Characterization of C415 Mutants of Neuronal Nitric Oxide Synthase†

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ABSTRACT: Nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine to citrulline and nitric oxide. C415H and C415A mutants of the neuronal isoform of NOS (nNOS) were expressed in a baculovirus system and purified to homogeneity for spectral analysis and activity measurements. UV−visible spectra of each mutant lacked an observable Soret peak, suggesting that neither mutant contained heme. When reduced in the presence of CO, however, a small Soret centered at 417 nm could be detected for the C415H mutant, further supporting the assignment of C415 as the axial ligand to the heme. In addition to a deficiency in bound heme, neither mutant had any detectable bound tetrahydrobiopterin, as compared to wild-type enzyme, which had a ratio of 0.84 mol of bound pteridine:1 mol of nNOS 160 kDa subunit. The C415H mutant contained bound FAD and FMN at levels of 1.0 ± 0.1 and 0.9 ± 0.1 mol/mol of nNOS subunit, respectively. UV−visible spectra of both nNOS mutants retained the distinctive absorbance due to tightly associated oxidized flavin prosthetic groups. Further, the spectra suggested the presence of a neutral flavin semiquinone. Ferricyanide oxidation of the C415A mutant yielded a spectrum that was essentially that of oxidized flavin. Ferricyanide titration showed that the C415A mutant contained approximately 1 reducing equiv. Circular dichroism spectra suggested that each mutant was folded properly, in that both spectra were found to be essentially identical to the spectrum of wild-type nNOS. Neither mutant could synthesize nitric oxide, and neither mutant had the ability to oxidize NADPH unless an exogenous electron acceptor was added. The rate of cytochrome *c* reduction by each mutant was found to be slightly less, but very similar to the rate (∼20 μmol mg^{-1} min^{-1}) observed with wild-type nNOS. In all cases, the rate of cytochrome *c* reduction increased approximately 15-fold with the addition of calmodulin. Overall, these spectral and activity data suggest that C415 is the axial heme ligand and that a point mutation at C415 prevents binding of heme and tetrahydrobiopterin without interfering with the global folding or the reductase function of nNOS.

Nitric oxide synthase (NOS; EC 1.14.13.39)[1] catalyzes the oxidation of L-arginine to nitric oxide (·NO)[1] and citrulline. ·NO continues to be the focus of intense interest due to an increased understanding of its significant role in mammalian physiology. The function of ·NO, including vasodilation, neurotransmission, and cytotoxicity, depends on the tissue and cellular location of its synthesis. Many of the effects of ·NO are mediated by the activation of soluble guanylate cyclase, the only known physiological receptor for ·NO. Given the toxicity of ·NO and its higher oxides, the production of ·NO by NOS must be carefully regulated; therefore, a mechanistic understanding of NOS continues to be an area of investigation. Three prototypic isoforms of NOS have been characterized [for reviews, see Nathan (1992) and Marletta (1993, 1994)]. The neuronal and endothelial forms are classified as constitutive enzymes, and their activity is regulated by the binding of a Ca^{2+}−calmodulin complex. The inducible isoform is regulated at the level of transcription by induction with a variety of cytokines (Nathan & Xie, 1994). This isoform copurifies with calmodulin (CaM) as a tightly associated subunit (Cho et al., 1992; Stevens-Truss & Marletta, 1995).

The cloning of the neuronal isoform (nNOS) provided a substantial amount of structural and mechanistic information about NOS. It was discovered that the carboxy-terminal domain of NOS had a high degree of identity and homology with cytochrome P450 reductase (Bredt et al., 1991). Work prior to and after the cloning documented the requirement for NADPH (Iyengar et al., 1987), the presence of tightly associated FAD and FMN (Hevel et al., 1991; Mayer et al., 1991; Stuehr et al., 1991), the presence of a bound protoporphyrin IX-type heme (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; McMillan et al., 1992), and the requirement for H_4B (Tayeh & Marletta, 1989; Kwon et al., 1989; Mayer et al., 1991). The mechanistic model provided by cytochrome P450 has been an important one for understanding NOS. It is now clear that NADPH supplies reducing equivalents to the reductase domain of NOS, where the flavins are bound. As with cytochrome P450 reductase,

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[1] Abbreviations: ·NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; H_4B, 6(*R*)-5,6,7,8-tetrahydro-L-biopterin; H_2B, 7,8-dihydrobiopterin; q-H_2B, quinonoid-7,8-dihydrobiopterin; DTT, dithiothreitol; Sf9, *Spodoptera frugiperda*; PCR, polymerase chain reaction; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; P450, cytochrome P450; CD, circular dichroism; CaM, calmodulin; MW, molecular weight; SOD, superoxide dismutase.

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the flavoprotein reductase domain transfers the electrons to the heme-binding, N-terminal domain where L-arginine is oxidized to citrulline and ·NO. Even though there is no sequence homology between the N-terminal domain of NOS and the family of cytochrome P450s, the identification of a thiolate−heme complex and a P450-like mechanism of catalysis in NOS has led to the description of NOS as a self-sufficient P450. NOS does not contain the highly conserved P450 consensus sequence (FxxGxxxCxG) found in greater than 95% of the P450s cloned (Nelson et al., 1993). However, one particular thiolate residue and its surrounding sequence in the N-terminal domain were identified as the likely candidate for the location of heme binding (McMillan et al., 1992; White & Marletta, 1993; Renaud et al., 1993). In nNOS, this sequence spans nine residues from W409 to G417, in which the putative thiolate ligand is C415.

Two reports have been published in which C415 was mutated to a histidine in overexpressed nNOS. Two different approaches were used. We expressed and purified the full-length enzyme containing a C415H mutation from a baculovirus system (Richards & Marletta, 1994). McMillan and Masters (1995) expressed in *E. coli* an N-terminal segment that includes the heme-binding domain and a C415H heme domain mutant. In our previous work, we were unable to detect any bound heme by UV−visible spectroscopy or hemochromagen assay, suggesting that C415 was the heme ligand and that the imidazole in the axial position prevented the heme from binding. The possibility still existed, however, that C415 was not the axial ligand, but that the C415H mutant had a structural perturbation that interfered with heme binding. McMillan and Masters reported a spectrum of a partially purified C415H mutant of the heme domain which had a Soret band at 412 nm. Their sample contained 100 mM imidazole, and its spectrum was similar to a spectrum of a bis(imidazole)−methemoglobin complex. Although their sample was only partially purified and unstable heme binding prevented the formation of a ferrous−CO complex, all evidence, including our own, suggests that C415 is the axial ligand.

In this paper, a C415A nNOS mutant was generated and expressed in baculovirus with the hope that an alanine in the axial position would allow heme binding. Since both the C415H and C415A mutations were made in the full-length enzyme, other characteristics of these mutants were studied as well: both spectral and activity comparisons with wild-type nNOS were made. Circular dichroism spectrometry was utilized to determine if each mutant retained its structural integrity. Finally, the function of the reductase domain was studied by measuring the rate at which each mutant could catalyze the oxidation of NADPH in the presence and absence of cytochrome *c*.

MATERIALS AND METHODS

Materials. Tissue culture flasks were obtained from Corning and Costar. Grace's supplemented insect cell culture medium was purchased from Gibco BRL. Characterized fetal bovine serum was obtained from HyClone Laboratories, Inc. DNA oligomers were synthesized by the DNA Core Facility at the University of Michigan Medical Center. All restriction enzymes were obtained either from Gibco BRL or from Boehringer Mannheim. *Taq* polymerase was purified for use in the polymerase chain reaction as previously reported (Engelke et al., 1990). dNTPs were obtained from Boehringer Mannheim. Baculogold viral DNA and pVL1393 were obtained from Pharmingen, Inc. The resins used in the purification were purchased from Bio-Rad. H_4B was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland) and was prepared in 50 mM Hepes (pH 7.4) containing 100 mM DTT. Research-grade CO (99.5%) was obtained from Matheson. Unless otherwise noted, all other chemicals and reagents were obtained from Sigma.

Preparation of nNOS Mutants for Expression. The cDNA for wild-type nNOS was kindly provided by Dr. Solomon Snyder (Johns Hopkins University) and modified for baculovirus expression as described previously (Richards & Marletta, 1994). The modifications to generate the nNOS cDNA for the C415H mutant have already been described (Richards & Marletta, 1994), and the polymerase chain reaction technique of overlap extension was repeated with the following modification to generate the nNOS cDNA for the C415A mutant. At position 1591, the codon was changed from TGT to the alanine codon, GCT. The reconstructed C415A cDNA was subcloned into the transfer vector pVL1393, and purified plasmid DNA was prepared using a Qiagen-tip 100 column (Qiagen, Inc.).

Production of Recombinant Baculovirus. Spodoptera frugiperda (Sf9) cells (American Type Culture Collection) were maintained in Grace's insect cell culture medium supplemented with 10% fetal bovine serum as described previously (Richards & Marletta, 1994). The generation of recombinant virus for the expression of the wild-type nNOS and C415H nNOS has already been described (Richards & Marletta, 1994). The same methods were used to generate recombinant baculovirus containing the C415A mutant for expression in Sf9 cells.

Two rounds of plaque purification led to the isolation of eight plaques for the mutant C415A nNOS recombinant virus. Virus was eluted overnight from each agar plug in 500 μL of Grace's media. The recombinant virus from two of the agar plugs was individually amplified to an approximate concentration of 5×10^7 particle forming units/mL, as described previously (Richards & Marletta, 1994).

Expression and Purification of Wild-Type nNOS and the C415 Mutants. Sf9 cells were infected in monolayers to generate the wild-type and both mutants of nNOS. Expression conditions have been previously reported (Richards & Marletta, 1994). Briefly, Sf9 cells were plated out in either 150 cm^2 or 225 cm^2 flasks at concentrations of 2×10^7 or 3×10^7 cells/flask, respectively. The appropriate recombinant virus was added at a multiplicity of infection of 5, and the infection was allowed to progress for 60−70 h before harvesting the cells. At 2 h post-infection, a hemin−albumin complex was added to the culture, which as shown previously (Richards & Marletta, 1994) is necessary to generate fully active preparations of enzyme. The final hemin concentration in the supplemented media was 20 μM. The cells were harvested, and 100000*g* supernatant was prepared as previously described (Richards & Marletta, 1994).

The purification of each form of nNOS followed the same procedure reported previously (Richards & Marletta, 1994) with a few minor modifications. The 100000*g* supernatant derived from three to five 150 cm^2 flasks (or the corresponding quantity of cells from 225 cm^2 flasks) was applied to a 1.5 g 2′,5′-ADP−Sepharose 4B (Pharmacia) column (3 cm × 1.5 cm) equilibrated with buffer A (10 mM K_2HPO_4, 100

mM NaCl, 0.5 mM L-arginine, 10% glycerol, and 10 μM H_4B at pH 7.4). The column was washed with 10 mL of buffer A containing 0.1 mM EDTA and 0.1 mM EGTA. A 20 mL salt wash followed, consisting of the same components as the previous wash but containing 300 mM NaCl instead of 100 mM NaCl. The column was then rinsed with 20 mL of buffer B (10 mM K_2HPO_4, 100 mM NaCl, 0.5 mM L-arginine, and 10 μM H_4B at pH 7.4). nNOS was eluted with 10 mL of buffer B containing 10 mM NADPH and 2 mM $CaCl_2$. The eluate was directly applied to a calmodulin affinity column (3 cm $\times$ 1 cm) equilibrated with buffer B, containing 2 mM $CaCl_2$ and prepared as previously reported (Richards & Marletta, 1994). The column was washed with 30 mL of buffer C (50 mM Hepes, 100 mM NaCl, and 10 μM H_4B at pH 7.4) containing 2 mM $CaCl_2$. nNOS was eluted in 10 mL of buffer C, containing 5 mM EGTA and 10% glycerol. The eluate was collected in an ultrafiltration cell (Amicon), equipped with a 70 kDa cutoff membrane (Filtron), and concentrated 10-fold under nitrogen. Depending on the final conditions required, the components of buffer C were modified for particular experiments. For experiments involving oxidation of C415A NOS by ferricyanide [potassium hexacyanoferrate(III)], the enzyme was isolated without added H_4B and DTT. The enzyme was stored at -80 °C with H_4B in excess of the enzyme concentration and glycerol added to a final concentration of 20%. The protein concentration of the purified enzyme was determined by the Bradford protein assay (Bio-Rad) using BSA as the standard.

Spectral Characterization. UV−visible spectral data were obtained on a Cary 3E spectrophotometer equipped with a Neslab RTE-100 temperature controller set at 25 °C. The ferrous−CO binding spectrum of C415H mutant was obtained as follows: the head-space in a quartz cuvette containing the enzyme sample at 4 °C was flushed with CO gas for 15 min, followed by reduction with sodium dithionite. Heme content was measured by the hemochromagen assay as described previously (White & Marletta, 1992).

Circular dichroism (CD) spectrometry was carried out at room temperature using an Aviv Model 62DS spectrometer with a 0.5 mm mountable cell. With gel filtration, as described below, or by changing buffer C in the purification, the enzyme samples were diluted in 10 mM K_2HPO_4 (pH 7.4) with 5% glycerol. Five spectra were taken of each sample and averaged to give the reported spectrum. Secondary structure predictions were made using three separate programs: SELCON (Sreerama & Woody, 1993) uses a modification of variable selection analysis called the self-consistent method to predict secondary structure. K2D (Andrade et al., 1993) uses a method of neural network analysis called proteinotopic mapping. Finally, CONTIN (Provencher & Glockner, 1981; Venyaminov et al., 1993) uses a method of analysis known as ridge regression to predict secondary structure.

Quantitation of Bound Biopterin. Unbound, excess H_4B was removed from purified enzyme by passing a 150 μL aliquot over a Bio-Gel P6-DG gel filtration column (25 cm $\times$ 0.7 cm) equilibrated with 10 mM K_2HPO_4 (pH 7.4) and 10% glycerol. Alternatively, the enzyme was purified with buffer C, changed to contain 10 mM K_2HPO_4 (pH 7.4) instead of Hepes. Bound biopterin in each sample was oxidized, applied to an Altex Ultrasphere-ODS 5 μm HPLC column (25 cm $\times$ 4.6 mm), and detected with a Waters 470 scanning fluorescence detector (λ_{ex} = 343 nm, λ_{em} = 438 nm) as described previously (Hevel & Marletta, 1992). Briefly, a 100 μL sample of enzyme containing 2.3 mM KI/I_2 and 9.0 mM NaOH was incubated in the dark for 5 min at 100 °C, followed by 55 min at room temperature. Samples were neutralized with 5 μL of 10 M H_3PO_4, and excess I_2 was scavenged with 5 μL of 1 M ascorbate, prior to HPLC analysis with 50 μL of sample. Under these conditions, H_4B and q-H_2B are oxidized to pterin, and H_2B is oxidized to biopterin. Authentic biopterin and pterin standards were used to quantify the oxidized H_4B released from the enzyme sample. An authentic H_4B standard was oxidized and subjected to HPLC analysis to verify that recovery of H_4B was quantitative.

Quantitation of Bound Flavins. Enzyme, frozen in 20% glycerol and 50 mM Hepes (pH 7.4), was thawed and denatured by the addition of an equal volume of 10 M urea. Each sample was analyzed for flavin content using a modification of Light et al. (1980) by application to a Nova-Pak C_{18} column (150 mm $\times$ 3.9 mm, 4 μm particle size, Waters), equipped with a C_{18} guard column (Alltech, Vydac, 7.5 $\times$ 4.6 mm), using an HP 1090 Series II instrument with a diode array detector. The column was maintained at 40 °C and equilibrated with 95% of 5 mM ammonium acetate (pH 6.0) and 5% methanol. The samples were eluted using a linear gradient to 100% methanol over 9 min at 0.5 mL/min and monitored at 375 and 450 nm. Authentic FAD and FMN standards were used to quantify the flavin content of the enzyme samples.

Activity Assays. The ability of wild-type nNOS and the two mutants to catalyze the formation of ·NO was measured using the hemoglobin assay, an initial rate assay ($\Delta\epsilon$ = 60 000 M^{-1} cm^{-1} at 401 nm) (Hevel & Marletta, 1994). Each assay contained 5 μM oxyhemoglobin, 50 μM L-arginine, 1 mM $CaCl_2$, 20 μg of calmodulin/mL, 120 μM NADPH, 12 μM H_4B, and 167 μM DTT in a final volume of 500 μL (50 mM Hepes, pH 7.4). Reactions were initiated with enzyme, usually 5−10 μg.

The initial rate of NADPH oxidation catalyzed by each form of nNOS was measured by monitoring the decrease in absorbance at 340 nm ($\Delta\epsilon$ = 6200 M^{-1} cm^{-1}) (Dawson et al., 1986). Each assay contained 1 mM $CaCl_2$, 12 μM H_4B, 167 μM DTT, 1−5 μg of enzyme, and 150 μM NADPH in a final volume of 500 μL (50 mM Hepes, pH 7.4). The absorbance change at 340 nm was monitored continuously before and after the addition of calmodulin, at a concentration of 20 μg of calmodulin/mL. The assays were done in the presence and absence of 50 μM L-arginine.

The initial rate of cytochrome *c* reduction catalyzed by each form of nNOS was measured by monitoring the increase in absorbance at 550 nm ($\Delta\epsilon$ = 21 000 M^{-1} cm^{-1}) (Massey, 1959). Each assay contained 1 mM $CaCl_2$, 50 μM cytochrome *c*, and 1−3 μg of enzyme. The assay was initiated with NADPH at a final concentration of 150 μM. After the rate was recorded, calmodulin was added at a concentration of 20 μg of calmodulin/mL, and the new rate was recorded. The assays were done in the presence and absence of 50 μM L-arginine. Each assay was done in a final volume of 500 μL (50 mM Hepes, pH 7.4). The three assays described above were conducted on a Perkin Elmer 553 Fast Scan UV−visible spectrophotometer equipped with a water bath set to 37 °C.

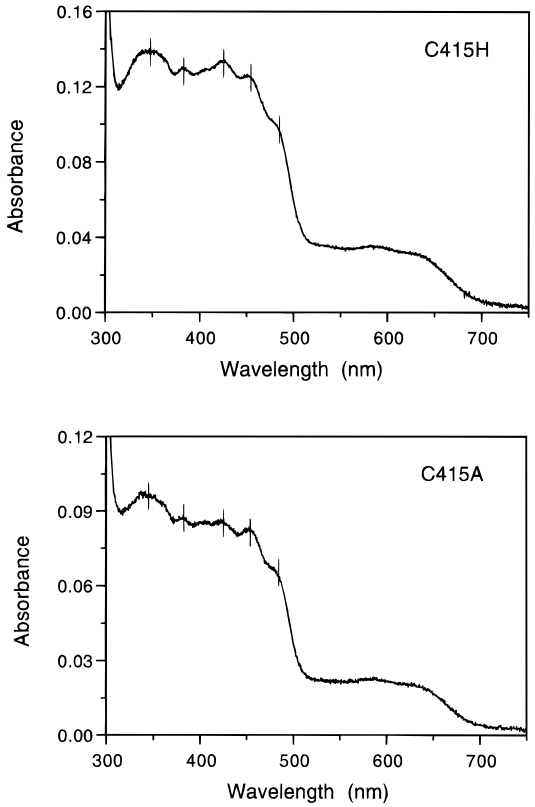

FIGURE 1: Absorbance spectra of the C415H and C415A nNOS mutants. Spectra were taken of freshly purified enzyme. Absorbance maxima are marked with vertical lines, and occur at 345, 382, 424, 453, and 483 nm.

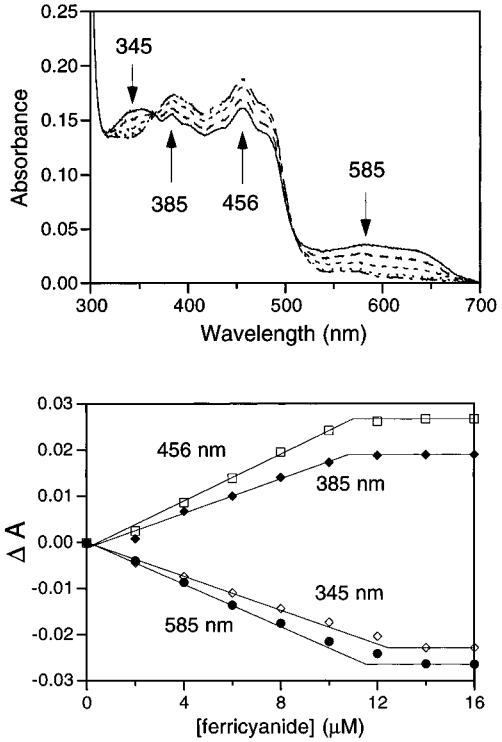

FIGURE 2: Oxidative titration of the flavin semiquinone species in the C415A nNOS mutant. (Top panel) An initial spectrum was taken of the C415A mutant (8.8 μM subunit) purified in the absence of H_4B (—). Titration with ferricyanide (4 μM increments shown) resulted in the oxidation of the neutral flavin semiquinone. Isosbestic points occur at 367 and 511 nm. (Bottom panel) ΔA as a function of added ferricyanide.

RESULTS

Spectral Characterization. UV−visible spectral analysis of both the C415H and C415A mutants as purified demonstrates the absence of a Soret band in the 400 nm region of the spectra (Figure 1), indicating that little or no protoporphyrin IX is bound. The C415H mutant was also analyzed with the hemochromagen assay, which did not detect any bound porphyrin (Richards & Marletta, 1994). The absorbance at 345 nm and the broad absorbance from 500 to 650 nm are characteristic of a neutral flavin semiquinone (Massey & Palmer, 1966). The small absorbance at 424 nm is of unknown origin, but consistently observed. Both of these absorbances are bleached by ferricyanide, as shown with the C415A mutant in Figure 2 (top panel). The remaining absorbance from approximately 380 to 500 nm is due to the bound, oxidized flavin prosthetic groups. Quantitative titration with ferricyanide was performed twice, yielding values of 1.05 and 1.3 mol of ferricyanide/mol of enzyme (Figure 2, bottom panel). The data in Figure 2 are from the second titration.

When the C415H mutant was reduced with dithionite in the presence of CO, the flavin absorbance decreased, revealing a well-defined peak at 417 nm (Figure 3). A substoichiometric concentration of ferrous protoporphyrin IX coordinated to a histidine in the axial position would cause this type of absorbance with CO bound (Adar, 1978). A similar experiment with the C415A mutant resulted in the same loss of oxidized flavin absorbance, but no peak due to bound heme was observed (data not shown).

Samples of wild-type, C415H, and C415A nNOS were prepared at equal concentrations of 240 μg/mL for analysis

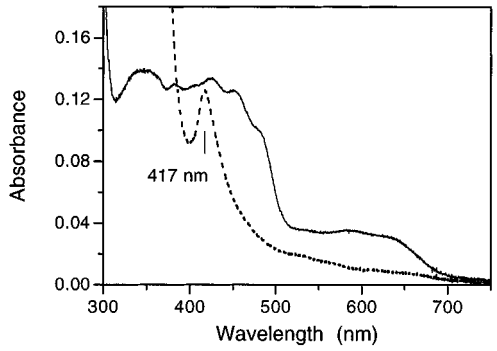

FIGURE 3: Absorbance spectra of the C415H nNOS mutant. A spectrum was taken of the freshly purified C415H mutant (—). The reduced-CO spectrum (- - -) was generated as described under Materials and Methods.

by CD spectrometry. Five spectra were generated for each sample and then averaged together to give the spectra in Figure 4. All three spectra have absorbance minima at 208 and 222 nm and an absorbance maximum at 192 nm. Differences among the spectra can be attributed to slight variations in protein concentration. Each averaged spectrum was subjected to a more rigorous analysis using three programs to predict the secondary structure elements. Results from this analysis are shown in Table 1.

Quantitation of Bound Biopterin. The C415H and C415A mutants were subject to biopterin analysis by reverse-phase separation using HPLC and fluorescence detection. For comparison, wild-type nNOS was analyzed in the same manner. The mutants did not contain detectable quantities of biopterin, while the wild-type contained 0.84 equiv/subunit (Table 2).

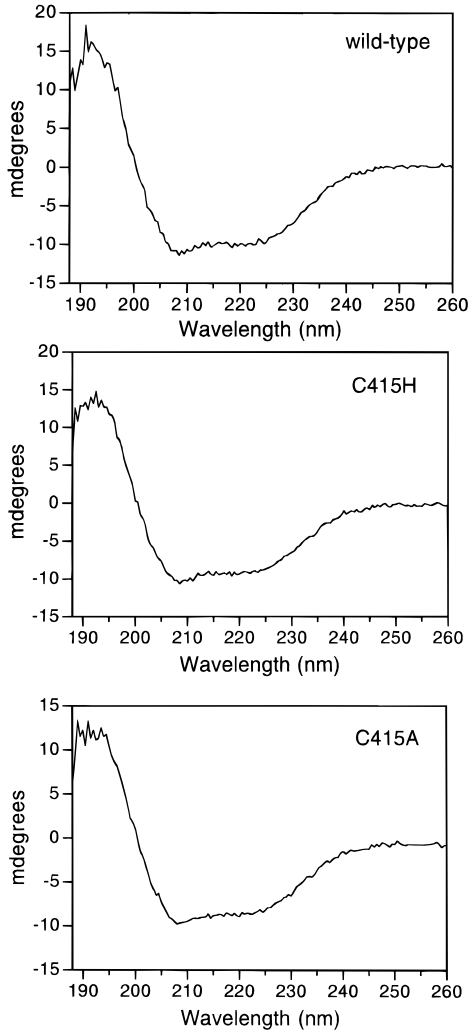

FIGURE 4: CD spectra of wild-type, C415H, and C415A nNOS. Equal concentrations (240 μg/mL) of each preparation of enzyme were used to acquire the spectra. Five spectra were taken of each enzyme sample and averaged together to generate the spectrum shown.

Table 1: Secondary Structure Predictions

program	wild-type nNOS[a]	C415H mutant	C415A mutant
SELCON			
α-helix	31	29	29
β-sheet	20	23	31
β-turn	27	28	32
K2D			
α-helix	28	31	31
β-sheet	15	10	10
β-turn	np[b]	np	np
CONTIN			
α-helix	35	28	26
β-sheet	28	27	22
β-turn	16	11	15

[a] The remaining percentage not predicted falls into the category of random coil or unspecified structure. [b] Not predicted.

Quantitation of Bound Flavins. The C415H mutant and wild-type NOS were analyzed for flavin content by reverse-phase separation using HPLC and absorbance detection. Both the wild-type and mutant enzymes contained 1.0 ± 0.1 equiv of FAD/subunit. The wild-type enzyme contained 0.8 ± 0.1 equiv of FMN/subunit, and the mutant contained 0.9 ± 0.1 equiv of FMN/subunit (Table 3).

Table 2: Pteridine Content of nNOS[a]

nNOS	protein (pmol)	biopterin[b] (pmol)	pterin[b] (pmol)	total (pmol)	stoichiometry (equiv)
wild-type	62.5	3.0	49.6	52.6	0.84
C415H	138.0	nd[c]	nd[c]		
C415A	75.0	nd[c]	nd[c]		

[a] Calculations were based on a calculated MW of 160 000. [b] Each measurement was made in duplicate. [c] None detected. The level of detection is 0.5 pmol/50 μL injection.

Table 3: Flavin Content of nNOS[a]

nNOS	protein (pmol)	FAD[b] (pmol)	stoichiometry (equiv)	FMN[b,c] (pmol)	stoichiometry (equiv)
wild-type	102	107 ± 1	1.0 ± 0.0	79 ± 8	0.8 ± 0.1
C415H	42	40 ± 4	1.0 ± 0.1	39 ± 4	0.9 ± 0.1

[a] Calculations were based on a calculated MW of 160 000. [b] Each measurement was made at least in triplicate. [c] A small amount of FMN was found to decompose to riboflavin in both enzyme and standard samples. The reported values were corrected to include the measured riboflavin: 6 ± 2 pmol in the wild-type and 4 ± 2 pmol in the mutant sample.

Table 4: NADPH Oxidation[a]

assay	wild-type nNOS (nmol mg^{-1} min^{-1})			C415H	C415A
enzyme + NADPH	*b*	*b*	*b*	*b*	*b*
+CaM	750 ± 30	1070 ± 50	1000 ± 130	*b*	*b*
+L-Arg	400 ± 20	450 ± 70	480 ± 20	*b*	*b*

[a] Each measurement was made in duplicate or triplicate. [b] Does not exceed the level of NADPH autoxidation of 20 nmol/min.

Activity of Wild-Type and C415 Mutants of nNOS. The rate at which the wild-type nNOS catalyzed the oxidation of NADPH was compared to each of the two mutants, C415H and C415A (Table 4). In each case, the assays were carried out with three different preparations of purified enzyme. Each of the three separate assays are shown for the wild-type enzyme. No NADPH oxidation was observed unless CaM was added. When L-arginine was added to the assay, the rate of oxidation decreased to approximately half of the original value. With the addition of L-arginine, the wild-type enzyme was able to proceed through normal turnover and synthesize ·NO. The C415H mutant and C415A mutant did not catalyze the oxidation of NADPH, although spectral studies demonstrated that NADPH was able to partially reduce the flavins bound to the enzyme (data not shown). The addition of CaM, and subsequent addition of L-arginine, did not affect either mutant's ability to catalyze the oxidation of NADPH. Neither C415 mutant could synthesize ·NO as detected by the hemoglobin assay.

With the addition of the electron acceptor cytochrome *c*, the two C415 mutants were able to utilize NADPH and reduce cytochrome *c* at rates similar to those of the wild-type nNOS. Assays were carried out with three independent preparations of each form of nNOS, and the mean specific activities of cytochrome *c* reduction are reported in Table 5. Initially the enzyme was incubated with cytochrome *c*, and no reduction was detected. Upon the addition of NADPH, a small rate of reduction was observed. When CaM was added after NADPH, the rate increased by a factor of approximately 15. The rate of reduction was decreased slightly upon the addition of L-arginine. This phenomenon was most obvious with the wild-type nNOS.

Table 5: Cytochrome *c* Reduction by NADPH[a]

assay	wild-type nNOS (μmol mg^{-1} min^{-1})	C415H nNOS (μmol mg^{-1} min^{-1})	C415A nNOS (μmol mg^{-1} min^{-1})
enzyme + cyt *c*	*b*	*b*	*b*
+NADPH	1.2 ± 0.2[c]	1.3 ± 0.2	1.0 ± 0.2
+CaM	21 ± 4	15 ± 4	16 ± 4
enzyme + Arg + cyt *c*	*b*	*b*	*b*
+NADPH	1.12 ± 0.02	1.4 ± 0.2	1.0 ± 0.1
+CaM	16 ± 6	14 ± 3	17 ± 5

[a] Each measurement was made in duplicate or triplicate. [b] Does not exceed the background level of cytochrome *c* reduction of 0.02 μmol mg^{-1} min^{-1}. [c] The errors shown are the standard deviations of the means for the three independent preparations of each enzyme. Within a single preparation, the average standard deviation for a given set of conditions was 6%, with a minimum of 0.9% and a maximum of 26%.

DISCUSSION

Site-directed mutagenesis studies with nNOS have been carried out in an effort to verify the assignment of C415 as the axial ligand to the heme moiety. As mentioned above, in this report and in a previous publication (Richards & Marletta, 1994), the focus has been on studying C415 mutants in the full-length enzyme expressed in a baculovirus system. In another site-directed mutagenesis report, a C415H mutation was made in an N-terminal heme-binding segment of nNOS expressed in *E. coli* (McMillan & Masters, 1995). The choice of C415 as the putative axial ligand was initially made by amino acid sequence comparisons with the other cloned NOSs and with the family of cytochrome P450s (McMillan et al., 1992; White & Marletta, 1993) and by molecular modeling (Renaud et al., 1993).

In this report, despite the apparent lack of bound heme in the C415H nNOS spectrum, reduction with dithionite in the presence of CO revealed a small Soret with a λ_{max} of 417 nm (Figure 3). This wavelength corresponds with the Soret bands at 419−424 nm reported for CO-complexed ferrous hemoglobins and myoglobins (Adar, 1979; Egeberg et al., 1990), which also contain histidine as the proximal ligand. As in the heme-binding domain mutant reported by Masters, the heme of the C415H mutant in the full-length enzyme presented here is quite labile. After subjecting the C415H enzyme to gel filtration, no bound heme could be detected using the pyridine hemochromagen assay (Richards & Marletta, 1994).

Because of the potential to do mechanistic studies with a C415 mutant with a bound but catalytically inert heme, a C415A mutant was generated and also expressed as the full-length enzyme in baculovirus. Because of its small size, alanine seemed unlikely to interfere with heme binding. A C415S mutant was also generated, which displayed properties that were essentially identical to the C415H and C415A mutants (unpublished results). As can be seen in Figure 1, the UV−visible spectra of both the C415H and C415A nNOS mutants are essentially identical. They both contain the general spectral features of an oxidized flavoprotein, namely, absorbance from approximately 380 to 500 nm, which is similar to that seen in cytochrome P450 reductase (Vermilion & Coon, 1978a; Yasukochi et al., 1979). In addition, the absorbance from 500 to 650 nm is characteristic of a neutral flavin semiquinone (Figure 1) (Massey & Palmer, 1966). Upon quantitative oxidative titration with ferricyanide, the absorbance due to the semiquinone species decreased while the absorbance of the oxidized flavins increased (Figure 2, top panel), suggesting that the enzyme as isolated is approximately one-electron-reduced from the fully oxidized form (Figure 2, bottom panel). The neutral flavin semiquinone absorbance has been observed in wild-type nNOS (Richards & Marletta, 1994) and in cytochrome P450 reductase (Vermilion & Coon, 1978a; Yasukochi et al., 1979). Complete reduction was effected by the addition of sodium dithionite, as evinced by the bleaching of the flavin spectrum. The properties and mechanistic implications will be discussed in detail below.

McMillan and Masters (1995) have reported the *E. coli* expression of the reductase domain of nNOS. The UV−visible spectrum of the reductase domain following ferricyanide oxidation is typical of an oxidized flavoprotein. Abu-Soud et al. (1994) recently published a spectrum of nNOS that had been dialyzed against 2 M urea for greater than 30 h. This enzyme preparation, labeled "apo-NOS," had lost the bound heme and H_4B which resulted in a spectrum with a characteristic oxidized flavoprotein absorbance. The spectrum of apo-NOS did not contain an absorbance representative of a neutral flavin semiquinone, as was observed with the C415 mutants reported here. The urea dialysis over an extended time period could have disrupted the protein and destabilized the semiquinone species. Activity comparisons between apo-NOS and the C415 mutants will be discussed below.

Since neither mutant had any significant level of bound heme, the influence of these single point mutations on the overall protein structure was investigated. CD spectra were taken of each mutant and compared to the wild-type enzyme in an effort to address potential folding abnormalities. When the spectra in Figure 4 are overlaid, they appear to be essentially identical except for slight differences in the absorbance magnitude, which can attributed to small variations in the protein concentrations of each sample. The spectral data were also analyzed by three different programs that predict secondary structure (Table 1). Each program was analyzed for its ability to accurately predict secondary structure for a select group of previously crystallized proteins (Dr. Norma Greenfield, UMDNJ, Piscataway, NJ, personal communication). The SELCON program generally gave good predictions, but often had errors in β-structure predictions. Therefore, the predictions generated by the K2D program (good for β-sheet predictions) and the CONTIN program (good for β-turn predictions) are also included. Two important conclusions can be drawn from the predictions listed in Table 1. First, within each program, very similar secondary structure predictions are made for each of the mutants compared to the wild-type nNOS. This provides quantitative evidence to support the observation that the three CD spectra in Figure 4 are essentially identical. The two mutants, therefore, do not appear to have undergone any significant structural perturbations as a result of the mutation

at C415 that were detectable by CD. Second, when the structural predictions are compared among programs, it is clear that the method of analysis affects the prediction outcomes, especially with β-sheet and β-turn predictions.

In addition to the lack of heme binding, both mutants had a dramatically reduced affinity for H_4B. It has been hypothesized (Hevel & Marletta, 1992; Campos et al., 1995) that H_4B plays a catalytic role in the conversion of L-arginine to ·NO and citrulline. An nNOS mutant with a nonfunctional or nonexistent heme moiety might have helped to answer this question if H_4B were still bound to the enzyme. Spectral work with the inducible isoform of NOS (White and Marletta, unpublished results) and nNOS (Wang et al., 1995) has shown that H_4B directly influences the spectral properties of the heme. Given the spectral evidence of a connection between H_4B and the heme, it was not clear whether a mutant deficient in heme binding would still bind H_4B. It was determined, in fact, that neither mutant contained detectable amounts of H_4B (Table 2). For comparison, the biopterin content of wild-type nNOS was measured. The highest previous reports of stoichiometry for wild-type nNOS have been 0.5 mol of biopterin per mole of 160 kDa subunit of enzyme (Schmidt et al., 1992; Harteneck et al., 1994), whereas the inducible isoform of NOS is reported to have an H_4B stoichiometry of 1 per 130 kDa subunit (Hevel & Marletta, 1992). After gel filtration, the wild-type nNOS had a stoichiometry of 0.84 mol of biopterin per nNOS monomer. Therefore, it appears that, like the inducible isoform, the constitutive nNOS also contains a 1:1 stoichiometry of pteridine to nNOS monomer.

Despite the observation that the C415 NOS mutants bound neither heme nor H_4B, HPLC analysis indicated that the enzyme still had both flavins tightly associated. The oxidation of C415A mutant NOS with ferricyanide resulted in the disappearance of the flavin semiquinone absorbances at 345 and 585 nm and an increase in the oxidized flavin absorbances (maxima at 385 and 456 nm and a shoulder at 482 nm), with isosbestic points at 367 and 511 nm. The absorbances at 345 and 585 nm are characteristic of a neutral flavin semiquinone. Complete oxidation occurred with a stoichiometry of approximately 1 mol of ferricyanide/mol of subunit (or 1 mol of ferricyanide/2 mol of flavin). The interpretation of these observations is facilitated by a comparison to extensive studies on cytochrome P450 reductase.

Cytochrome P450 reductase forms an air-stable, one-electron-reduced species (Vermilion & Coon, 1978a; Yasukochi et al., 1979) that has been identified as a neutral radical of FMN (FAD−FMNH·). This identification was made by comparison to the neutral radical formed from FMN-depleted reductase (FADH·), which shows an absorbance band at 592 nm (rather than 585 nm) and no shoulder at 630 nm (Vermilion & Coon, 1978b). Anaerobic reduction of oxidized cytochrome P450 reductase with 2 equiv of NADPH results in the three-electron-reduced enzyme (FAD·−$FMNH_2$) (Vermilion & Coon, 1978b). The inability of stoichiometric NADPH to reduce cytochrome P450 reductase to the four-electron-reduced state and the relative stability of the one-electron-reduced species to oxidation led to the mechanistic proposal that the reductase cycles between one- and three-electron-reduced states (FAD−FMNH· and FAD·−$FMNH_2$) and transfers electrons to cytochrome P450 from fully reduced FMN ($FMNH_2$). Fully reduced reductase was prepared by reduction with an NADPH-generating system or dithionite. Aerobic oxidation of partially or fully reduced enzyme restores the spectrum of the air-stable semiquinone (Vermilion & Coon, 1978a; Yasukochi et al., 1979).

This behavior and the mechanistic scheme are consistent with the preliminary results for the C415 mutants. As with cytochrome P450 reductase, reduction of C415A NOS with dithionite results in a largely featureless electronic spectrum with a shoulder at 427 nm (data not shown). Reoxidation of the fully reduced enzyme by air restores the basic features of the one-electron-reduced enzyme, although somewhat more semiquinone is evident a short time after oxidation (<2 h) when compared to that in the enzyme as isolated (data not shown). Addition of NADPH to C415 mutant NOS (with CaM bound) partially reduces the enzyme (data not shown), consistent with the mechanistic scheme described for cytochrome P450 reductase. Comparison of the electronic spectroscopy of P450 reductase (Vermilion & Coon, 1978a; Yasukochi et al., 1979) and C415A NOS (Figure 2) does not permit the assignment of the neutral semiquinone specifically to one of the flavins.

The ability of wild-type nNOS to catalyze the reduction of exogenous electron acceptors by NADPH has been reported from several laboratories (Klatt et al., 1992; Baek et al., 1993; Sheta et al., 1994). To determine if the C415H and C415A mutants retained the reductase activity of wild-type NOS, cytochrome *c* assays were performed. While there were small variations from one enzyme preparation to another, the two mutants retained most of the cytochrome *c* reductase activity seen with the wild-type nNOS (Table 5). Without the addition of CaM, the reduction of cytochrome *c* was quite slow, approximately 1 μmol mg^{-1} min^{-1} for both the wild-type and mutants. When CaM was added, the rate increased by a factor of approximately 15. The addition of 50 μM L-arginine to the cytochrome *c* reduction assays had different effects on the wild-type and mutant enzymes (Table 5). For the wild-type nNOS, the rate of reduction of cytochrome *c* was decreased, in agreement with observations made previously (Sheta et al., 1994). By contrast, the addition of L-arginine to assays with the C415 mutants did not change the rate of cytochrome *c* reduction.

A reasonable hypothesis for the increase in the rate of cytochrome *c* reduction caused by the binding of CaM to wild-type nNOS had been that CaM enabled the enzyme to transfer electrons from the flavins in the reductase domain to the heme, where an accelerated reduction of cytochrome *c* could take place. However, this mechanism is impossible with the two mutants because no heme is present, yet a similar increase in the rate of reduction was observed. This would suggest that in both the wild-type and the mutants essentially all of the catalytic reduction of cytochrome *c* is mediated via the flavins. In the presence of CaM, the wild-type enzyme does reduce cytochrome *c* at a slightly faster rate than the mutants. This increase could be due to the small contribution of a heme-dependent pathway. We conclude from these new data that the conformational change induced by CaM (Sheta et al., 1994) does more than couple the reductase domain with the heme-binding domain. It appears that some redox or conformational change takes place in the reductase domain, increasing the rate of electron transfer from NADPH via the flavins to cytochrome *c*.

When the cytochrome *c* assay was made partly anaerobic, the rate was substantially decreased, and the addition of CaM

had no further effect on the rate (data not shown). The addition of superoxide dismutase, 600 units/mL, to an aerobic assay had no effect on the rate of cytochrome *c* reduction by NADPH. At a higher concentration of SOD, 1200 units/mL, a small decrease (~20%) in the rate of reduction was observed. The role of superoxide in the reduction of cytochrome *c* has been somewhat controversial: SOD has both inhibited and failed to inhibit NOS-catalyzed cytochrome *c* reduction by NADPH (Sheta et al., 1994; Abu-Soud et al., 1994). The interpretation of these results with SOD is complicated by the fact that SOD is present in concentrations that far exceed that of wild-type or mutant NOS. Despite the variability of the SOD results, however, the dramatic decrease in cytochrome *c* reduction in the absence of oxygen suggests that an oxygen product does mediate the reduction of cytochrome *c* by the nNOS reductase domain.

In the absence of an exogenous electron acceptor, neither mutant could catalyze the aerobic oxidation of NADPH (Table 4). With the wild-type nNOS, the addition of CaM initiated the oxidation of NADPH, by coupling the flavins with the heme (Abu-Soud & Stuehr, 1993) where the electrons could be transferred to molecular oxygen. The addition of L-arginine enabled the enzyme to produce citrulline and •NO, while consuming NADPH at a rate which is roughly half that observed in the absence of L-arginine. The C415 mutants did not contain heme or pteridine, and they were unable to catalyze the aerobic oxidation of NADPH, even in the presence of CaM. The apparent requirement for heme in the catalytic aerobic oxidation of NADPH is in marked contrast to the lack of dependence on heme for cytochrome *c* reduction: both reactions require molecular oxygen, but the interaction of oxygen and the NOS flavins is not sufficient to effect catalytic oxidation of NADPH unless cytochrome *c* is present. These results suggest that either cytochrome *c* induces a minor structural change in the reductase domain, which allows molecular oxygen access to the flavins, or cytochrome *c* drives the oxidation of the flavins by trapping superoxide. Cytochrome P450 reductase also has very low levels of NADPH oxidase activity unless an exogenous electron acceptor is present (Vermilion & Coon, 1978a).

It is interesting to note that by making apo-NOS, Abu-Soud et al. (1994) observed similar NADPH oxidase activity and cytochrome *c* reduction rates as were seen with the C415 mutants. The mutagenesis approach and the urea denaturation approach both generated an NOS deficient in heme. Results from these preparations suggest a new role for CaM. Not only does CaM couple electron transfer between the reductase domain and the heme domain (Abu-Soud & Stuehr, 1993), but it also accelerates the rate by which NADPH reduces the flavins. In addition to cytochrome *c* reduction, which gives indirect evidence of this accelerated rate, Abu-Soud et al. (1994) used stopped-flow spectrophotometry to monitor directly the increased rate of flavin reduction catalyzed by NOS in the presence of CaM.

In conclusion, the generation of a C415A mutant resulted in an enzyme that did not have the ability to bind heme. Both the C415H and C415A mutants bound FAD and FMN and displayed UV−visible spectra typical of a flavoprotein, such as the analogous cytochrome P450 reductase. The CD spectra provided important evidence that mutating C415 did not induce major structural perturbations. The ability of the mutants to catalyze the reduction of cytochrome *c* demonstrated that the reductase portion of each mutant is intact. The increase in the rate of cytochrome *c* reduction upon the addition of CaM revealed that CaM affects the rate of electron transfer within the reductase domain, a conclusion based on the fact that the mutants have little or no heme bound. Finally, the small Soret detected at 417 nm, when the purified C415H mutant was reduced in the presence of CO, provides further evidence that C415 is the axial ligand to the heme.

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