The FAD-shielding Residue Phe$^{1395}$ Regulates Neuronal Nitric-oxide Synthase Catalysis by Controlling NADP$^+$ Affinity and a Conformational Equilibrium within the Flavoprotein Domain*

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Phe$^{1395}$ stacks parallel to the FAD isooxalazine ring in neuronal nitric-oxide synthase (nNOS) and is representative of conserved aromatic amino acids found in structurally related flavoproteins. This laboratory previously showed that Phe$^{1395}$ was required to obtain the electron transfer properties and calmodulin (CaM) response normally observed in wild-type nNOS. Here we characterized the F1395S mutant of the nNOS flavoprotein domain (nNOSr) regarding its physical properties, NADP$^+$ binding characteristics, flavin reduction kinetics, steady-state and pre-steady-state cytochrome c reduction kinetics, and ability to shield its FMN cofactor in response to CaM or NADP(H) binding. F1395S nNOSr bound NADP$^+$ with 65% more of the nicotinamide ring in a productive conformation with FAD for hydride transfer and had an 8-fold slower rate of NADP$^+$ dissociation. CaM stimulated the rates of NADPH-dependent flavin reduction in wild-type nNOSr but not in the F1395S mutant, which had flavin reduction kinetics similar to those of CaM-free wild-type nNOSr. CaM-free F1395S nNOSr lacked repression of cytochrome c reductase activity that is typically observed in nNOSr. The combined results from pre-steady-state and EPR experiments revealed that this was associated with a lesser degree of FMN shielding in the NADP$^+$-bound state as compared with wild type. We conclude that Phe$^{1395}$ regulates nNOSr catalysis in two ways. It facilitates NADP$^+$ release to prevent this step from being rate-limiting, and it enables NADP(H) to properly regulate a conformational equilibrium involving the FMN subdomain that controls reactivity of the FMN cofactor in electron transfer.

Nitric-oxide synthases (NOS)$^1$ are homodimeric enzymes that synthesize NO via oxidation of L-Arg and participate in various physiological and pathological settings (1–6). In the NOS polypeptide, an N-terminal oxygenase domain is linked to a C-terminal reductase domain by a calmodulin (CaM)-binding sequence. The NOS oxygenase domain contains binding sites for iron protoporphyrin IX (heme), (6R)-5, 6, 7, 8-tetrahydro-L-biotin (H$_4$B), and L-Arg and is the site where oxidative catalysis takes place. The NOS reductase domain (NOSr) contains binding sites for FMN, FAD, and NADPH and functions to transfer reducing equivalents from NADPH to the oxygenase domain.

NOSr belongs to a small family of structurally related dual-flavin reductases that also includes cytochrome P450 reductase (CYP) (7, 8), methionine synthase reductase (9), and novel reductase-1 (10). These proteins are comprised of separate FMN and FAD/NADPH modules attached by a flexible hinge region (11, 12). It is believed that these reductases are the product of gene fusion because their FMN and FAD/NADPH subdomains show a high similarity to flavodoxins (13) and ferredoxin NADP$^+$ reductases (FNR) (14), respectively. In NOSr and related flavoproteins, the FAD receives electrons from NADPH via hydride transfer and then sequentially passes the electrons to the FMN cofactor. Ultimately, it is the 2-electron reduced FMN hydroquinone that transfers an electron to the ferric heme in the NOS oxygenase domain (15). This electron transfer allows the heme to bind dioxygen and initiate its reductive activation in conjunction with H$_4$B as is required for NO synthesis (16). The FMN hydroquinone in NOSr can also reduce external electron acceptors such as cytochrome c (17), and this activity is widely used as a tool to study electron transfer by NOSr. The binding of CaM to NOSr elicits responses in the enzyme that include activation of the ferric heme reduction (18, 19) and increasing the rate of cytochrome c reduction. The rate of ferric heme reduction by NOSr is very important because it and the rates of ferric heme-NO dissociation and ferrous heme-NO oxidation are the three key kinetic parameters identified by this laboratory that define the characteristics of NO release by any given NOS (20, 21).

The mechanism through which structural elements in NOSr regulate its electron transfer is a topic of current interest. The related flavoprotein CYP has been extensively characterized with respect to its electron transfer mechanisms (22–26). Although these studies serve as a guide, it is important to appreciate that in NOSr there are additional complexities that do not exist in related flavoproteins. For example, rates of NOSr electron transfer reactions are repressed relative to most other related flavoproteins, but this repression is relieved upon CaM binding (27). Studies of NOSr and its unique regulatory elements typically utilize purified NOSr domains constructed to include their adjacent CaM-binding motifs so that the native electron transfer and CaM-response profiles of the proteins are retained (28–33). Besides CaM binding, other components involved in controlling NOSr electron transfer include an auto-

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‡ The abbreviations used are: NOS, nitric-oxide synthase; H$_4$B, 6(R)-tetrahydrobiotin; CaM, calmodulin; nNOS, neuronal NOS; NOSr, isolated NOS reductase domain; DytIII-HEDTA, dysprosium-hydroxyethylglycineethylethylendiaminotriacetic acid complex; mW, milliwatt; EPPS, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid; CYP, cytochrome P450 reductase.
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inhibitory insert within the FMN module (34–37), a C-terminal extension (38–40), multiple phosphorylation sites (41–46), a loop within the connecting domain (47), and NADP⁺ binding (30). Although their mechanisms of action remain speculative, these elements are thought to act in concert to positively and negatively regulate electron transfer by NOSr.

This laboratory recently reported that Phe₁₁₃⁹⁵ in rat neuronal nNOS is involved in redox regulation (48). Phe₁₁₃⁹⁵ is representative of a conserved group of aromatic amino acids that shield the re face of the FAD isoalloxazine ring in NOS, related flavoproteins, and simpler FNR proteins (12, 49, 50). The aromatic side chains of these shielding residues must move away from the FAD isoalloxazine ring to allow a productive binding interaction with the nicotinamide ring of NADPH that is essential for hydride transfer to FAD (12). In addition to an influence on NADPH binding specificity, we found that the aromatic side chain of Phe₁₁₃⁹⁵ was needed to repress electron transfer in CaM-free nNOS and to fully relieve the repression upon CaM binding (48). The unexpected significance of Phe₁₁₃⁹⁵ prompted us to study our most interesting mutation, F1395S, in the isolated nNOSr in order to understand better how Phe₁₁₃⁹⁵ helps regulate electron transfer and catalysis by nNOS.

EXPERIMENTAL PROCEDURES

General Methods and Materials—UV-visible spectra and steady-state kinetic data were obtained by using a Hitachi U-2000 or Cary 100 Bio-spectrophotometer. Single wavelength stopped-flow kinetic experiments were performed by using a Hi-Tech (Salisbury, UK) SF-51MX instrument equipped for anaerobic work and photomultiplier detection. Full spectra stopped-flow experiments were performed by using a Hi-Tech SF-61 instrument equipped for anaerobic work and rapid-scanning diode array detection. Data from multiple identical stopped-flow experiments were averaged to improve the signal-to-noise ratio and fit exponential functions using software (IS-2) provided by the instrument manufacturer. The buffer used for all experiments and protein purifications (Buffer A), unless noted otherwise, contained 40 mM potassium phosphate, pH 7.6, 10% glycerol, and 150 mM NaCl. Samples were made anaerobic when necessary by repeated cycling of vacuum followed by a positive pressure of catalyst-deoxygenated nitrogen in an airtight anaerobic cuvette or by the addition of glucose oxidase (10 units/ml), catalase (134 units/ml), and glucose (2–3 mM) to the sample. Larger volumes of buffer were made anaerobic by extended bubbling of catalyst-deoxygenated nitrogen through the liquid. Wild-type nNOSr was prepared for use by oxidizing the purified air-stable semiquinone form with potassium ferricyanide followed by passing the mixture through a PD-10 desalting column. F1395S nNOSr was prepared for use by briefly incubating with 1 mM 2'-AMP and passing the mixture through a PD-10 column, unless noted otherwise. The concentration of nNOSr samples was determined by using an extinction coefficient of 22,900 M⁻¹ cm⁻¹ at 457 nm for the fully oxidized form (29). Graphing of all data and curve fitting of data not obtained in stopped-flow experiments was done by using Origin software. Human CaM was expressed with the pACYC plasmid and purified with standard procedures utilizing phenyl-Sepharose chromatography. 5-Deazariboflavin, prepared using the method of Yoneda and co-workers (51, 52), was obtained from Amershams Biosciences. NADPH was obtained from Alexis Biochemicals. All other reagents were obtained from Sigma and used without further purification.

Generation of Wild-type and F1395S nNOSr Proteins—The wild-type rat nNOSr construct (Met₆⁹₅ to Ser₁₄₂⁹) was created by using a PCR-ligation approach (primer 1 (AGATTCCATCATGGTCACATATAAC) and primer 2 (CCCCCAGCGACAGACATCACACG)) that inserted an NdeI site adjacent to the translation start site at Met₆⁹₅ and primer 2 (CCCCCAGCGACAGACATCACACG) that inserted an NdeI site adjacent to the translation start site at Met₆⁹₅ and primer 2 (CCCCCAGCGACAGACATCACACG). The PCR product fragment so generated and pCWori vector containing full-length rat nNOS DNA were both digested with NdeI and SpHl, and the resulting 256-bp PCR fragment was cloned into the double-digested pCWori/nNOS vector at the restriction sites NotI and XbaI. For the F1395S mutant, rat nNOSr cDNA was inserted into the pCWori vector by using its 5' NdeI and 3' XbaI restriction sites. The F1395S substitution in the nNOSr cDNA was engineered by subcloning a PCR-generated fragment from pCWori/nNOSr with a 5'-oligonucleotide containing the desired mutation. This mutated nNOSr cDNA fragment, coding from the KpnI unique restriction site at position 4170 to the XbaI restriction site at position 4441, was amplified using the following primers: F1395S forward primer, AAGGTTACCAAGGGA-CATCTGGAGGTCCCTCAGAACG; F1395S reverse primer, AAA-TCTAGAACGGACGAGACACAGGACGACGACAG. The restriction sites are indicated by underlines, and the mutation site is denoted with boldface. The PCR product and wild-type pCWori/nNOSr were digested by both KpnI and XbaI restriction enzymes. The double-digested fragment of wild-type pCWori/nNOSr was replaced with the double-di- gested PCR fragment to generate the recombinant plasmid. After DNA sequencing to confirm the desired results, E. coli BL21 (DE3) cells were transformed with the nNOSr plasmids and selected with ampicillin for protein expression. When transformants were available, the cells were also transformed with a pACYC plasmid containing human CaM and selected with chloramphenicol.

Protein Expression and Purification—The nNOSr proteins and CaM (when present) were expressed in E. coli. The cells were lysed and centrifuged, and the protein was precipitated from the supernatant with ammonium sulfate in the presence of CaCl₂ using a procedure described previously (53). The resulting protein pellets were resuspended in Buffer A containing phenylmethylsulfonyl fluoride (1 mM) and applied to a 2',5'-ADP-Sepharose column pre-equilibrated with Buffer A plus β-mercaptoethanol (5 mM), FAD (2 μM), FMN (2 μM), and EDTA (0.5 mM) when necessary to remove the co-expressed CaM. The bound protein was washed extensively with the equilibration buffer and then eluted with the same buffer containing 10 mM NADP. The eluted protein sample was immediately brought to a final concentration of 2 mM CaCl₂ and applied to a column of CaM-Sepharose pre-equilibrated with Buffer A plus 2 mM CaCl₂. The bound protein was washed extensively with the equilibration buffer and then eluted with Buffer A containing 3 mM EDTA. The resulting protein sample was concentrated and stored frozen in aliquots at −80 °C. The flavin content of the nNOSr proteins was obtained by boiling a known amount of nNOSr protein for 3 min followed by centrifugation and determination of the flavin concentration in the supernatant by using an extinction coefficient of 12.2 mM⁻¹ cm⁻¹ at 447 nm.

Steady-state Cytochrome c Reduction Assays—Cytochrome c reductase activities of the nNOSr proteins were determined by using an assay procedure as described previously (53) without the addition of superoxide dismutase or catalase.

Interaction between the nNOSr Proteins and NADP⁺—UV-visible spectral changes resulting from the association of NADP⁺ with 20 μM nNOSr proteins were observed by calculating a difference spectrum between the proteins in solution and the same protein sample after treatment with a 3-fold excess of NADP⁺. Determination of NADP⁺ apparent dissociation constant (Kₐ) values was done by titrating the nNOSr proteins (20 μM) with aliquots of a known concentration of NADP⁺ and recording the visible spectrum after a short (3–4 min) anaerobic delay at 20 °C. The mathematical derivation of Kₐ values attempted by plotting Δ(A₁₆₂ nm − A₇₀₀ nm) versus NADP⁺ concentration for each titration point and fitting the data by using nonlinear regression analysis to the protein-ligand complex model developed by Wang et al. (54). Rate constant (kₐ) values for the dissociation of NADP⁺ from the oxidized nNOSr proteins were determined by rapidly mixing a solution containing nNOSr (30–40 μM) and NADP⁺ (100 μM) with a solution of 2',5'-ADP (4 mM) in the stopped-flow instrument at 10 or 23 °C and fitting the absorbance decreases at 510 nm to single exponential functions.

An aerobic Bioluminescence Photoreduction—The nNOSr protein samples with and without a saturating amount of NADP⁺ (based on Fig. 2) were diluted to a final concentration of 3 μM in Buffer A containing 2 mM CaCl₂ and a catalytic amount of 5-deazariboflavin with care taken to shield the sample from ambient light. Each titration point was obtained by illuminating the sample with a commercial slide projector bulb for a fixed length of time (3–10 s) and then acquiring a visible spectrum after a 2–3-min equilibration. The titration was complete when further irradiation did not result in absorbance changes. The absorbance reduction of F1395S nNOSr in the presence of NADP⁺ yielded a stable reduced nNOSr/NADP⁺ charge transfer complex. The extinction coefficient of this complex (1200 μM⁻¹ cm⁻¹ at 700 nm) was estimated from a 25 μM sample of F1395S nNOSr protein that gave Δ(A₁₆₂ nm) = 0.030 at the end of the titration (1-mm cuvette).

The absorbance changes associated with NOSr flavin reduction by NADPH were recorded by rapidly mixing a solution of oxidized nNOSr (6–10 μM) containing either EDTA (1 mM) or CaCl₂ (2 mM) + CaM (18–24 μM) with a solution of 60–100 μM NADPH (excess NADPH) or a single molar
equivalent of NADPH at 10°C in the stopped-flow instrument. The maximum absorbance value for a given protein sample at 457 nm during single wavelength experiments was obtained by replacing the NADPH solution in one of the stopped-flow syringes with buffer only and recording additional mixing events. The individual rate constants associated with absorbance changes at 457 nm were first estimated by analysis of experiments of varying lengths. The final reported values were obtained by fitting an experiment on a time scale capturing all four rate constants to a quadruple exponential function such that the residuals were minimized and contained little or no systematic deviation between the fit curve and the actual data. Percent absorbance changes were calculated based on the percent absorbance change in the instrument dead time and the relative proportions of the ΔA values for each case obtained from the fitting program. Analysis of absorbance changes observed at 600 nm and correlation of these data with rapid-scanning stopped-flow diode array spectra were done as described (see “Results”).

Anaerobic Pre-steady-state Cytochrome c Reduction—A solution of nNOSr (16 μM), glycine (3 mM), 5-deazariboflavin (catalytic), and either EDTA (1 mM) or CaCl₂ (2 mM) was completely photoreduced in an anaerobic cuvette by using a commercial slide projector bulb until no changes in the UV-visible spectrum of the sample were observed upon further irradiation of the sample. The pre-reduced protein sample was rapidly mixed with a solution of cytochrome c (4 μM) at 10°C, and the absorbance changes at 550 nm were recorded. In some cases 1 mM NADPH was added to the pre-reduced protein sample, and the mixture was incubated at 10°C for at least 15 min prior to mixing. Absorbance data were fit to a single exponential function.

Oxidation of Reduced F1395S nNOSr—A solution of F1395S nNOSr protein (8 μM) containing either EDTA (1 mM) or CaCl₂ (2 mM) + CaM (20 μM) in air-saturated buffer was reduced by adding NADPH (160 μM) and then allowed to autoxidize at room temperature in an open cuvette while following the process at 457 nm and recording visible spectra at the indicated time points during the experiment (see “Results”).

Preparation of EPR Samples—All EPR experiments were carried out in 20 mM HEPES buffer, pH 7.4, with 25% glycerol (v/v). The nNOS protein concentrations used were 40 μM (20 μM) containing either EDTA (1 mM) or CaCl₂ (2 mM) + CaM (30 μM) was completely photoreduced in an anaerobic cuvette by using a commercial slide projector bulb until no changes in the UV-visible spectrum of the sample were observed upon further irradiation of the sample. The pre-reduced protein sample was rapidly mixed with a solution of cytochrome c (4 μM) at 10°C, and the absorbance changes at 550 nm were recorded. In some cases 1 mM NADPH was added to the pre-reduced protein sample, and the mixture was incubated at 10°C for at least 15 min prior to mixing. Absorbance data were fit to a single exponential function.

PREPARATION OF EPR SAMPLES—All EPR experiments were carried out in 20 mM HEPES buffer, pH 7.4, with 25% glycerol (v/v). The nNOS protein concentrations used were 40–60 μM. The dyrosprosin(III) complex with HEDTA (Dy(III)-HEDTA) was prepared by chelating DyCl₃·6H₂O with an excess (5:4) of HEDTA in the same buffer as the nNOS samples. The nNOS samples were prepared in the presence or absence of CaM (Sigma) with different concentrations of Dy(III)-HEDTA. The full-length nNOS samples contained 1 mM N₂-nitro-t-arginine methyl ester and H₂D with a molar ratio of 2:1 H₂D to nNOS. For all samples containing CaM, the molar ratio of CaM to nNOS was 2:1, and the Ca²⁺ concentration was 1–2 mM. Protein samples were treated with NADPH followed by air oxidation to generate the flavin semiquinone radical and then transferred to sealed EPR tubes. Sperm whale myoglobin (MbNO) was prepared as described previously (55) and used as a reference.

EPR Spectroscopy—EPR spectra were recorded at 35 K using a Varian E-9 EPR spectrometer equipped with an Oxford Instruments ESR-9 flowing helium cryostat. The microwave frequency was 9.28 GHz, modulation amplitude was 4 G at 100 kHz, and the field center was set as 3300 G. Microwave power saturation data were plotted and fit to the following empirical equation as described by Rupp et al. (56):

\[ S = KP₀ \left( 1 + \frac{P₀}{P₀ + b} \right)^{-\frac{1}{2}} \]  

(Eq. 1)

where S is the signal height; K is a proportionality factor; P is the microwave power; P₀ is the power required for half-saturation; and b is the inhomogeneity parameter which was set at 1 in this case (55).

RESULTS

Protein Expression and Purification—Given recent data contradicting the effects of CaM on flavin reduction kinetics (31), special consideration was given to the protein expression and purification methods used in this work. Proteolytic degradation of the CaM-binding site in nNOSr is often observed during its expression and purification (28, 31). One strategy to prevent the CaM-binding site co-expression of the nNOSr with CaM (30, 31) and subsequent purification in the presence of Ca²⁺. A final purification step involving CaM affinity chromatography can also increase homogeneity of the protein and ensure that the purified protein can reversibly bind CaM. The effectiveness of these principles is illustrated in Table I. WT nNOSr was expressed in the absence of CaM and purified using only 2',5'-ADP-Sepharose chromatography. Its steady-state cytochrome c reductase activity was then measured both before and after an additional purification step with a CaM-Sepharose affinity column. The 2',5'-ADP purified sample exhibited only a modest rate enhancement upon CaM binding, whereas the CaM affinity column purified fraction showed a CaM effect nearly double that of the original sample. Not surprisingly, the nNOSr protein fraction that did not bind to the CaM-Sepharose resin showed little or no CaM effect in this assay. Finally, qualitative analysis of nNOSr protein samples by SDS-PAGE showed that those prepared using the two sequential affinity chromatography steps had the greatest level of purity (data not shown).

To answer the question of whether or not co-expression with CaM has an effect on the properties of the final nNOSr produced, multiple batches of proteins were prepared that either had or had not been co-expressed with CaM. After their purification via sequential 2',5'-ADP-Sepharose and CaM-Sepharose affinity chromatography, no differences were found between the two types of proteins significant enough to account for the discrepancies suggested in the literature regarding the effect of CaM on stimulation of flavin reduction kinetics (31). Because the nNOSr proteins co-expressed with CaM were obtained with higher yields and had a slightly higher CaM-dependent rate enhancement in the cytochrome c reductase assay, only nNOSr proteins that were co-expressed with CaM and purified according to the dual affinity chromatography method were used for the remainder of this work. Yields of purified proteins were at least 10 mg/liter culture, and flavin analysis showed that they contained the proper 2:1 flavin-to-protein stoichiometric ratio.

Steady-state Cytochrome c Reductase Activities—Cytochrome c reductase activities of WT and F1395S nNOSr proteins prepared according to the final procedure described above are listed in Table II. The WT protein exhibited a 9–10-fold rate enhancement upon CaM binding. In contrast, the activity of the CaM-free F1395S mutant was nearly 4-fold greater than that of WT, indicating a poor repression of this catalytic activity in the mutant. Additionally, there was little or no rate enhancement upon binding CaM to F1395S nNOSr. Similar behavior was observed previously for the full-length F1395S nNOS (48).

Interaction of NADP⁺ with nNOSr Proteins—Full-length F1395S nNOS has altered nicotinamide cofactor binding properties as reflected by decreased apparent Kₘ values for NADPH and NADH (48). In oxidized FNR (49, 57) and CYPR (50) enzymes, occupancy of the nicotinamide binding adjacent to the FAD isoxaloazine in a productive conformation for hydride transfer causes a shift in the flavin absorbance spectrum. Difference spectra between the NADP⁺-bound and -free forms exhibit a characteristic peak centered near 510 nm. We characterized the binding of NADP⁺ to oxidized WT and F1395S nNOSr and then utilized the observed spectral changes to compare their NADP⁺ affinities, amounts of productive binding, and binding kinetics. Difference spectra obtained from the binding of NADP⁺ to the nNOSr proteins are shown in Fig. 1.

### Table I

| Protein | CaM (ratio) | Activity | S.D. in |
|---------|-------------|----------|---------|
| Original ADP-purified | 481 ± 79 | 1504 ± 140 (3.1) |
| CaM-binding fraction | 711 ± 114 | 4188 ± 358 (5.9) |
| Non-CaM-binding fraction | 755 ± 34 | 859 ± 67 (1.1) |

Activities were determined at 23°C as described under "Experimental Procedures" and reported as turnover number (min⁻¹) ± S.D. in each case.
Table II

| Turnover numbers for steady-state cytochrome c reductase activities of nNOSr proteins |
|---------------------------------|------------------|
|                                 | −CaM             | +CaM             |
| WT nNOSr                        | 451 ± 23         | 4082 ± 488       |
| F1395S nNOSr                    | 1771 ± 89        | 2044 ± 300       |

Panel A represents a control experiment that contained FAD in solution and no protein. As shown, a spectral perturbation occurred in the 400–500-nm range upon addition of NADP⁺. This provides a measure of the intrinsic interaction between the two compounds in the absence of protein. Thus, any additional absorbance changes occurring upon addition of NADP⁺ to nNOSr can be attributed to its binding with the enzyme. In addition, some mutant FNR enzymes that have an increased affinity for NADP⁺ co-purify with an equivalent of NADP⁺ and thus require treatment with excess 2'-AMP and gel filtration to remove the bound cofactor (49). Therefore, as shown in Fig. 1, spectral properties of the nNOSr proteins were examined with and without preincubation with 2'-AMP to determine whether either of them also contained bound NADP⁺ after purification.

Fig. 1, panel B, shows that a spectral shift centered near 510 nm occurred in WT nNOSr along with some modulation of the spectrum between 400 and 500 nm when excess NADP⁺ was added regardless of whether nor not the sample was treated with 2'-AMP. A similar difference spectrum was obtained for the F1395S mutant in Fig. 1, panel C, but only after it had been incubated with 2'-AMP and passed through a PD-10 desalting column. Thus, both WT and F1395S nNOSr proteins exhibited similar spectral shifts that are characteristic of NADP⁺ binding in the productive conformation to FNR-like proteins. However, the F1395S mutant also showed an increased affinity for NADP⁺ as evidenced by its being purified fully in the NADP⁺-bound form.

The WT and F1395S nNOSr proteins at equal concentration (20 μM) were then titrated with NADP⁺, and the absorbance increase at 510 nm was recorded. Fig. 2 (panel A) shows that the F1395S mutant achieved an ~3-fold greater total absorbance change than WT upon saturation, indicating that the mutant bound NADP⁺ with a greater percentage of the nicotinamide ring held in the productive conformation with the FAD isoalloxazine ring. CaM binding did not affect the fraction of WT nNOSr having NADP⁺ bound in the productive conformation (Fig. 2, panel B). An attempt was made to fit the spectral titration curves to a model for protein-ligand binding (54) that does not place constraints on relative protein and ligand concentrations. The data from the WT nNOSr fit well to this model giving an apparent Kd value of 5.9 ± 1.1 μM for NADP⁺ (Fig. 2, panel A), consistent with values previously reported for nNOSr and plant FNR (33, 57). Note that this apparent Kd value is based only on the fraction of NADP⁺ bound in the productive conformation and thus reflects an equilibrium between productively bound NADP⁺ and all other forms (nonproductively bound, free in solution, etc.) of NADP⁺. The affinity of F1395S nNOSr toward binding NADP⁺ in the productive conformation was so high that its titration curve was essentially linear, and the protein was saturated with the addition of 1 eq of NADP⁺ (Fig. 2, panel A). These results were also observed in similar mutants of plant FNR (57), and under such conditions an apparent Kd value using the ligand binding model cannot be calculated. Regardless, it is clear that F1395S nNOSr has a higher affinity toward binding NADP⁺ in the productive conformation and does so to a significantly greater extent than WT nNOSr.

Stopped-flow kinetic measurements of NADP⁺ binding were attempted to complement the equilibrium data. Unfortunately, when we tried to measure the association rate (k_on) of NADP⁺ with the nNOSr proteins at 10 °C, much of the spectral gain at 510 nm associated with NADP⁺ binding took place in the dead time of the instrument. Measurement of the NADP⁺ release rate (k_off), however, was successful. As shown in Fig. 3, mixing the NADP⁺-bound proteins with an excess of 2',5'-ADP resulted in time-dependent absorbance decreases at 510 nm. The amount of 2',5'-ADP needed for this experiment was deter-
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Fig. 3. Kinetics of dissociation of NADP⁺ from WT and F1395S nNOSr. The NADP⁺-saturated nNOSr proteins were rapidly mixed with an excess of 2',5'-ADP in the stopped-flow instrument, and the absorbance decrease at 510 nm was recorded as described under “Results” and “Experimental Procedures.” The curves were fit to single exponential functions yielding the following kₛₒₜ rates: WT (10 °C), 64.1 ± 0.6 s⁻¹; F1395S (10 °C), 8.2 ± 0.01 s⁻¹. The calculated best fit curves are plotted as dotted lines. Inset, titration curve for the displacement of NADP⁺ from 26 μM F1395S nNOSr with 2',5'-ADP and the calculated best fit solid line to a single exponential decay.

Fig. 4. Anaerobic equilibrium photoreductions of WT and F1395S nNOSr. Panel A, complete set of visible spectra obtained during a titration of F1395S nNOSr in the absence of NADP⁺ with normalization at 700 nm. Panels B–E, visible spectra without normalization obtained during four other photoreductions of WT (panels B and D) and F1395S (panels C and E) nNOSr in the presence and absence of a saturating concentration of NADP⁺ at the following points: 1) initial spectrum, dashed line; 2) point of maximum 600-nm absorbance buildup, dash-dot line; 3) final spectrum of the titration, solid line. The maximum absorbance value at 600 nm calculated as a percentage of the absorbance at 457 nm in the corresponding oxidized protein spectrum is indicated for each set of conditions.
absence of such a charge transfer absorbance in the corresponding spectrum of WT nNOSr (Fig. 4, panel D) under these equilibrium conditions is consistent with the lower affinity of NADP$^+$ for the WT enzyme and smaller percentage of NADP$^+$ bound in the productive conformation when Phe$^{1395}$ is present. Finally, the apparent inability to completely reduce the nNOSr proteins with ease at the end of two titrations (Fig. 4, panels D and E) can be explained through partial oxidation of the flavins by the excess NADP$^+$ present in these two experiments.

Kinetics of Flavin Reduction by NADPH—Flavin reduction data from this laboratory using full-length nNOS (see Refs. 18 and 60 for examples) and independent results obtained by others (29, 30, 33) using the isolated nNOSr established a role for CaM in providing a significant activating effect in this reductive reaction of nNOSr. Recently, Knight and Scrutton (31) reported a new detailed examination of flavin reduction and electron transfer in nNOSr. Interestingly, they did not observe a significant effect of CaM binding on flavin reduction kinetics. To examine the issue further in this laboratory, the flavin reduction of oxidized WT and F1395S nNOSr proteins by NADPH was examined. Single wavelength stopped-flow traces obtained at 457 nm for the reduction of WT and F1395S nNOSr in the CaM-free and CaM-bound states are shown in Fig. 5. For each experiment, the initial absorbance value representing no flavin reduction was also obtained. In all cases a significant amount of the total absorbance change (typically near 30%) took place in the dead time of the instrument, consistent with results published previously (30). The magnitude of absorbance decrease at 457 nm was eventually the same in the CaM-bound and CaM-free forms of both enzymes, as judged from experiments that monitored flavin reduction over a longer period (data not shown). The recorded absorbance changes at 457 nm (Fig. 5) fit well to a quadruple exponential function (31) using the process described under “Experimental Procedures,” and thus four rate constants were obtained in each experiment. Representative values for these rate constants ($k_1$ to $k_4$) are given in Table III. Flavin reductions were repeated with multiple independent batches of protein and although, not surprisingly, some minor variations were observed between batches, the overall results and relative trends reported here (Fig. 5 and Table III) were consistently reproduced.

We observed a significant effect of CaM binding on the flavin reduction kinetics of WT nNOSr. This is apparent through visual inspection of the traces in Fig. 5 and is quantified by ~4–6-fold increases in the catalytically relevant rate constants $k_1$, $k_2$, and $k_3$ (Table III). Thus, our current data reaffirm the stimulatory effect of CaM on the NADPH-dependent flavin reduction kinetics in nNOSr. In contrast, CaM had no significant effect on the kinetics of flavin reduction in F1395S nNOSr. In both the CaM-bound and CaM-free states, the rate constants associated with flavin reduction in F1395S nNOSr most closely resembled those obtained with WT nNOSr in its CaM-free state. Thus, Phe$^{1395}$ appears to be required for CaM binding to stimulate the rate of NADPH-dependent flavin reduction in nNOSr.

Absorbance change at 600 nm during the reduction of NOSr and related flavoproteins corresponds to the formation and/or decay of flavin semiquinone species as well as charge transfer complexes. We utilized a combination of single wavelength and rapid-scanning diode array stopped-flow experiments to characterize the absorbance changes observed at 600 nm during the reduction of WT and F1395S nNOSr by NADPH. Reduction of both proteins by a single molar equivalent of NADPH was examined first, and the results are given in Fig. 6. There were no significant differences caused by the F1395S mutation on the overall qualitative behavior of the proteins under these conditions. The absorbance increases observed at 600 nm can be described as an initial fast phase taking place within the first 200 ms (Fig. 6, panels A and B) followed by a much slower phase that required nearly a minute for completion (Fig. 6, panels C and D). The amount of absorbance increase taking place in the slow phase was approximately double the amount of increase in the fast phase for each protein. The absorbance traces were fit to single exponential functions, and the calculated rates are given in Table IV. CaM binding increased the rates of absorbance changes at 600 nm for WT nNOSr, although these increases were relatively small, whereas CaM had no measurable effect on the rates obtained with F1395S nNOSr (data not shown). Visible spectra corresponding to the beginning and end of each phase obtained by rapid-scanning diode array detection are given in Fig. 6, inset of each panel. In Fig. 6, panels A and B, the decrease in absorbance at 457 nm and the similarity of the broad absorbance increases at longer wavelengths to those observed previously (Fig. 4, panel E) indicate that the fast phase of absorbance increase at 600 nm corresponds mainly to hydride transfer and formation of an FADH$_2$–NADP$^+$ charge transfer complex in both proteins. The ~3-fold faster formation of this complex in the F1395S enzyme when compared with WT nNOSr (Table IV) is consistent with the mutant having greater affinity and a higher percentage of NADPH productive binding. Based on the extinction coefficient for the reduced nNOSr-NADP$^+$ charge transfer complex, the absorbance increases observed in Fig. 6, panels A and B, are consistent with the protein in each sample accumulating as a charge transfer complex in the initial fast phase. The subsequent slow phase corresponds to a relaxation of the system into a thermodynamically stable state that clearly involves the formation of a significant population of flavin semiquinone species in both proteins. For F1395S nNOSr, the absorbance at 600 nm
Properties of F1395S nNOS Reductase

Reductions were carried out in the stopped-flow instrument at 10 °C with a 10-fold excess of NADPH while monitoring the absorbance changes at 457 nm. Data were fit to a quadruple exponential function as described under “Experimental Procedures.”

| Protein   | Conditions | Dead time<sup>a</sup> | k<sub>1</sub> (%) | k<sub>2</sub> (%) | k<sub>3</sub> (%) | k<sub>4</sub> (%) |
|-----------|------------|------------------------|------------------|------------------|------------------|------------------|
| Wild type | −CaM       | 27                     | 40 ± 5.3 (12)    | 3.0 ± 0.16 (29)  | 0.71 ± 0.04 (21) | 0.026 ± 0.006 (11) |
| Wild type | +CaM       | 25                     | 151 ± 9.4 (13)   | 13.0 ± 0.60 (26) | 4.4 ± 0.41 (20)  | 0.090 ± 0.017 (16)  |
| F1395S    | −CaM       | 33                     | 43 ± 4.1 (15)    | 3.7 ± 0.72 (12)  | 1.1 ± 0.13 (23)  | 0.022 ± 0.013 (17)  |
| F1395S    | +CaM       | 35                     | 40 ± 4.5 (14)    | 4.9 ± 1.3 (13)   | 1.3 ± 0.1 (24)   | 0.023 ± 0.010 (14)  |

<sup>a</sup> Percentage of the total absorbance change occurring in the instrument dead time.

<sup>b</sup> Individual rate constants are reported as follows: calculated rate (s<sup>−1</sup>) (% of the total absorbance change for this process).

Fig. 6. Kinetics of anaerobic flavin reduction in WT and F1395S nNOSr by a single molar equivalent of NADPH (600 nm).

Initial rapid absorbance increase at 600 nm followed by a slower partial decrease (Fig. 7, panel A). Each phase was fit to a single exponential function, and the results are reported in Table IV. Rapid-scanning diode array spectra corresponding to the three time points indicated in Fig. 7, panel A, are given in panels B and C. The spectra show that the initial absorbance increase at 600 nm is part of a broader absorbance increase extending past 700 nm into the long wavelength region and a decrease in absorbance in the 450–500-nm range. This indicates that the spectral intermediate observed to build up at 600 nm under these conditions contains a charge transfer complex, most likely between FADH<sub>2</sub> and NADP<sup>+</sup>. Analysis of the observed absorbance increases (Fig. 7) based on the extinction coefficient of the reduced nNOSr-NADP<sup>+</sup> charge transfer complex indicates that at least two-thirds of the WT protein samples exist in charge transfer complexes at the point of maximum 600 nm absorbance buildup. The shapes of the spectra corresponding to time point 2 (Fig. 7, panels B and C) also imply the existence of some flavin semiquinone species, most noticeable in the CaM-free sample of this particular data set. However, the magnitude of these signals is small when compared with that observed in equilibrium experiments (Fig. 4). The spectra also imply that decay of the 600-nm signals corresponds to further reduction of the flavins beyond the 2-electron level, consistent with our kinetic results obtained at 457 nm (Fig. 5). For F1395S nNOSr, the absorbance trace at 600 nm recorded during reduction by excess NADPH (Fig. 8, panel A) showed essentially no change in amplitude in the first 0.5 s followed by a slow gain in absorbance (Fig. 8, panel A, inset) that is also known to accompany the reduction of WT nNOSr by excess NADPH on a similar time scale (31). CaM binding did not alter these results (data not shown). Spectra collected at the three time points indicated are given in Fig. 8, panel B. They show that little or no flavin semiquinone species were present at either 0.07 or 0.5 s after mixing and that near-complete flavin reduction was achieved within 0.5 s. The presence of some flavin semiquinone species was indicated at the end of the 10-s slow phase, perhaps due to thermodynamic equilibration within the system.

**Pre-steady-state Cytochrome c Reduction**—Recently, Craig et al. (30) described a stopped-flow experiment to investigate the kinetics of cytochrome c reduction by an excess of pre-reduced nNOSr. Use of this method enabled us to evaluate how CaM binding, occupancy of the NADPH-binding site, and the F1395S mutation influence the kinetics of electron transfer between the FMN hydroquinone and cytochrome c. The rate of electron transfer between the photoreduced nNOSr proteins and cytochrome c was determined by monitoring the absorbance gain at 550 nm under pseudo-first order conditions (excess nNOSr proteins), and the effects of CaM and/or NADPH binding on these rates were evaluated. Fig. 9 contains representative stopped-flow traces demonstrating the effect of binding NADPH in the absence of CaM on the kinetics of cytochrome c reduction by photoreduced WT and F1395S nNOSr. All absorb-
Reductions by a single molar equivalent (Fig. 6) or an excess (Figs. 7 and 8) of NADPH were carried out in the stopped-flow apparatus at 10 °C while monitoring the absorbance changes at 600 nm as described under “Experimental Procedures.” All rates are the result of fitting absorbance changes to a single exponential function.

### Table IV

| Sample type   | NADPH     | Initial phase | Slow phase, increase |
|---------------|-----------|---------------|----------------------|
|               | Increase  | Decrease      |                      |
| WT + CaM      | 19.4 ± 0.7| 0.08 ± 0.005 |                      |
| WT + CaM      | 25.5 ± 1.0| 0.14 ± 0.007 |                      |
| F1395S        | 61.4 ± 7.2| 0.06 ± 0.006 |                      |
| WT + CaM      | 36.1 ± 4.3| 3.7 ± 0.3     |                      |
| WT + CaM      | 98.2 ± 13.0| 7.1 ± 0.5    |                      |
| F1395S        | 0.38 ± 0.03|              |                      |

CaM-free WT and F1395S nNOSr proteins. Adding NADPH to photoreduced WT nNOSr slowed its rate of electron transfer to cytochrome c, reproducing the observation by Craig et al. (30) that NADPH binding stabilizes a conformation of WT nNOSr that impedes electron transfer from the FMN hydroquinone. In contrast, adding NADPH to photoreduced F1395S nNOSr actually increased its rate of cytochrome c reduction to an extent even greater than that achieved with CaM binding alone. This indicates that Phe1395 has an essential function in the mechanism by which NADPH binding regulates nNOS electron transfer to cytochrome c and suggests that when Phe1395 is absent NADPH binding stabilizes a conformation of nNOSr that enables electron transfer from the FMN hydroquinone.

### Solvent Accessibility of the FMN Semiquinone—Measuring the effect of the soluble electron-spin relaxing agent Dy(III)-HEDTA on the microwave power saturation of a protein-bound radical species is an established EPR method for determining the average solvent accessibility of the radical (55, 61, 62). We
applied this technique to examine further the characteristics of how NADPH, CaM, and Phe1395 may influence the shielding of the FMN cofactor in nNOSr. Because the EPR method requires that the FMN semiquinone radical be present, it was important to establish its formation and lifetime in F1395S nNOSr as shown in Fig. 11. We collected visible spectra at key time points (Fig. 11, inset) both before and after mixing the enzyme with excess NADPH in air-saturated buffer while continuously monitoring the absorbance at 457 nm. The time points are as follows: 1) prior to NADPH addition; 2) during steady-state NADPH oxidation; and 3) after all NADPH had been oxidized.

Upon addition of NADPH most of the absorbance in the 400–500 nm range was lost, indicating that a significant proportion of each flavin was in its 1- and 2-electron reduced forms during steady-state NADPH oxidation. The broad absorbance centered near 600 nm indicates that F1395S nNOSr contained some flavin semiquinone species during this time. After approximately 2 min, there was a re-oxidation of enzyme flavins leading to the relatively stable species 3. Based on its absorbance levels at 457 nm, the broad absorbance at 600 nm, and analogy to WT nNOSr, species 3 is assigned as containing a large proportion of the FMN semiquinone. The slight shift in absorbance toward longer wavelengths most obvious near 510 nm in spectrum 3 is consistent with the enzyme containing bound NADP⁺ at this point. Although species 3 was stable within the time frame of our study, it underwent further oxidation over the next 60 min regenerating fully oxidized F1395S nNOSr (data not shown). CaM binding had minimal effects on the flavin oxidation kinetics. These results show that the F1395S mutation increased the O₂ reactivity of the reduced flavins relative to WT nNOSr and establish that the transient FMN semiquinone radical in F1395S nNOSr is sufficiently air-stable to perform the desired EPR experiments.

Full-length nNOS, WT nNOSr, and F1395S nNOSr were each treated with a slight excess of NADPH and then allowed to air-oxidize until they reached their 1-electron reduced FMN semiquinone forms. The samples were frozen, and the effects of Dy(III)-HEDTA on the microwave power saturation properties of the FMN semiquinone radicals were evaluated. Representative power saturation curves for full-length nNOS in the presence of four different concentrations of Dy(III)-HEDTA are shown in Fig. 12. In each case, the microwave power saturation of the FMN semiquinone radical was relieved by the addition of Dy(III)-HEDTA in a concentration-dependent manner. A set of experiments was performed for each of the three proteins to determine how CaM binding to each NADP⁺-bound protein would affect the ability of Dy(III)-HEDTA to alter their power saturation curves. The curves were fit as described under “Experimental Procedures” and characterized by the parameter $P_{1/3}$.

The values of $P_{1/3}$ obtained in the absence of Dy(III)-HEDTA...
were subtracted from each $P_{1/2}$ value obtained with added Dy(III)-HEDTA to obtain $\Delta P_{1/2}$, a parameter that reflects only the influence of Dy(III)-HEDTA on the FMN semiquinone radical power saturation curves. The $\Delta P_{1/2}$ values determined in the presence and absence of CaM for each of the three NADP$^+$-bound nNOS proteins are plotted versus the concentration of Dy(III)-HEDTA in Fig. 13. In each case, there was a linear relationship between $\Delta P_{1/2}$ and Dy(III)-HEDTA concentration. Linear regression analysis of the data gave the slopes of the lines, $m$ (mW/mM), which are reported in Table V. The values of $m$ provide a measure of the physical accessibility of the Dy(III)-HEDTA molecules in solution to the protein-bound FMN semiquinone radical. A larger value for the slope indicates greater dependence of $\Delta P_{1/2}$ on added Dy(III)-HEDTA and thus greater accessibility of the FMN semiquinone radical to the solvent. Both full-length nNOS and WT nNOSr exhibited a significant increase of $m$ upon binding CaM. In contrast, F1395S nNOSr in the CaM-free state had an $m$ value that was comparable with both of the WT proteins in their CaM-bound forms and showed only a slight increase in $m$ with CaM binding. These data are consistent with NADP$^+$ stabilizing a conformation of WT nNOSr characterized by a less accessible FMN in the absence of CaM, but being incapable of doing so and actually having the opposite effect in CaM-free F1395S nNOSr. The data also show that CaM binding reverses the effect of NADP$^+$ and thus promotes greater FMN accessibility.

**DISCUSSION**

Phe$^{1395}$ is of interest because of its participation in the unique and complex regulatory mechanism of nNOS (48). The impact of the F1395S mutation on different aspects of nNOSr function and control revealed in this work are discussed individually and then collectively to explain the role of Phe$^{1395}$ in determining the steady-state catalytic activities of nNOSr.

**Phe$^{1395}$ and the Interaction of nNOSr with NADP(H)**—Our data indicate that F1395S nNOSr has an increased affinity for NADP$^+$ and, by extension of FNR structural data (49), binds NADP$^+$ with a significantly higher percentage of its nicotinamide ring occupying a productive conformation relative to WT enzyme. If one accepts that the absorbance change obtained at 510 nm upon saturating the proteins with NADP$^+$ is directly proportional to the fraction of productively bound cofactor and that the F1395S mutation, as assumed with plant FNR mutants (57), enables 100% occupancy of the productive binding mode in nNOSr, then ~35% of the NADP$^+$ bound to WT nNOSr is in the productive conformation. A similar fraction for NADP$^+$ productive binding was observed in plant FNR (57) and CYPR (50). These relatively low percentages are not surprising given that the nicotinamide ring is bound in a nonproductive conformation away from the FAD in the crystal structures of both these enzymes (8, 14) and in the FNR fragment of WT nNOSr (12). In fact, the productive NADP$^+$-binding mode has only been observed in crystal structures of FNR and CYPR mutants whose flavin-shielding residues corresponding to Phe$^{1395}$ were mutated to remove the aromatic side chain (49, 50). Our findings in nNOSr are consistent with the multiple-interaction bipartite binding model for NADP(H) (57, 63, 64), where dis-
placement of Phe^{1395} from the FAD isoalloxazine to achieve nicotinamide ring binding in the productive conformation carries it with an energetic cost. When that cost is lowered by mutation of Phe^{1395}, a greater fraction of bound NADP(H) can achieve the productive conformation, and the resulting increase in noncovalent binding interactions between the nicotinamide and FAD isoalloxazine rings creates an overall higher affinity toward NADP(H). Most important, this mutational effect can slow catalysis by the flavoprotein if it causes NADP^{+} dissociation to become rate-limiting in steady-state catalysis. Such a phenomenon was observed in the form of a dramatic decrease in cytochrome c reduction activity by the structurally related W676H and W676A mutants of CYPR (63) and also becomes relevant with F1395S nNOSs as discussed below, although the two proteins are fundamentally different in the sense that the F1395S mutation in CaM-free nNOSs causes a net increase in its cytochrome c reduction activity.

Although a productive nicotinamide-FAD interaction is prerequisite for flavin reduction by NADPH, the degree of productive NADP^{+} binding in nNOSs did not correlate with the rates of flavin reduction. For example, CaM binding increased the rate of flavin reduction in nNOSs without changing the percentage of productive NADP^{+} binding. Also, the F1395S mutant had equivalent (∼CaM) or slower (+CaM) rates of NADPH-dependent flavin reduction relative to WT nNOSs despite its exhibiting a 3-fold greater percentage of productive NADP^{+} binding. This may suggest that productive and nonproductive NADP^{+} conformers are in rapid equilibrium within these flavoproteins. Thus, the specific ability of Phe^{1395} to control the distribution of NADP^{+}-binding modes does not appear to be a mechanism by which this residue influences flavin reduction kinetics or its regulation by CaM in WT nNOSs.

Flavin Reduction—In general, reduction of nNOSs and related flavoproteins begins with hydride transfer from NADPH to FAD followed by an interflavin FAD to FMN electron transfer yielding a 2-electron reduced di-semiquinone form of the protein. Subsequently, and possibly corresponding, events include NADP^{+} dissociation, binding of a second molecule of NADPH, additional interflavin electron transfer, and a second hydride transfer to FAD. The stopped-flow traces we collected at 457 nm during reduction of fully oxidized nNOSs by excess NADPH span the entire process described above and fit well to a multiple exponential function yielding four distinct rate constants. Although our kinetic analysis of the stopped-flow data was purposely identical to that recently published for nNOSs by Knight and Scrutton (31), CaM binding caused significant increases in each of the catalytically relevant rate constants ($k_1$, $k_2$, and $k_3$, Table III) only in the present work. It is noted that our protein construct was 38 amino acids longer at the N-terminal end than the one used by Knight and Scrutton (31). Interestingly, however, the rat nNOS protein used by Iyanagi and co-workers (29) was also truncated relative to ours at the N-terminal end by over 20 amino acids, but they observed a stimulation of flavin reduction kinetics so fast that it occurred in the instrument dead time. The buildup and/or decay of flavin semiquinone species is expected only if interflavin electron transfer is fast relative to events such as NADP^{+} release and hydride transfer from a second NADPH molecule. The lack of a large semiquinone buildup demonstrated here and the slow equilibration to semiquinone species in the absence of excess NADPH observed in Fig. 6 both support the previously proposed concept (30, 31) that our rat nNOSs contains a kinetic limitation on interflavin electron transfer which is relieved, at least in part, by NADP^{+} release.

The lack of absorbance change at 600 nm during the first 0.5 s of F1395S nNOSs flavin reduction by excess NADPH is interesting given that we observed rapid charge transfer complex formation when the mutant protein was mixed with a single equivalent of NADPH. The increased NADPH affinity of F1395S nNOSs may have made charge transfer complex formation so fast that it occurred in the instrument dead time when the protein was mixed with an excess of NADPH. This could explain the lack of rapid absorbance gain at 600 nm. The lack of subsequent absorbance decay at 600 nm may indicate that charge transfer complexes are maintained throughout the reduction process by a first and second molecule of NADPH. A more conclusive explanation for this phenomenon must await future studies.

These reduction experiments also illustrate a difference between F1395S nNOSs and related CYPR mutants (W676H and W676A). When the CYPR mutants are mixed with excess NADPH, the product is a stable 2-electron reduced protein due to NADP^{+} remaining tightly bound and preventing further reduction (65). In contrast, absorbance changes obtained during reduction of F1395S nNOSs by excess NADPH indicate that it is reduced beyond the 2-electron level, revealing that...
Properties of F1395S nNOS Reductase

Regulating nNOSr Conformational States Relevant to Electron Transfer—On the basis of steady-state cytochrome c reductase activities alone, it appears that the CaM-free F1395S mutant lacks repression and its CaM-bound form lacks the ability to become fully activated, or de-repressed, when compared with WT nNOSr. Previous work with three related flavoproteins (50, 66, 67) led to the idea that some type of FMN subdomain reorientation relative to the rest of the flavoprotein must occur during catalytic reduction of an external electron acceptor. Such a model is illustrated in Fig. 14. A crystal structure of CYP1 depicts a conformation where the isoalloxazine rings of FMN and FAD are positioned next to each other (8) such that electron transfer between the two flavins is enabled (Fig. 14, species A), similar to what is also observed in a recent crystal structure of a complete nNOSr.2 Once the FMN hydroquinone is formed in this conformation, however, it is thought to be incapable of delivering electrons to an acceptor such as the NOS heme or cytochrome c without first adopting a more open conformation (Fig. 14, species B) (50, 66, 67).2

Conformations A and B (Fig. 14) likely represent limits of a dynamic continuum that are both essential for catalytic turnover by nNOSr and related flavoproteins. Conformation A is required to generate FMN hydroquinone, whereas conformation B is necessary to transfer electrons from the FMN hydroquinone to external acceptors. Thus, the steady-state activity of NOSr is determined at least in part by the position and dynamics of the equilibrium between conformations A and B. Efficient catalysis results when the energy barrier for interconversion between the two states of the FMN module is low enough to allow facile electron transfer through the nNOSr via the FMN cofactor. Conversely, a NOSr with either form significantly stabilized relative to the other will appear to be catalytically repressed with respect to reduction of external electron acceptors such as cytochrome c. To the extent that external ligands (CaM and NADPH) and the NOSr regulatory elements influence the relative energies, and thus the equilibrium, between these two forms of the FMN subdomain, they can control the overall catalytic activity of the NOSr. This model assumes that electron transfer between the FMN hydroquinone and the acceptor is fast, as appears to be the case when excess cytochrome c is the acceptor in the presence or absence of CaM (30).

This conceptual model of NOSr regulation via control of a conformational equilibrium (Fig. 14) mandates that surface or solvent accessibility of the FMN cofactor should directly correlate with the observed rate of electron transfer to external acceptors, and any exceptions to this rule require further explanation. Measuring the kinetics of pre-steady-state cytochrome c reduction by an excess of pre-reduced nNOSr is one way to determine the relative proportion of FMN hydroquinone accessible to an electron acceptor under various conditions (30). In complementary experiments, the average solvent accessibility of the FMN semiquinone radical under each condition can be determined by using EPR spectroscopy. The data we obtained for WT and F1395S nNOSr (Fig. 10 and Table V) are consistent with the model and reveal a key difference between these two proteins with regard to ligand control of their FMN subdomain equilibria. Upon binding NADPH, a greater proportion of the FMN subdomain in CaM-free WT nNOSr becomes shielded as judged by the pre-steady-state cytochrome c reduction rate decreasing by $\sim 75\%$. Remarkably, the very same conditions ($-\text{CaM}$, $+\text{NADPH}$) shifted the conformational equilibrium of F1395S nNOSr toward the FMN de-shielded form, as judged by its greater than 2-fold increase in cytochrome c reduction rate over the original value. Binding of CaM alone to either nNOSr protein favors their FMN de-shielded forms, and in the case of WT nNOSr this overrides the shielding effect of NADPH binding. These effects are fully supported and verified by the EPR experiments. Based on these data, the following can be concluded. (a) Structurally, the F1395S mutant has a normal CaM response since CaM binding appears to induce conformational changes similar in nature to those observed with WT nNOSr. Therefore, any catalytic properties of F1395S nNOSs that fail to reflect a normal CaM effect cannot simply be explained by a defective CaM response. (b) Phe1395 is critically involved in the NADPH-dependent regulatory mechanism controlling the equilibrium, and thus relative energies, between FMN-shielded and -de-shielded forms of nNOSs. Interestingly, the F1395S mutation did not simply eliminate NADPH-dependent regulation, but it actually reversed the response to NADPH such that it became an activating ligand for electron transfer from the CaM-free mutant protein. (c) The excellent correlation between the EPR data and pre-steady-state cytochrome c reduction results validates the use of the latter for evaluating FMN accessibility.

Implications for Steady-state Catalysis—Craig et al. (30) described the nNOSr as being poised on a conformational “seesaw” with regard to repression of its electron transfer with

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2 Garcin, E. D., Bruns, C. M., Lloyd, S. J., Hosfield, D. J., Tiso, M., Gachhui, R., Stuehr, D. J., Tainer, J. A., and Getzoff, E. D. (2004) J. Biol. Chem., in press.
NAD(P)H acting on one side and CaM on the other. Our data support their description and suggest that the “see-saw” actually corresponds to the relative positioning of a dynamic equilibrium between shielded and de-shielded states of the FMN subdomain (Fig. 14). As the relative energies of the two conformations change due to CaM binding, the equilibrium shifts toward the less shielded conformation, and the FMN module becomes more catalytically active. Of course, if the equilibrium shifts too far toward the de-shielded conformation, the protein is predicted to lose catalytic activity due to an inability to reform the FMN hydroquinone at a sufficient rate.

Craig et al. (30) proposed that the repressed steady-state cytchrome c reduction of CaM-free nNOSr is due to NADP(H)-dependent conformational effects because a substantial proportion of nNOSr is NADP(H)-bound during turnover. This concept is consistent with our current data. A similar NADP(H) occupancy must occur in CaM-free F1395S nNOSr during its catalytic turnover, but in this case NADP(H) binding favors FMN de-shielding and results in a higher steady-state cytochrome c reductase activity relative to CaM-free WT. Thus, the steady-state catalytic difference between the two CaM-free nNOSr proteins is due to NADP(H) having an opposite effect on a conformational equilibrium rather than any intrinsic energetic difference between them. The divergent NADPH response in the mutant may also explain our observation of some NO synthesis by CaM-free full-length F1395S nNOS (48). Thus, our results extend the existing hypothesis (48) that movement of the Phe1395 side chain upon binding NADP(H) helps trigger conformational changes involving the adjacent C-terminal control element of nNOSr, which together with the autoinhibitory insert may control the equilibrium between FMN shielded and de-shielded forms (12, 30).

If CaM binding de-shields the FMN subdomain of F1395S nNOSr to a similar level as in WT enzyme (Fig. 10 and Table V), then why is the steady-state cytchrome c reductase activity of CaM-bound F1395S nNOSr only 50% that of CaM-bound WT enzyme? The answer is the slow dissociation of NADP+ from F1395S nNOSr. The F1395S koff for NADP+ at 23 °C is ~31 s⁻¹ (1860 min⁻¹), in excellent correlation with the steady-state cytochrome c turnover rate of CaM-bound F1395S nNOSr at 23 °C (2044–300 min⁻¹). Although our measured koff rates are from the fully oxidized protein and not the partially reduced forms existing during steady-state catalysis, we believe that collective consideration of all the data presented gives strong support to this conclusion. Thus, the increased NADP+ affinity in the mutant results in slower NADP+ release, and this step becomes rate-limiting for steady-state catalysis as evidenced by the relatively slow cytochrome c reduction activity relative to CaM-bound WT nNOSr. In addition, since NADPH binding to CaM-free F1395S nNOSr de-shields its FMN module nearly as much as binding CaM, the steady-state cytochrome c reductase activity of CaM-free F1395S nNOSr appears to be limited by NADP+ release as well. In other words, the steady-state cytochrome c reduction activities of CaM-free and CaM-bound F1395S would be significantly higher if not for this kinetic limitation caused by removal of Phe1395. These conclusions are supported by analysis of NADH versus NADP(H) dependent cytchrome c reduction by CaM-bound F1395S nNOS (48). Because NADH lacks the 2'-phosphate group, its affinity for the protein is lower, and its dissociation rate (koff) is expected to be faster than that of NADPH. Thus, the Vmax cytchrome c reductase activity of F1395S nNOSr should be greater for NADH than for NADPH, which was indeed observed (48).

Our data may help explain how Phe1395 impacts NOS heme reduction and NO synthesis. Although CaM binding de-shields the FMN subdomain in F1395S nNOSr to an extent comparable with WT nNOS, its rates of heme reduction and NO synthesis are much lower (48). Thus, without Phe1395 side chain movement, the nNOSr regulatory elements may not fully relieve the block on NOS heme access in response to CaM, which appears to be a separate function (19) occurring along with the de-shielding of the FMN subdomain. This possibility can now be explored.

Conclusions—Our data suggest a regulatory model for nNOSr in which its observed catalytic activity is influenced by an equilibrium between relatively shielded and de-shielded conformations of the FMN subdomain. Phe1395 is required to stabilize the shielded conformation when NADP(H) binds in the absence of CaM, and this is associated with catalytic repression. CaM binding shifts the equilibrium toward a de-shielded conformation giving the FMN hydroquinone greater access to cytchrome c, but Phe1395 is not required for this effect of CaM. Once the protein has been conformationally activated by CaM binding, however, Phe1395 is required to prevent NADP+ release from limiting the rate of cytchrome c reduction in the steady state. Phe1395 appears to have distinct roles in regulating NADP(H) affinity, transmitting the effect of NADPH binding on the conformational equilibrium of CaM-free nNOSr and facilitating reduction of flavin and heme centers in CaM-bound nNOS. Its phenyl side chain controls the extent of productive NADP(H) binding, and this explains its effect on NADP(H) affinity and related catalytic impact. However, we speculate that the other roles of Phe1395 are manifested through phenyl side chain movement away from FAD upon productive NADP(H) binding, as it is this movement that is expected to influence the behavior of an adjacent regulatory element in nNOSr.

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