the perturbant concentration. Linear transformation of titration data was performed by linear regression analysis ($r > 0.97$).

RESULTS

The absolute spectrum of rat brain NOS is shown in panel A of Figure 1. The presence of a shoulder at ~410 nm in the Soret region of the spectrum indicates the presence of some low-spin-state heme iron in the predominantly high-spin heme iron enzyme preparation. Figure 1B shows the absolute spectrum of NOS after addition of L-arginine, in which the disappearance of the shoulder at 410 nm has occurred, indicating the loss of the low-spin-state component. While the absolute spectral changes that occur within the Soret region of the spectrum of NOS upon substrate perturbation are subtle, the technique of difference spectrophotometry allows ready observation of heme–substrate interactions when sufficient concentrations of enzyme are used. It is important to note that the substrate perturbation effects were observed in the absence of calmodulin and thus brain NOS heme–substrate interactions are calmodulin-independent.

Figure 2 shows the substrate perturbation difference spectra obtained for the native substrate L-arginine with rat brain NOS. This difference spectrum is similar to the "type I" difference spectrum obtained for substrates with cytochromes

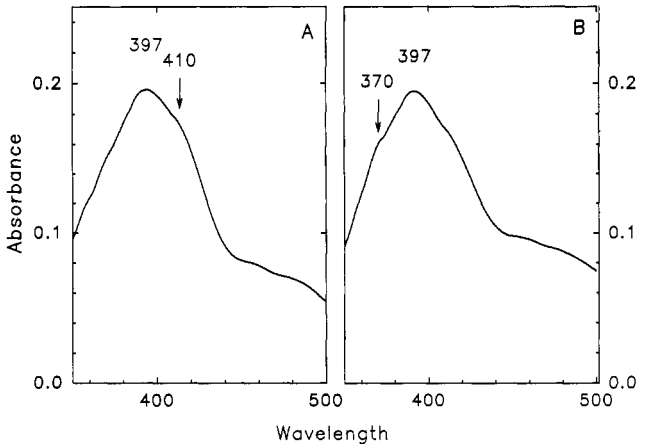

FIGURE 1: Absolute spectrum of NO synthase. Panel A: Spectrum of 1.9 μM NOS samples. Panel B: Spectrum of NOS after addition of 1.8 μM L-arginine in 50 mM Tris-HCl, pH 7.5, and 10% glycerol, with a final sample volume change of <1%. Spectra were normalized to zero absorbance at 700 nm.

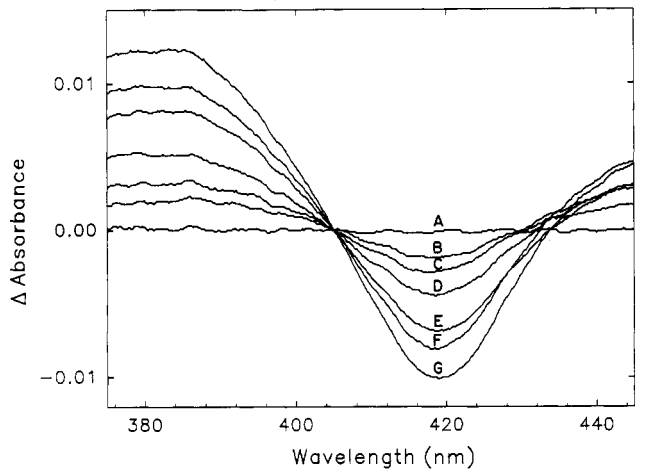

FIGURE 2: L-Arginine perturbation difference spectra. Aliquots of 0.50 mL of 2.2 μM NOS were placed in the sample and reference cuvettes, which were maintained at 10 °C in the spectrophotometer. The baseline was adjusted to zero absorbance. Additions of L-arginine in 50 mM Tris-HCl, pH 7.5, and 10% glycerol were made to the sample cuvette using a Hamilton syringe. The spectrum was recorded from 350 to 500 nm following each addition. The final concentrations of L-arginine (from a stock solution of 200 μM) were (A) baseline, (B) 0.20 μM, (C) 0.40 μM, (D) 0.60 μM, (E) 1.0 μM, (F) 1.4 μM, and (G) 2.0 μM, respectively. The total sample volume change was <2%. All spectra were normalized to zero absorbance at 405 nm. The data shown are representative of duplicate titration experiments.

P450, *e.g.*, hexobarbital with hepatic microsomal cytochromes P450 (Schenkman *et al.*, 1967). The spectral perturbation was characterized by the appearance of a peak at ~380 nm, a trough with an absorbance minimum at ~420 nm, and an isosbestic point at ~405 nm. These spectral changes are in the region of the Soret band of the *b*-type heme prosthetic groups and reflect interaction of the perturbant with the heme iron prosthetic group of NOS. The spectral binding constant, K_s, obtained by plotting the absorbance difference against perturbant concentration, is the concentration of perturbant resulting in half the maximal theoretical spectral change. The value of the spectral binding constant for L-arginine, K_s = 2.5 μM, obtained from the double-reciprocal plot of the absorbance differences versus substrate concentration, is shown in Figure 3. This spectral binding constant was independent of enzyme concentration. This value is in agreement with the reported kinetic constant for L-arginine, K_m = ~2 μM (Bredt & Snyder, 1991; Schmidt *et al.*, 1991). Further, addition of D-arginine, which is neither a substrate nor an inhibitor of NOS, resulted

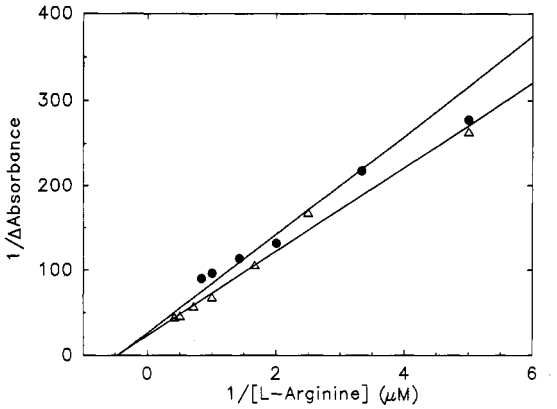

FIGURE 3: Determination of the spectral binding constant (K_s) for L-arginine. Difference spectra of NOS L-arginine titrations were recorded using 1.1 μM (circles) and 2.2 μM (triangles) enzyme samples as described in Figure 2. The L-arginine concentrations ranged from 0.20 to 2.4 μM and from 0.10 to 1.2 μM for the 2.2 and 1.1 μM NOS samples, respectively. Enzyme substrate saturation was observed over the concentration ranges used. The absorbance differences ($A_{380} - A_{420}$) versus [L-arginine] was plotted as a double-reciprocal plot. All linear transformation of titration data was performed by linear regression analysis ($r > 0.97$). The value of K_s = 2.5 μM was derived from the *x*-intercept.

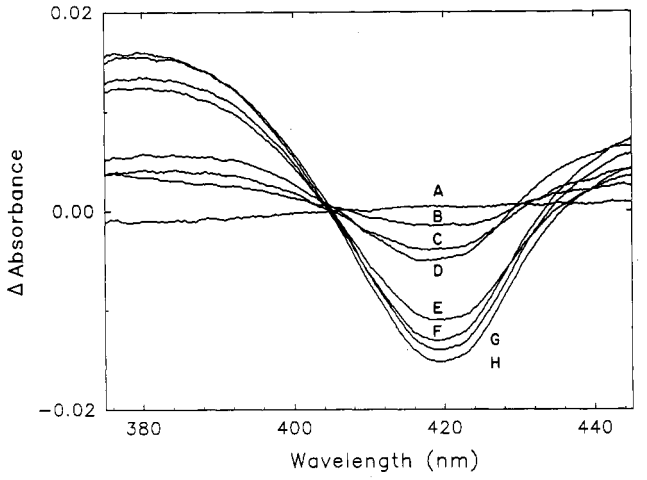

FIGURE 4: N^G-Methyl-L-arginine perturbation difference spectra. Titration of 2.2 μM NOS with NMA was performed, at 15 °C, as described in Figure 2. The final concentrations of NMA (from a stock solution of 200 μM) were (A) baseline, (B) 0.20 μM, (C) 0.40 μM, (D) 0.60 μM, (E) 1.0 μM, (F) 1.4 μM, (G) 1.8 μM, and (H) 2.2 μM, respectively. All spectra were normalized to zero absorbance at 405 nm.

in no spectral perturbation at concentrations up to 10 μM (data not shown).

The interaction of N^G-methyl-L-arginine (NMA), a potent inhibitor of NOS, produced spectral changes with the heme prosthetic group, shown in Figure 4, similar to those observed with L-arginine. The spectral binding constant for NMA, K_s = 2.3 μM, was also independent of enzyme concentration, as shown in Figure 5. Bredt and Snyder (1990) reported a K_i of 1.4 μM for this compound. Significantly, the oxygenated reaction intermediate N^G-hydroxy-L-arginine (NOHArg) was demonstrated to interact with the heme prosthetic group of NOS. Figure 6 shows the spectral perturbation obtained upon titration of NOS with NOHArg, which is similar to that obtained with L-arginine. The spectral binding constants obtained from the double-reciprocal plot shown in Figure 7, K_s = 0.4 μM (2 μM NOS) and 0.8 μM (3.5 μM NOS), were enzyme concentration-dependent and thus are "apparent K_s" values, although these differences were not appreciable.

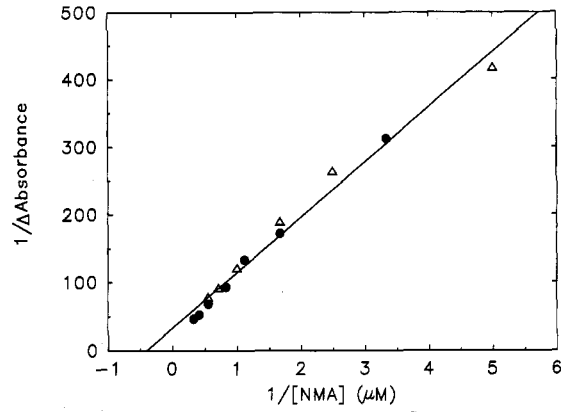

FIGURE 5: Determination of the K_s for N^G-methyl-L-arginine. Difference spectra were recorded using 3.5 μM (circles) and 2.0 μM NOS (triangles), as described in Figure 4. The concentrations of NMA ranged from 0.3 to 3.0 μM and from 0.2 to 1.8 μM for the 3.5 and 2.0 μM NOS samples, respectively. The difference in absorbance ($A_{380} - A_{420}$) versus [NMA] was plotted as a double-reciprocal plot and the K_s value obtained from the x-intercept.

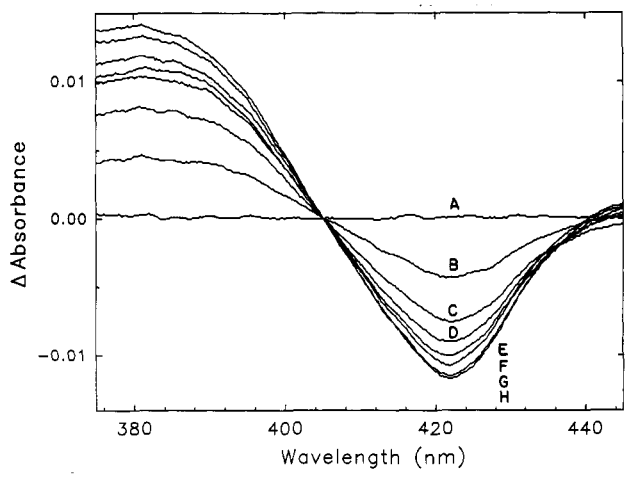

FIGURE 6: N^G-Hydroxy-L-arginine perturbation difference spectra. Titration of 3.5 μM NOS with NOHArg was performed at 15 °C, as described in Figure 2. The final concentrations of NOHArg (from a 300 μM stock solution) were (A) baseline, (B) 0.30 μM, (C) 0.60 μM, (D) 0.90 μM, (E) 1.2 μM, (F) 1.8 μM, (G) 2.4 μM, and (H) 3.0 μM, respectively.

Stuehr *et al.* (1991a) reported an apparent K_m of 6.6 μM for NOHArg.

In all experiments, titrations of NO synthase with L-arginine, NMA, and NOHArg displayed saturation at or near equimolar enzyme and substrate concentrations (data not shown). However, the absorbance changes associated with these titrations represent only about 10–20% of the total enzyme present, based on an extinction coefficient for NOS of ϵ_{397} ~0.07 μM^{-1} (Stuehr & Ikedo-Saito, 1992). To address this issue, NOS was converted to a predominantly low-spin-state species, which possesses a wavelength maximum at ~428 nm, by the addition of imidazole, as shown in the inset of Figure 8A. The titration of the 2.8 μM samples of NOS with imidazole resulted in conversion of about 75% of the total enzyme present to the low-spin state, as shown in Figure 8A. The spectral binding constant for imidazole was determined to be 160 μM, which is in agreement with the reported apparent K_i of 200 μM (Wolff *et al.*, 1993). The difference spectra obtained for NOS upon addition of imidazole are similar to the "type II" difference spectra observed for cytochromes P450 in the presence of various nitrogenous compounds, such as aniline (Schenkman *et al.*, 1967). These spectral perturbations are characterized by the appearance of a peak at about 430 nm and a trough at 395 nm. Titration of the imidazole-induced low-spin-state NOS with L-arginine resulted in restoration of 70% of the enzyme present to the high-spin state, as shown in Figure 8B. The spectral binding constant for L-arginine in the presence of 1.0 mM imidazole was determined to be 2.9 μM.

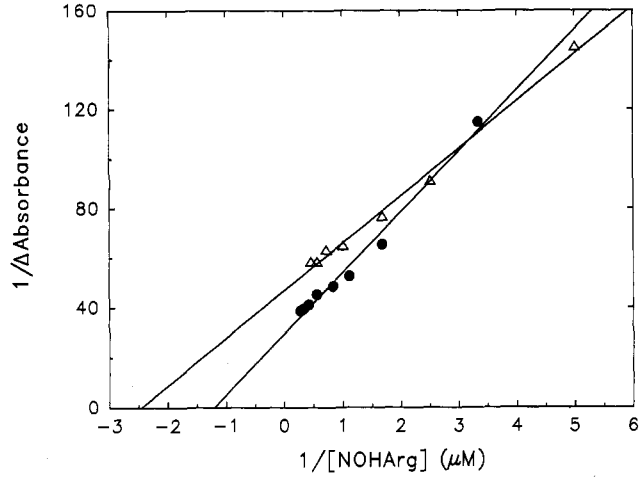

FIGURE 7: Determination of the K_s for N^G-hydroxy-L-arginine. Difference spectra were recorded using 3.5 μM (circles) and 2.0 μM (triangles) NOS, as described in Figure 6. The concentrations of NOHArg ranged from 0.3 to 3.6 μM and from 0.2 to 2.2 μM for the 3.5 and 2.0 μM samples, respectively. The difference in absorbance ($A_{380} - A_{420}$) versus [NOHArg] was plotted as a double-reciprocal plot and the K_s value obtained from the x-intercept.

DISCUSSION

NO synthase catalyzes two calcium/calmodulin-dependent, NADPH-dependent monooxygenations of the substrate L-arginine to yield the final products NO and citrulline. We report in this paper that the substrate L-arginine, the intermediate N^G-hydroxy-L-arginine, and the inhibitor N^G-methyl-L-arginine interact with the heme prosthetic group in a specific and titratable manner, indicating that the substrate binds in the vicinity of the heme prior to electron transfer. The binding of these compounds is independent of calcium/calmodulin and tetrahydrobiopterin and may represent an initial event in catalysis. Titration of NOS with L-arginine and NOHArg in the presence of calcium/calmodulin showed no effects on the properties of the observed spectral perturbation or the respective binding constants (data not shown). In addition, it is important to note that the spectral perturbation of NOS observed with the oxygenated intermediate compound NOHArg is attributable to interaction with the heme prosthetic group and is similar to that obtained with L-arginine. These results suggest further that the heme prosthetic group serves as the reaction center for the incorporation of oxygen into both products.

The heme iron of the NO synthase, as purified, is a predominantly high-spin, pentacoordinate ferric species. This property may reflect the presence of an enzyme–substrate complex in which L-arginine is bound to a major portion of the enzyme. The addition of L-arginine, NMA, or NOHArg results in the conversion of the remaining fraction of low-spin, hexacoordinate heme iron remaining to the high-spin state. Spectral binding constants for these compounds were in the ~0.5–2.5 μM concentration range, indicating a high enzyme–substrate affinity. The spectral binding constants obtained for NOHArg were dependent on enzyme concentration and thus represent apparent K_s values. The enzyme concentration dependence for this compound suggests that NOHArg may

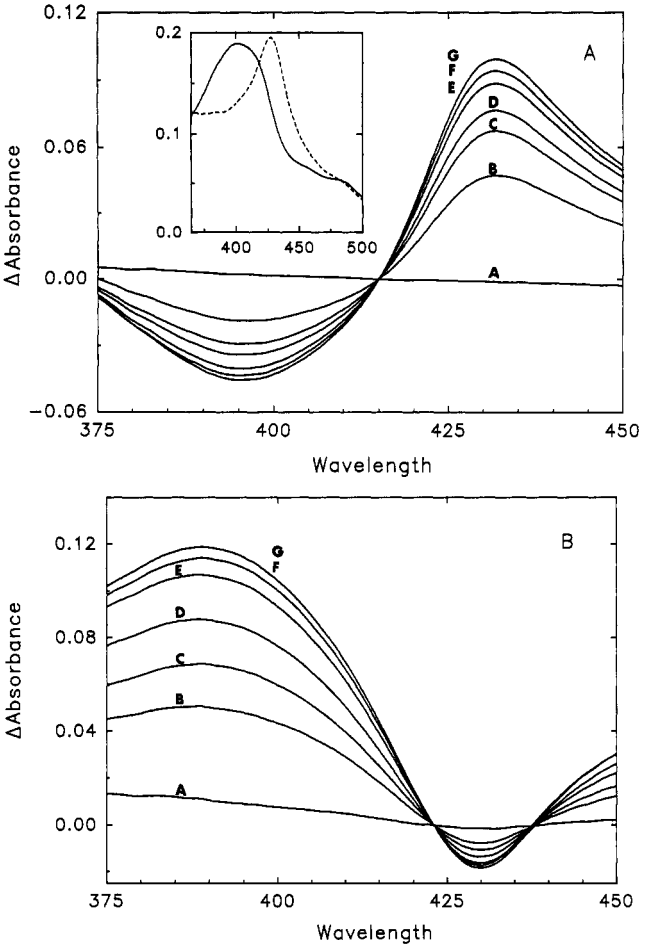

FIGURE 8: Panel A: Imidazole spectral perturbation of NOS. Titration of 2.8 μM NOS with imidazole was performed at 15 °C, as described in Figure 2. The final concentrations of imidazole were (A) baseline, (B) 100 μM, (C) 200 μM, (D) 300 μM, (E) 500 μM, (F) 700 μM, and (G) 1.0 mM, respectively. The difference spectra were normalized to zero absorbance at 415 nm. The spectral binding constant for imidazole, K_s = 160 μM, was obtained from a double-reciprocal plot of the difference in absorbance ($A_{430} - A_{390}$) versus [imidazole]. The inset shows the absolute spectra of 2.8 μM NOS in the absence (solid line) and presence (dashed line) of 1.0 mM imidazole. Panel B: Titration of imidazole-induced low-spin-state NOS with L-arginine. Imidazole was added to a final concentration of 1.0 mM to both the sample and reference cuvettes, and the baseline was recorded. Additions of L-arginine were made to the sample cuvette, with resulting final concentrations of (A) baseline, (B) 2 μM, (C) 4 μM, (D) 8 μM, (E) 20 μM, (F) 40 μM, and (G) 60 μM, respectively. The spectra were normalized to zero absorbance at 423 nm. The spectral binding constant for L-arginine in the presence of imidazole, K_s = 2.9 μM, was obtained from a double-reciprocal plot of the difference in absorbance ($A_{390} - A_{430}$) versus [L-arginine].

be capable of displacing bound substrate. The apparent K_s values, which are <1 μM, further support this argument.

The addition of imidazole to NOS results in titration of the predominantly high-spin-state species to the low-spin state. Approximately 75% conversion of the total enzyme present to the low-spin-state species was obtained in the presence of 1 mM imidazole. This conversion likely occurs by formation of a hexacoordinate heme iron, in which imidazole serves as the distal sixth ligand, with a concomitant displacement of bound substrate from the active site. The restoration of a high-spin-state, enzyme–substrate complex upon the addition of L-arginine (~70% conversion to high-spin state in the presence of 6 μM L-arginine) was independent of the presence of imidazole, based on the spectral binding constant for L-arginine of 2.9 μM. The effects of imidazole on substrate binding are in accordance with the noncompetitive inhibition of citrulline formation reported for imidazole and related compounds (Wolff *et al.*, 1993). Thus, this indicates that a major fraction of the purified NOS (>80%) contains a bound uncharacterized ligand, which accounts for the predominantly high-spin-state spectral properties of the freshly isolated enzyme, that can be displaced in the absence of L-arginine by imidazole.

The recent reports that rat brain NO synthase contains a heme prosthetic group (McMillan *et al.*, 1992; Stuehr *et al.*, 1992), which forms a reduced carbon monoxide–heme adduct with an absorbance wavelength maximum at 445 nm, suggested the presence of an axial thiolate ligand for the heme iron and thus has prompted comparison to the cytochrome P450 monooxygenases. While no sequence similarities have been observed between the NOS and cytochrome P450 families, particularly within the putative cysteinyl peptides and the conserved I-helix of cytochromes P450 (Nelson *et al.*, 1993), it is appropriate to consider the mechanism of cytochrome P450 substrate oxygenation (Dawson, 1988) as a model for NOS in light of the data presented here. It is interesting to note that the soluble cytochrome $P450_{BM-3}$, from *Bacillus megaterium*, possesses both a flavoprotein domain, containing FAD and FMN, and a heme-containing domain, which functions as the oxygenation reaction center (Nahri & Fulco, 1986). Formation of an enzyme–substrate complex, characterized by a type I difference spectrum, is the initial event in catalysis with the various mammalian cytochromes P450 and the soluble, bacterial proteins $P450_{CAM}$ and $P450_{BM-3}$. This is in contrast to peroxidase reactions where the oxidant (hydrogen peroxide) reacts first to form higher valency state heme iron complexes followed by discharge on addition of substrates. In the cytochrome P450 mediated reactions, the ferric heme iron is subsequently reduced to Fe(II) by an electron derived from NADPH, via the flavins, and binds molecular oxygen. The transfer of the second electron from NADPH to the heme iron results in fission of the bound dioxygen and oxygenation of the substrate. The heme–substrate interaction, observed in the present studies by difference spectrophotometry for L-arginine and NOHArg, offers support for the analogy of the catalytic mechanisms of cytochromes P450 and NO synthase, at least in the primary substrate binding event(s). Further experimentation is required to determine the mode(s) of interaction of this heme-binding, substrate-interactive domain of the NOS molecule with the C-terminal flavoprotein domain and the catalytic events controlling these interactions.

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