THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 32, Issue of August 6, pp. 22313–22320, 1999

Printed in U.S.A.

Role of Reductase Domain Cluster 1 Acidic Residues in Neuronal Nitric-oxide Synthase

CHARACTERIZATION OF THE FMN-FREE ENZYME*

(Received for publication, April 9, 1999)

Subrata Adak‡, Sanjay Ghosh‡, Husam M. Abu-Soud‡, and Dennis J. Stuehr‡§

From the ‡Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195

The nNOS reductase domain is homologous to cytochrome P450 reductase, which contains two conserved clusters of acidic residues in its FMN module that play varied roles in its electron transfer reactions. To study the role of nNOS reductase domain cluster 1 acidic residues, we mutated two conserved acidic (Asp^{918} and Glu^{919}) and one conserved aromatic residue (Phe^{892}), and investigated the effect of each mutation on flavin binding, conformational change, electron transfer reactions, calmodulin regulation, and catalytic activities. Each mutation destabilized FMN binding without significantly affecting other aspects including substrate, cofactor or calmodulin binding, or catalytic activities upon FMN reconstitution, indicating the mutational effect was restricted to the FMN module. Characterization of the FMN-depleted mutants showed that bound FMN was essential for reduction of the nNOS heme or cytochrome *c*, but not for ferricyanide or dichlorophenolindolphenol, and established that the electron transfer path in nNOS is NADPH to FAD to FMN to heme. Steady-state and stopped-flow kinetic analysis revealed a novel role for bound FMN in suppressing FAD reduction by NADPH. The suppression could be relieved either by FMN removal or calmodulin binding. Calmodulin binding induced a conformational change that was restricted to the FMN module. This increased the rate of FMN reduction and triggered electron transfer to the heme. We propose that the FMN module of nNOS is the key positive or negative regulator of electron transfer at all points in nNOS. This distinguishes nNOS from other related flavoproteins, and helps explain the mechanism of calmodulin regulation.

Synthesis of nitric oxide (NO)[1] by the neuronal NO synthase (nNOS) can activate as well as modulate many functions in mammalian physiology (1–3). The nNOS is inactive in its native form and requires Ca^{2+}-promoted calmodulin (CaM) binding for activation (4). This enables nNOS to participate in signal transduction cascades by generating NO in response to increases in intracellular Ca^{2+} (2). nNOS is a bidomain enzyme containing an N-terminal oxygenase domain with binding sites for heme, tetrahydrobiopterin (H_4B), and L-arginine (Arg), and a C-terminal reductase domain with binding sites for FMN, FAD, and NADPH (5–9). A ~20 amino acid consensus CaM-binding site is located between the nNOS reductase and oxygenase domains (4, 10). During NO synthesis the reductase domain transfers electrons from NADPH to the heme. This enables heme-dependent oxygen activation and stepwise conversion of Arg to NO and citrulline, with *N*-hydroxy-L-arginine (NOHA) being formed as an intermediate (3, 11, 12). CaM performs a critical role in the process by triggering electron transfer from the reductase domain flavins to the oxygenase domain heme (13, 14). Recent evidence suggests this transfer occurs between reductase and oxygenase domains that are located on different subunits of the NOS homodimer (15). However, it is still unclear what protein residues facilitate electron transfer, and how CaM controls the domain interaction.

The NOS reductase domain actually belongs to a subset of related reductases that contain a N-terminal FMN-containing flavodoxin module that is linked to a C-terminal NADPH- and FAD-binding ferridoxin-like module (FNR) (16–18). Other similar dual-flavin reductases include NADPH cytochrome P450 reductase, sulfite reductase flavoprotein, and the cytochrome P450BM3 reductase domain. Because of the structural homology, work done with these proteins serves to guide investigation of nNOS. In general, the FNR and FMN modules of these proteins appear to fold separately and function when expressed independently or after being separated by proteolysis (16–18). A crystal structure of cytochrome P450 reductase has recently revealed the interactions that occur between the FNR and FMN modules of that enzyme (17).

All of these proteins (or their isolated reductase domains) share biochemical similarities in transferring NADPH-derived electrons to either hemeprotein acceptors or attached hemeprotein domains (11, 18–21). Electron transfer typically proceeds from NADPH to FAD to FMN to hemeprotein, although this path has not been definitively demonstrated for nNOS. In cytochrome P450BM3, recent work suggests that its FMN module is capable of interacting with both the FNR and attached hemeprotein domain by means of a flexible linker, and this enables the FMN module to shuttle electrons between the FNR and heme during catalysis (22). All of these flavoproteins also transfer NADPH-derived electrons to artificial acceptors including cytochrome *c*, ferricyanide, and dichlorophenolindolphenol (DCIP) (11, 18–21).

Despite the similarities, nNOS is distinguished from these flavoproteins by its ability to increase electron transfer rates to acceptors upon CaM binding (11, 14, 18). CaM binding is associated with an increase in tryptophan and flavin fluorescence (19, 23), suggesting that CaM acts by inducing a conforma-

* This work was supported by National Institutes of Health Grant GM51491. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Established Investigator of the American Heart Association. To whom correspondence should be addressed: Immunology NB-3, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-445-6950; Fax: 216-444-9329; E-mail: stuehrd@ccf.org.

[1] The abbreviations used are: NO, nitric oxide; DCIP, dichlorophenolindolphenol; DTT, dithiothreitol; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FNR, ferridoxin $NADP^+$ reductase; H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; NADPH, reduced β-nicotinamide adenine dinucleotide; NOHA, N^{ω}-hydroxy-L-arginine; nNOS, neuronal NO synthase.

eNOS(bovine)	662	**F**CAFARAVDTRLEELGGERLLQLGQG**DE**LCGQEEAFRGWA
eNOS(human)	659	**F**CAFARAVDTRLEELGGERLLQLGQG**DE**LCGQEEAFRGWA
iNOS(chicken)	631	**F**CAFAHAIDQKLSQLGALQLTPVGEG**DE**LNGQEEAFRTWA
iNOS(mouse)	628	**F**CAFAHDIDQKLSHLGASQLAPTGEG**DE**LSGQEDAFRSWA
nNOS(rat)	892	**F**CAFGHAVDTLLEELGGERILKMREG**DE**LCGQEEAFRTWA
NOS(drosophila)	826	**F**CAFGQYVDNILGELGGERLLRVAYG**DE**MCGQEQSFRKWA
Cyt.P450 reductase(rat)	181	**F**NAMGKYVDQRLEQLGAQRIFELGLG**DD**DGNLEEDFITWR

FIG. 1. **Sequence alignment of different NOS and a cytochrome P450 reductase.** The conserved Phe and cluster 1 acidic residues are in *bold*.

tional change within the reductase domain. Stopped-flow analysis (9, 14) and work with partially active CaM mutants (24, 25) show that CaM stimulates electron transfer to the acceptors primarily by increasing the rate of NADPH-dependent flavin reduction. Importantly, CaM's ability to speed flavin reduction can occur independent of its triggering nNOS heme reduction (24–26), suggesting that the effects involve different structural elements of CaM. Thus, while nNOS and related flavoproteins display many structural and biochemical similarities, the CaM activation component makes nNOS unique and suggests significant structure-function differences do exist.

Studies investigating the interaction between cytochrome P450 reductase and its cytochrome P450 or cytochrome *c* acceptor hemeproteins have implicated two clusters of acidic residues within the FMN module ^{207}Asp-Asp-Asp^{209} (cluster 1) and ^{213}Glu-Glu-Asp^{215} (cluster 2) in controlling interactions important for electron transfer (27). Both clusters reside in the FMN module of the reductase and are highly conserved among related flavoproteins including the NOSs (Fig. 1). Mutagenic (27) and chemical cross-linking (28) studies with cytochrome P450 reductase suggested Asp^{208} of cluster 1 is important for electron transfer to its P450 acceptor hemeprotein. For example, *N*-demethylase activity of the Asn^{208} mutant was inhibited by 63% without changing the reductase K_m toward cytochrome P450 or NADPH (27). The mutation did not affect cytochrome *c* or ferricyanide reductase activities, indicating interaction between the reductase and these molecules is distinct from its interaction with cytochrome P450. Similar observations were reported in *Anabaena* flavodoxin (29), where mutagenic analysis of cluster I acidic residues Asp^{144} and Glu^{145} showed they were involved in flavodoxin-supported P450c$_{17}$ progesterone 17a-hydroxylase activity but not involved in cytochrome *c* reduction. Crystal structures of cytochrome P450 reductase (17) and *Anabaena* flavodoxin (30) show that cluster 1 residues are located near the surface, presumably positioned to interact with a positive surface patch on their hemeprotein acceptor.

Because protein sequence and functional data suggest that the nNOS reductase domain and cytochrome P450 reductase have a similar secondary and tertiary structure, we hypothesized that cluster 1 residues may also be important in controlling reductase-oxygenase domain interaction and electron transfer in nNOS. We utilized site-directed mutagenesis to assess the importance of conserved amino acids Phe^{892}, Asp^{918}, and Glu^{919} with respect to FMN binding, electron transfer reactions, and catalytic activities of nNOS. Surprisingly, our data show that these three residues impact nNOS function primarily by stabilizing FMN binding to the reductase. Their mutation resulted in FMN-depleted forms of nNOS, which we used to investigate reductase domain function and how the FMN module participates in nNOS response to CaM.

EXPERIMENTAL PROCEDURES

Materials—Superoxide dismutase was obtained from Calbiochem and was of the ferrous manganese type. All other regents and materials were obtained from Sigma or from sources previously reported (9, 15, 31).

Molecular Biology—Wild type and mutant nNOS with a His$_6$ tag attached to the N-terminal of the protein were overexpressed in *Escherichia coli* strain BL21 (DE3) using a modified PCWori vector and purified as described (31, 32). Restriction digestions, cloning, bacterial growth, and transformation and isolation of DNA fragments were performed using standard procedures (33). Site-directed mutagenesis was done using the Altered Sites I *in vitro* Mutagenesis Kit from Promega. Wild-type nNOS cDNA was cut from PCWori with *Nde*I and *Xba*I and cloned into the *Xba*I site of the pAlter-I mutagenesis vector. Incorporated mutations were confirmed by DNA sequencing at the Cleveland Clinic core facility. DNAs that contained the desired mutations were cloned into the *Nde*I and *Xba*I sites of the PCWori vector and transformed into *E. coli* BL21(DE3). Oligonucleotides used to construct site-directed mutants in the nNOS were synthesized by Life technologies. Silent mutations that incorporate unique restriction sites were also added to aid in screening. Mutations and corresponding oligonucleotides are listed below, with silent mutations underlined and mutagenic codons in bold. The silent restriction site incorporated in the first three oligonucleotides was *Afl*II and in the fourth was *Xho*I. D918A, pGGAGAGGATTCTTAAGATGAGGGAGGGG**GCT**GAGCTTTGCGGAC; E919A: pGGAGAGGATTCTTAAGATGAGGGAGGGGGAT**GCG**CTTTGCGGGAC; D918A, E919A: pGGAGAGGATTCTTAAGATGAGGGAGGGG**GCTGCG**CTTTGCGGAC; F892A: pGTACCCCCACGCCTGTGCCTTTGGGCATGCGGTGGACACCCTCCTCGAGGAACTGGGA.

Expression and Purification of Wild-type and Mutant nNOS—Transformed bacteria were grown at 37 °C in 3 liters of terrific broth supplemented with 125 mg/liter ampicillin and 20 mg/liter chloramphenicol. Protein expression was induced when the cultures reached an OD_{600} of 0.8 to 1 by adding 1 mM isopropyl-β-D-thiogalactoside, and the cultures were supplemented with 0.4 mM δ-aminolevulinic acid. After further growth at room temperature for 24 h, the cells were harvested and resuspended in buffer A (40 mM HEPPS, pH 7.6, with 10% glycerol, 1 mM Arg, 150 mM NaCl, 10 μM H_4B, 3 mM ascorbic acid) containing 1 mM EDTA, 0.5 mg/ml each of leupeptin and pepstatin, 1 mg/ml lysozyme, and phenylmethylsulfonyl fluoride. Cells were lysed by freeze-thawing three times in liquid nitrogen followed by sonication for three 25-s pulses with a 1-min rest on ice between pulses, using a medium probe Sonicator Cell Disrupter (Model W-220F, Heat systems, Ultrasonics. Inc.). The cell lysate was centrifuged at 4 °C for 30 min and the cell-free supernatant was precipitated by adding 50% (w/v) ammonium sulfate. The precipitant was centrifuged at 4 °C for 30 min at 16,000 rpm in a JA-17 rotor and kept at −70 °C. The ammonium sulfate precipitate was resuspended in a buffer A containing 1 mM phenylmethylsulfonyl fluoride. The resuspended solution was loaded onto a Ni^{2+} nitrilotriacetic acid-Sepharose CL-4B column that had been charged with 50 mM $NiSO_4$ and equilibrated with buffer A containing 1 mM phenylmethylsulfonyl fluoride. The column was washed with 5 times of column buffer and 5 times of column buffer containing 40 mM imidazole. The nNOS protein was eluted with 160 mM imidazole in buffer A and active fractions were pooled and stored at 4 °C overnight in the presence of 1 mM DTT. The fractions were next loaded onto a 2′,5′-ADP-Sepharose column equilibrated with 40 mM HEPPS buffer, pH 7.6, containing 10% glycerol, 0.5 mM Arg, 3 mM DTT, and 2 μM H_4B. After adsorption the column was washed with column buffer containing 450 mM NaCl, and the protein was eluted with column buffer containing 10 mM NADPH. Selected fractions were concentrated using a Centriprep-50, dialyzed against 40 mM HEPPS, pH 7.6, containing 10% glycerol, 2.5 mM DTT, and 2 μM H_4B, and stored in aliquots at −70 °C.

UV-Visible Spectroscopy—Spectral data was recorded on a Hitachi U3110 Spectrophotometer in the presence of H_4B and Arg. Scans of the dithionite-reduced CO-bound proteins were taken in 40 mM HEPPS, pH 7.6, containing 10% glycerol, 1 mM DTT, 1 mM Arg, and 20 μM H_4B. The ferrous-CO adduct absorbing at 444 nm was used to quantitate the

heme protein content using an extinction coefficient of 74 mM^{-1} cm^{-1} (A_{444}-A_{500}) (34).

Determination of Bound FAD and FMN—Bound FAD and FMN were released from nNOS or mutants by heat denaturation of the enzyme (95 °C for 5 min in the dark). It is essential to use well sealed vials for this procedure in order to avoid loss of sample volume. Subsequently samples were cooled to 4 °C and filtered to remove denatured protein. Samples were injected into a Microsorb Cyano Analytical HPLC Column (5 mm × 4.6 mm × 15 cm) and subjected to isocratic elution with 5 mM ammonium acetate, pH 6.0, containing 20% (v/v) methanol at a flow rate of 1 ml/min. FAD and FMN had retention times of 4.1 and 7.6 min and the peaks were completely resolved. Flavins were detected by fluorescence emission and quantitated based on authentic freshly prepared FAD and FMN standards.

NO Synthesis—The initial rate of NO synthesis by nNOS and mutants was quantitated at 37 °C using the oxyhemoglobin assay for NO (34). The nNOS (~25 nM) was added to a cuvette containing 40 mM HEPPS, pH 7.6, containing 15 μg/ml CaM, 0.62 mM $CaCl_2$, 0.3 mM DTT, 5 mM Arg, 4 μM each of FAD and H_4B, 100 units/ml catalase, and 10 μM oxyhemoglobin to give a final volume of 0.7 ml. The reaction was started by adding NADPH to give 0.2 mM. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an absorbance increase at 401 nm and quantitated using the extinction coefficient of 38 mM^{-1} cm^{-1} .

NADPH Oxidation—The initial rate of NADPH oxidation at 25 °C was quantitated spectrophotometrically at 340 nm using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The composition of the assay mixture was similar to that of the NO synthesis measurement except that oxyhemoglobin was absent unless specified otherwise.

Reduction of External Electron Acceptors—Wavelengths and extinction coefficient used to quantitate the NADPH-dependent reduction of cytochrome *c*, DCIP, and ferricyanide were 550 nm (21 mM^{-1} cm^{-1}), 600 nm (20.6 mM^{-1} cm^{-1}), and 420 nm (1.2 mM^{-1} cm^{-1}), respectively. The composition of the assay mixture was 40 mM HEPPS, pH 7.6, 4 μM FAD, 0.1 mg/ml bovine serum albumin, 10 μg/ml CaM, 0.6 mM EDTA, 10 units/ml catalase, 10 units/ml superoxide dismutase, and cytochrome *c*, DCIP, or ferricyanide at 0.1, 0.1, or 1 mM, respectively. In some cases, 0.83 mM Ca^{2+} was added to promote CaM binding to nNOS. After the addition of nNOS, the reaction was initiated by adding 0.1 mM NADPH. K_m values for cytochrome *c*, CaM, and FMN were determined from experiments in which the concentration of these molecules was varied and by reciprocal analysis of the velocity *versus* concentration data.

Reduction of Heme Iron—All samples were equilibrated at 25 °C under anaerobic conditions in buffer saturated with CO. The cuvette contained 3 μM wild-type nNOS or mutants in CO-saturated 40 mM HEPPS buffer, pH 7.6, containing 0.5 mM DTT, 4 μM H_4B, 0.6 mM EDTA, 1 mM Arg, and 6 μM CaM. Concentrated anaerobic NADPH solution was added to the sample to give 0.1 mM. CaM binding and heme reduction were initiated by adding 1 mM $CaCl_2$.

Fluorescence Spectroscopy—Flavin fluorescence measurements were done using a Hitachi model F-2000 spectrofluorometer as described previously (9) with modifications. A 1-ml quartz cuvette with a path length of 1 cm was used for the experiments. The nNOS proteins were diluted to 2 μM in 40 mM HEPPS, pH 7.6, containing 0.6 mM EDTA, 1 mM DTT, and 3 μM CaM. Proteins were irradiated with 450–460 nm light using an in-line 8% filter and their emission spectra were recorded between 450 and 700 nm. In some experiments flavin fluorescence emission at 530 nm was monitored *versus* time before and after consecutive addition of 1 mM Ca^{2+} and 3 mM EDTA.

Flavin Reduction Kinetics—The kinetics of flavin reduction were analyzed under anaerobic conditions as described previously (14), using a stopped-flow apparatus (Hi-tech Ltd., model SF-51) equipped for anaerobic work. Wild-type nNOS and mutants were briefly treated with ferricyanide and desalted prior to use in these experiments in order to oxidize the residual air-stable flavin semiquinone that is present in nNOS after purification (9, 34). Measurements were made under pseudo first-order conditions and initiated by rapid mixing a solution of 0.1 mM NADPH with a solution containing 3 μM CaM-free or -bound nNOS or mutants in 40 mM HEPPS buffer, pH 7.6, containing 0.5 mM EDTA, 6.0 μM CaM and in some cases, 1 mM Ca^{2+}. The absorbance change was monitored at 485 nm. Signal to noise ratios were improved by averaging the 10 individual scans. The time course of absorbance change was best fit to a single or double exponential equation by use of a nonlinear least-squares method provided by the instrument manufacturer (14).

RESULTS

Mutant nNOS Expression and Prosthetic Group Content—The two-step enzyme purification typically yielded about 8 mg of full-length heme-containing nNOS mutants per liter of culture, which is similar to our yield of wild-type nNOS expressed in the same system. Spectroscopic analysis showed that all mutants contained heme in a predominantly low spin state. The heme iron of each mutant was observed to shift to high spin upon addition of 20 μM H_4B and 1 mM Arg. Dithionite reduction of each mutant in the presence of Arg, H_4B, and CO produced the expected 444-nm absorbance peak for the ferrous-CO complex in all cases (data not shown). These data show that the reductase domain mutations did not alter expression of the full-length enzyme or affect the properties of the heme-containing oxygenase domain. Flavin analysis showed that the mutants contained normal quantities of FAD (~1 per subunit) but contained below normal or practically undetectable levels of FMN (Table I). The D918A,E919A double mutant and single mutants F892A and D918A had almost no bound FMN, while E919A contained almost half the saturating level of FMN. Thus, the cluster I point mutations reduced or prevented stable binding of FMN by the nNOS reductase domain.

NO Synthesis and NADPH Oxidation by the nNOS Mutants—Table II compares the catalytic activities (expressed as turnover number per heme) of wild-type and mutant nNOS enzymes with regard to NO synthesis and NADPH oxidation. NO synthesis from either Arg or NOHA was slower or absent in the mutants in relation to their FMN content, consistent with FMN being a critical component for electron transfer during NO synthesis in nNOS. CaM-stimulated NADPH oxidation by each mutant was also reduced or blocked in a pattern identical to NO synthesis. Because CaM stimulation of NADPH consumption requires heme reduction in nNOS when O_2 is the electron acceptor (14, 35), this suggested that the FMN-de-

TABLE I
Flavin content per heme of wild-type and mutant nNOS

The values represent the mean and S.E. for three measurements each.

Enzyme	FAD	FMN
Wild-type	0.97 ± 0.02	1.0 ± 0.02
D918A	0.87 ± 0.02	ND[a]
E919A	1.02 ± 0.06	0.49 ± 0.07
D918A,E919A	0.92 ± 0.05	ND
F892A	1.03 ± 0.09	0.09 ± 0.06

[a] ND, not detectable.

TABLE II
Comparative analysis of NO synthesis and NADPH oxidation of nNOS and mutant proteins

Turnover number (k_{cat}) is express in nanomole of product formation per nanomole of protein/min. NO synthesis from Arg and NADPH oxidation rates were determined in FMN-free assay mixture in the presence or absence of CaM as described under "Experimental Procedures." The values represent the mean and S.E. for three measurements each.

Activity	nNOS		D918A		E919A		D918A,E919A		F892A	
	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM
NO synthesis from Arg	70 ± 5	ND[a]	ND	ND	37 ± 2	ND	ND	ND	4 ± 1	ND
NO synthesis from NOHA	93 ± 5	ND	ND	ND	55 ± 4	ND	ND	ND	6 ± 1	ND
NADPH oxidation (+Arg, +H_4B)	146 ± 10	3.0 ± 1	4.0 ± 1	2.7 ± .5	70 ± 5	3.5 ± .5	5.5 ± 1	2.3 ± .5	11 ± 1	2.9 ± .5

[a] ND, not detectable.

pleted mutants have defective electron transfer between their reductase domain and heme in response to CaM.

Heme Iron Reduction—To test this possibility we compared NADPH-dependent heme iron reduction in the mutant and wild-type nNOS under anaerobic conditions. Heme iron reduction was followed spectrally over time as a buildup of the ferrous-CO complex, whose Soret peak absorbs maximally at 444 nm (8, 13). The percentage of NADPH-dependent heme iron reduction was determined relative to complete reduction achieved by adding dithionite to the sample at the end of each experiment. As shown in Fig. 2, heme reduction in wild-type nNOS is fast and complete under these conditions as previously reported (35). Heme reduction rates in each mutant were slower and approached maximum levels that were approximately in proportion to their bound FMN content. Thus, inhibition of heme iron reduction due to FMN depletion likely explains why CaM-induced NO synthesis and NADPH oxidation is slow in the mutants.

Mutant Reduction of Cytochrome c, DCIP, and Ferricyanide—CaM binding speeds electron transfer from the nNOS reductase domain to artificial acceptors such as cytochrome *c*, ferricyanide, and DCIP. As summarized in Table III, CaM increased rates of cytochrome *c* reduction in all cases, although basal and CaM-stimulated rates were markedly lower in the mutants compared with wild-type. This suggests that the mutants respond to CaM, but can only reduce cytochrome *c* in proportion to their bound FMN content. In contrast, mutant basal rates of DCIP reduction were only slightly lower than wild-type, and instead of increasing in response to CaM they actually decreased in the three mutants that contained the least amount of bound FMN. Moreover, mutant basal rates of ferricyanide reduction were 2- or 3-fold greater than wild-type basal level, and did not change significantly in response to CaM. These findings indicate that reduction of DCIP or ferricyanide by nNOS do not require bound FMN, and for ferricyanide bound FMN inhibits its reduction.

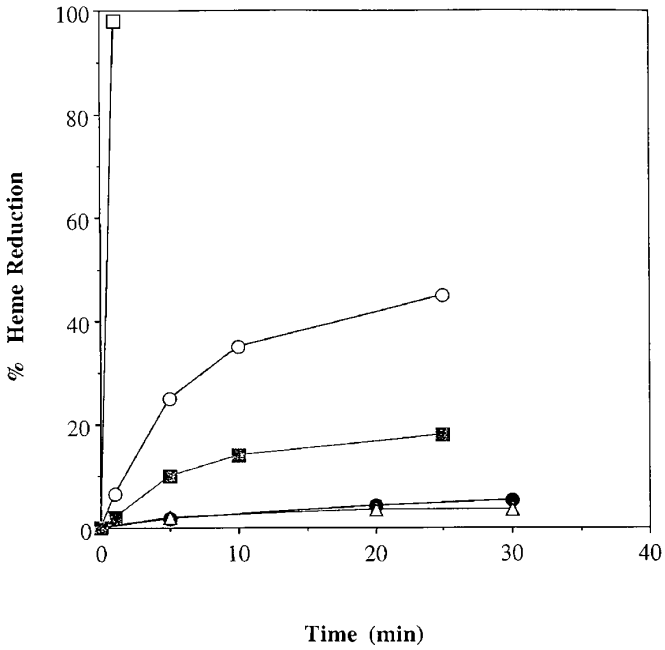

FIG. 2. **NADPH-dependent heme iron reduction in wild-type and mutant nNOS proteins.** Heme reduction was monitored *versus* time at room temperature under anaerobic conditions and in the presence of CO as described under "Experimental Procedures." Wild-type (□), E919A (○), D918A (●), D918A,E919A double mutant (Δ), and F892A (■).

Reconstitution of Catalytic Activities with Exogenous FMN—Cytochrome P450 reductase and cytochrome P450BM3 preparations that are rendered FMN-free by mutagenesis or dialysis can often be reconstituted with exogenous FMN (20, 36, 37). We attempted reconstitution of the FMN-depleted nNOS mutants by incubating the proteins for 2 min in buffer containing various concentrations of FMN, followed by assaying their NO synthesis or CaM-induced cytochrome *c* reductase activities (Fig. 3, *A* and *B*). Wild-type nNOS increased its NO synthesis and cytochrome *c* activities by about 30% in the presence of added FMN. Added FMN also enabled each mutant to recover NO synthesis and cytochrome *c* reduction activities. The percentage recovery achieved for NO synthesis *versus* cytochrome *c* reduction were similar for any given mutant, consistent with both reactions requiring bound FMN. When assayed at the highest FMN concentration the three mutants with lowest native FMN content reached maximal activities that were ~40–50% the level of wild-type nNOS, while the mutant retaining 50% native FMN content (E919A) reached 85% wild-type activity. Incubating the mutants with FMN for longer times prior to assay did not increase the amount of recovered activity (data not shown). Similar results have been reported for FMN binding mutants of cytochrome P450 reductase and cytochrome P450BM3, which also did not fully reconstitute their activities in response to added FMN (36, 37).

Apparent K_m and V_{max} values for mutant FMN reconstitution were determined by double reciprocal analysis of the NO synthesis data from Fig. 3 and are listed in Table IV. The apparent K_m for FMN in wild-type nNOS was 90 nM. Mutant K_m values were approximately 2 orders of magnitude greater than wild-type except for the partially FMN-replete E919A mutant, whose K_m was increased by a factor of 10. Thus, the mutants all have reduced binding affinity for FMN, explaining why they contain little or no FMN after purification. As summarized in Table V, adding FMN to the mutants enabled them to respond more normally regarding each catalytic activity in the presence or absence of CaM. Ferricyanide reduction rates in CaM-free nNOS were lowered in the presence of added FMN,

TABLE III
Reductase activities of wild type nNOS and mutant proteins

Turnover number (k_{cat}) is expressed as nanomole of product formation per nanomole of protein/min. Each value represents the mean ± S.D. for two protein preparations each assayed in triplicate. Measurements were done at 27 °C without added FMN as described under "Experimental Procedures."

Activity	nNOS		D918A		E919A		D918A,E919A		F892A	
	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM
Cytochrome *c* reduction	5000 ± 478	412 ± 41	94 ± 10	ND[a]	2506 ± 306	250 ± 20	116 ± 20	ND	666 ± 55	50 ± 5
Ferricyanide reduction	5083 ± 505	1750 ± 220	4900 ± 545	4629 ± 512	5045 ± 502	3812 ± 400	4950 ± 450	5016 ± 510	4900 ± 413	5125 ± 585
DCIP reduction	1427 ± 102	441 ± 50	258 ± 20	387 ± 26	767 ± 75	450 ± 30	150 ± 15	325 ± 20	249 ± 22	358 ± 50

[a] ND, not detectable.

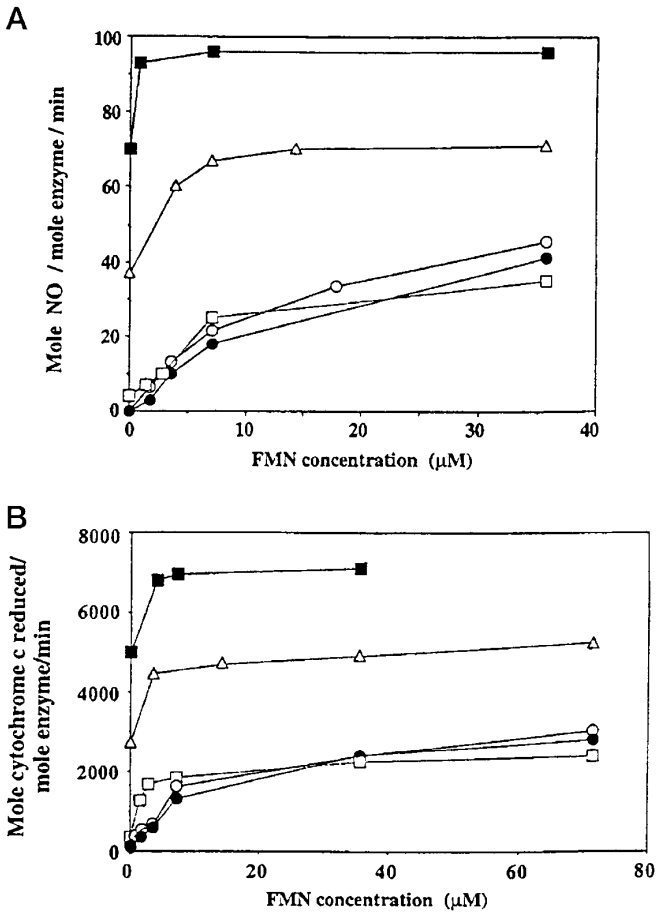

FIG. 3. **Enzymatic activities as a function of added FMN concentration.** NO synthesis (*panel A*) and cytochrome *c* reductase activity (*panel B*) of wild-type nNOS and mutant proteins were measured as described in the presence of the indicated concentrations of FMN. The proteins were incubated for 2 min with various concentrations of FMN in the assay mixtures prior to starting the reaction by adding 0.2 mM NADPH. Wild-type nNOS (■), E919A (△), D918A (○), D918A,E919A double mutant (●), and F892A (□).

suggesting bound FMN inhibits ferricyanide reduction in this circumstance.

K_m for CaM and Cytochrome c—We next investigated whether cluster 1 mutations effect nNOS affinity toward CaM or cytochrome *c*. Fig. 4 contains representative reciprocal plots of cytochrome *c* reduction rate *versus* cytochrome *c* concentration by wild-type nNOS and the D918A,E919A double mutant. Similar data were obtained for CaM titrations, using cytochrome *c* reduction as a marker for activity. Table VI summarizes apparent K_m values for cytochrome *c* and CaM with wild-type and mutant nNOS proteins. The close similarity between mutants and wild-type indicate that our cluster 1 mutations did not effect nNOS interaction with either CaM or cytochrome *c*.

CaM-induced Flavin Fluorescence—CaM binding to nNOS or its isolated reductase domain causes a partially reversible increase in flavin fluorescence (9). We utilized our FMN-deficient mutants to determine if bound FMN was involved in the CaM-induced fluorescence increase. As shown in Fig. 5, wild-type nNOS increased its flavin fluorescence by a factor of 2 after Ca^{2+}-promoted CaM binding, and the increase was partly reversed by dissociating CaM with excess EDTA, consistent with previous work (9). The E919A mutant underwent a similar change in fluorescence upon binding and dissociation of CaM, although the magnitude of change was less compared with control, consistent with this mutant being 50% FMN-deficient.

TABLE IV
Apparent K_m for FMN and V_{max} values for nNOS and mutant proteins

Apparent K_m and apparent V_{max} values were estimated by NO synthesis assay with different concentrations of FMN using double reciprocal analysis. The values represent the mean and standard error for three measurements each.

Enzyme	Apparent K_m for FMN	Apparent V_{max}
	μM	min^{-1}
Wild-type	0.09 ± 0.01	100 ± 4
D918A	13.9 ± 1.5	46 ± 4
E919A	0.8 ± 0.1	73 ± 2
D918A,E919A	13.3 ± 1.3	53 ± 2
F892A	9.0 ± 1.0	48 ± 3

The three nNOS mutants that were mostly devoid of FMN did not increase their flavin fluorescence upon CaM binding under the same circumstances. Thus, bound FMN is essential for the fluorescence change associated with CaM binding to nNOS, suggesting the conformational change that occurs is restricted to the FMN module of the reductase.

Stopped-flow Analysis of Flavin Reduction—Because CaM binding causes a 15-fold increase in the rate of nNOS flavin reduction by NADPH (14), we measured flavin reduction rates in the nNOS mutants in the presence or absence of CaM. Experiments were done under pseudo first-order conditions using a 33-fold excess of NADPH, and flavin reduction was monitored at 485 nm. As shown in Fig. 6, the absorbance trace representing flavin reduction in our CaM-free wild-type nNOS best fit to a biphasic process with apparent rate constants of 9.2 s^{-1} (k_1) and 1.6 s^{-1} (k_2), respectively. CaM binding to wild-type nNOS increased the apparent rates to 54 and 14 s^{-1} (Table VII), consistent with earlier reports (9, 14). The first phase of the reaction (k_1) can be attributed to NADPH reduction of FAD, and the second phase (k_2) to electron transfer from FAD to FMN (38). In CaM-free nNOS the rate of FAD reduction is relatively slow and comparable to FAD reduction in monoamine oxygenase (2.5 s^{-1} at 4 °C) (39), whereas in CaM-bound nNOS the rate of FAD reduction is comparable to mammalian cytochrome P450 reductase (55 s^{-1} at 20 °C) (40), but slower than spinach ferridoxin (105 s^{-1} at 4 °C) (41) or bacterial cytochrome P450BM3 (130 s^{-1} at 4 °C) (38).

Also shown in Fig. 6 is the absorbance trace for the CaM-free D918A,E919A double mutant, which best fit to a monophasic process giving an apparent rate of 39 s^{-1}. The monophasic nature of the reaction is consistent with only FAD being bound in the protein and represents the rate of FAD reduction by NADPH. The apparent rate constants for all CaM-free and CaM-bound nNOS mutants are listed in Table VII. These data show that in the CaM-free state the three mutants devoid of FMN had rates of FAD reduction (k_1) that are four times faster than FAD reduction in wild-type nNOS, and their rates did not increase further with CaM. The partially FMN-replete E919A mutant had a CaM-free rate of FAD reduction that is between the two extremes, and the rate was slightly increased after CaM binding. Together, this indicates that bound FMN has a significant negative effect on FAD reduction rate in the CaM-free basal state.

DISCUSSION

Our work reveals that reductase domain cluster 1 residues Asp^{918}, Asp^{919}, and Phe^{892} help stabilize FMN binding in nNOS. In related flavoproteins such as rat cytochrome P450 reductase and *Anabaena* flavodoxin, analogous cluster 1 residues facilitate electron transfer between the FMN module and hemeprotein partners, and mutations typically inhibit catalysis without affecting FMN binding (27, 29). Indeed, loss of FMN from these flavoproteins either requires special dialysis (20, 42,

TABLE V
Catalytic activities of wild-type nNOS and FMN-reconstituted mutants

Measurements were done at 25 °C as detailed under "Experimental Procedures." The values represent the mean and S.E. for three measurements each.

Activity	nNOS		D918A		E919A		D918A/E919A		F892A	
	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM	+CaM	−CaM
NO synthesis (+Arg)	90 ± 5	ND[a]	48 ± 4	ND	74 ± 5	ND	46 ± 5	ND	44 ± 4	ND
NO synthesis (+NOHA)	115 ± 10	ND	75 ± 5	ND	100 ± 5	ND	72 ± 5	ND	57 ± 5	ND
NADPH oxidation (+Arg, +H_4B)	190 ± 18	12.7 ± 1.0	101 ± 8	12.4 ± 1.0	150 ± 12	11.7 ± 2.0	108 ± 5	12.0 ± 1.0	97 ± 5	13.0 ± 1.0
Cytochrome *c* reduction	7000 ± 500	610 ± 50	2800 ± 200	430 ± 35	5240 ± 404	478 ± 22	3020 ± 250	493 ± 25	2406 ± 200	200 ± 15
Ferricyanide reduction	4900 ± 200	952 ± 300	5050 ± 200	3800 ± 210	5049 ± 500	1500 ± 150	4900 ± 300	3700 ± 200	4675 ± 500	2450 ± 200
DCIP reduction	1702 ± 250	489 ± 35	754 ± 50	448 ± 42	1800 ± 217	435 ± 50	760 ± 80	490 ± 30	1060 ± 80	407 ± 20

[a] ND, not detectable.

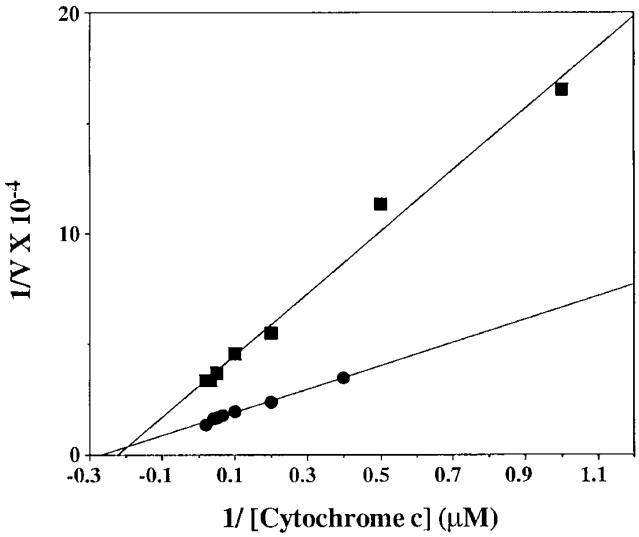

FIG. 4. **Double-reciprocal plots of cytochrome *c* reductase activity *versus* cytochrome *c* concentration for wild-type nNOS (●) and the FMN-reconstituted D918A mutant (■).**

TABLE VI
Apparent K_m values for cytochrome c and CaM in wild-type nNOS and FMN-reconstituted mutants

Each measurement was made in triplicate, according to the assay conditions described under "Experimental Procedures." The nNos was 0.5 nM.

nNOS protein	K_m of cytochrome *c*	K_m of calmodulin
	μM	*nM*
Wild-type	3.7 ± 0.1	3.3 ± 0.1
D918A	4.5 ± 0.2	3.1 ± 0.1
E919A	4.1 ± 0.2	3.5 ± 0.1
D918A,E919A	4.4 ± 0.2	3.2 ± 0.1
F892A	4.7 ± 0.2	3.3 ± 0.1

43) or mutation of distinct residues that directly contact or shield the FMN (36, 37). Thus, cluster 1 may function differently in nNOS as compared with other flavoproteins of its class. Crystal structures of cytochrome P450 reductase and *Anabaena* flavodoxin show that cluster 1 acidic residues and the conserved Phe (analogous to nNOS Phe^{892}) are positioned near the surface of the FMN module and away from the bound FMN (17, 29). If nNOS cluster 1 residues are similarly positioned, they would need to influence FMN binding through relatively long-range interactions.

Our cluster 1 mutants contained normal quantities of FAD

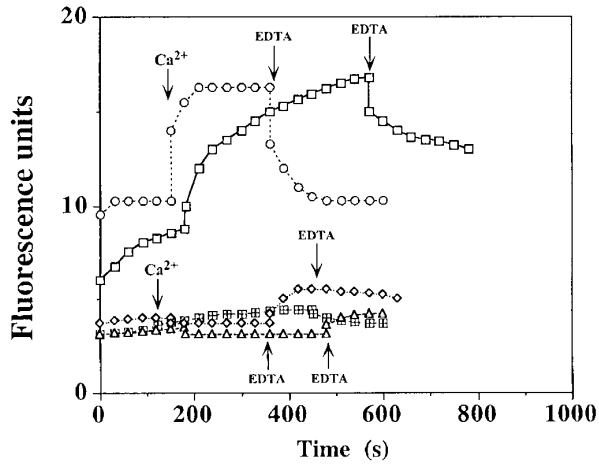

FIG. 5. **Kinetics of the CaM-induced change in flavin fluorescence at 15 °C.** CaM binding to wild-type nNOS or mutants was induced by adding excess Ca^{2+} at the times indicated by the *arrows*. After several minutes CaM was dissociated from the nNOS proteins by adding excess EDTA, as indicated by the *arrows*. Buffer composition and enzyme concentrations are described under "Experimental Procedures." Wild-type nNOS (□), E919A (○), D918A (◇), D918A,E919A double mutant (⊞), and F892A (△).

and heme, and could bind NADPH, H_4B, Arg, and CaM. Moreover, they all recovered considerable NO synthesis and cytochrome *c* reductase activity when supplied with exogenous FMN. This suggests the functional defects caused by mutation were primarily due to loss of FMN, and were restricted to the FMN module. However, because mutant activities approached but did not achieve wild-type levels even at the highest FMN concentrations, cluster 1 residues might have another effect on nNOS catalysis besides lowering FMN affinity. NO synthesis is related to the heme reduction rate in nNOS (9, 44), thus a slower electron transfer rate to the heme could explain the residual inhibition we observe. Cluster 1 residues might effect heme reduction rate by stabilizing the interaction between the FMN module and hemeprotein domain, or by modulating the redox properties of the bound FMN, as occurs in some related flavoproteins (22). However, the relatively minor residual inhibition that we observed in our FMN-reconstituted mutants clearly indicates that these effects would be secondary relative to the mutational effect on FMN binding. We conclude that cluster 1 residues are not as critical for electron transfer functions of the nNOS FMN module as for other flavoproteins of the same class.

Because cluster 1 mutations destabilized FMN binding in nNOS without greatly affecting other properties of the protein,

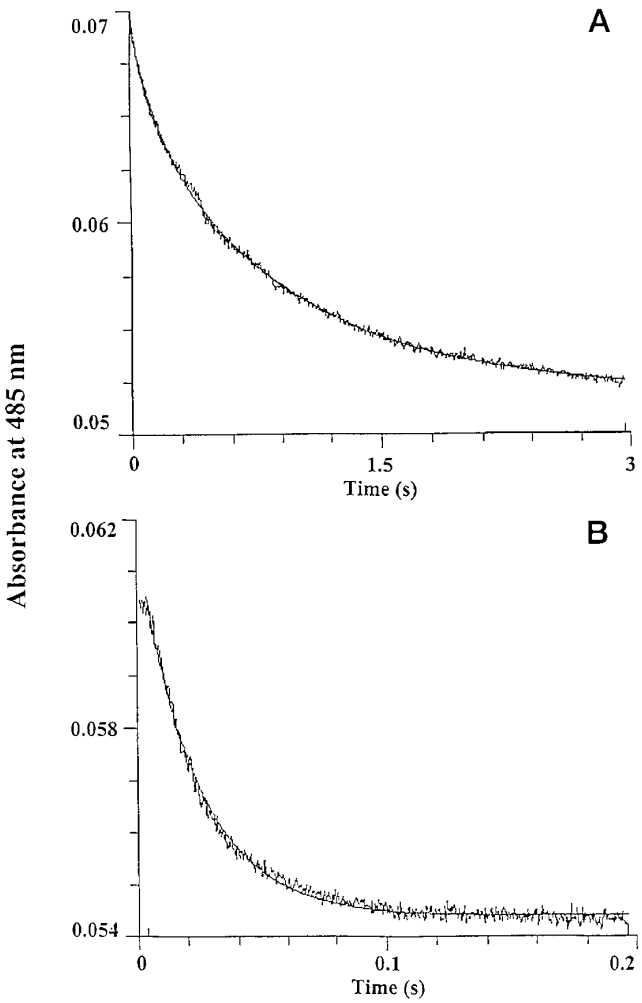

FIG. 6. **Kinetics of NADPH-dependent flavin reduction in the absence of CaM as measured by stopped-flow spectroscopy.** Flavin reduction was followed at 485 nm under anaerobic conditions at 10 °C. One syringe contained wild-type nNOS (3 μM, *panel A*) or mutant D918A (3 μM, *panel B*) in 40 mM HEPPS buffer, pH 7.6, containing 4 μM H_4B, 0.2 mM DTT, 50 mM NaCl, and 0.6 mM EDTA and was mixed rapidly with a syringe containing the above solution (minus enzyme) plus 1.2 mM NADPH. The *smooth line* running through each experimental trace is the line of best fit according to a two (*A*) or one (*B*) exponential equation.

TABLE VII
Observed rate constants for NADPH-dependent flavin reduction in wild-type nNOS and mutant proteins

Measurements were done under anaerobic conditions at 10 °C as described under "Experimental Procedures." The values are the average obtained with two or three preparations. The data were best fit to a monophasic rate for FMN-deficient mutants and a biphasic rate for wild-type nNOS, to generate two rate constants.

Protein	−CaM		+CaM	
	k_1	k_2	k_1	k_2
	s^{-1}		s^{-1}	
nNOS	9.2	1.6	54	14
D918A	43		38	
E919A	17		25	
D918A,E919A	39		48	
F892A	37		38	

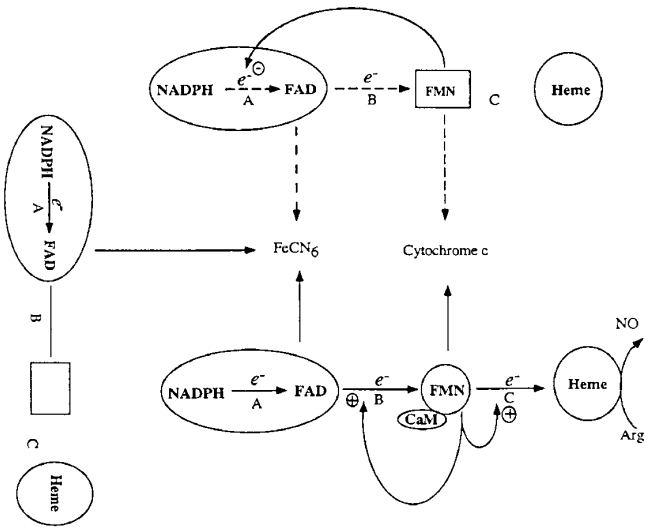

FIG. 7. **Key roles of the FMN module in nNOS function and CaM regulation.** In the absence of CaM (*upper horizontal figure*), the FMN module suppresses electron entry into the FNR module (*A*), and although it accepts electrons from the FNR module (*B*), it cannot transfer them to the nNOS heme (*C*). In the FMN-free nNOS (*left vertical figure*), the FMN module can no longer suppress FAD reduction in the FNR domain. When CaM binds to nNOS (*lower horizontal figure*), the FMN module undergoes a conformational change (illustrated by *square* to *circle* transition) that relieves its repression of the FNR domain (*A*), increases the rate of FMN reduction (*B*), and triggers electron transfer to the heme (*C*). These changes are associated with increased electron transfer from the indicated modules to ferricyanide ($FeCN_6$) and to cytochrome *c* (*dashed* to *solid arrow* transition), and initiation of NO synthesis.

they can help define reductase domain function. For example, we observed that bound FAD was still reduced by NADPH in the FMN-free mutants while electron transfer to the nNOS heme or cytochrome *c* was blocked. Also, CaM binding to the FMN-free mutants did not enable reduction of these acceptors. This establishes: 1) electron flow from NADPH to FAD, FMN, then heme in nNOS, as occurs in structurally related flavoproteins that do not bind CaM (18, 20, 45). 2) The FMN module of nNOS is the exit point for electron transfer to the nNOS heme or to other hemeproteins whether CaM is bound or not. Regarding electron transfer to smaller molecules like DCIP and ferricyanide, rates in the FMN- and CaM-free nNOS mutants were either not inhibited or in the case of ferricyanide were much faster than wild-type. Thus, the NADPH-FAD module can transfer electrons to these smaller molecules independent of the FMN module, as occurs in related flavoproteins (18, 20, 45), and is likely an important electron donor to these molecules in CaM-free nNOS.

In the CaM-free state, stopped-flow analysis showed that FMN-depleted mutants had faster rates of FAD reduction than wild-type nNOS. Their observed rates were as much as 4-fold faster, and varied in reverse proportion to their residual FMN content. Because NADPH-derived electrons are transferred first to FAD and then to FMN, our work shows that bound FMN can actually inhibit an "upstream" electron transfer step in the nNOS reductase. This effect has not been observed previously in the related proteins cytochrome P450 reductase, sulfite reductase, or cytochrome P450BM3, and so may be unique to the NOS. Our finding that FMN removal increased the rate of ferricyanide reduction in CaM-free nNOS, while it either lowers or does not change the rate in related flavoproteins (20, 36, 37, 43, 46, 47), supports this concept.

It is interesting that CaM binding to wild-type nNOS increased FAD reduction to a rate that was equivalent to the FMN-free nNOS mutants. Thus, CaM binding is functionally equivalent to FMN removal when one's view is restricted to FAD reduction. Two mechanisms are possible: bound CaM influences FAD reduction through a process that either relies on or does not involve the FMN module. The fact that CaM

binding did not further increase the rate of FAD reduction in FMN-free nNOS supports the first possibility. This suggests that bound CaM does not directly interact with the FNR module to increase FAD reduction, but instead acts indirectly through an effect on the FMN module that is functionally equivalent to FMN removal. This mechanism is consistent with CaM increasing flavin fluorescence only in the FMN module of nNOS.

Our current results suggest that the FMN module is the key response element in nNOS that regulates electron transfer at all points (summarized in Fig. 7). In the absence of CaM, the FMN module interacts with the FNR module to repress NADPH reduction of FAD (*point A*). In this circumstance, the FMN module accepts electrons from FAD (*point B*), but is unable to transfer electrons to the heme located in the oxygenase domain (*point C*). When CaM binds it causes a conformational change in the FMN module such that: 1) suppression of electron transfer in the FNR domain (*point A*) is relieved; 2) the rate of electron transfer from the FNR module to FMN (*point B*) is increased; and 3) the FMN module is able to transfer electrons to the oxygenase domain heme (*point C*). Removing FMN from its module by mutagenesis also relieves supression of FAD reduction within the FNR module, suggesting that the FMN module must be replete to have its effect. Regarding effects on catalysis, the relief of suppression at point A is associated with faster ferricyanide reduction, the increase in electron transfer at point B is associated with faster cytochrome *c* reduction, and the initiation of electron transfer at point C is associated with NO synthesis from Arg, or superoxide production in the absence of substrate. Together, these functions distinguish the nNOS reductase domain, and the FMN module in particular, from all other flavoproteins.

Our current model raises several interesting questions. For example, why does the nNOS FMN module slow electron transfer into the upstream FNR module of nNOS? Apparently, it must be important for the enzyme to slow electron flux through the reductase until a Ca^{2+} influx into cells occurs and CaM binding signals the time for heme reduction and NO synthesis. Is suppression of FAD reduction linked to the FMN module's inability to transfer electrons to the heme in the absence of CaM? This is unlikely, because previous work with CaM mutants indicates that repression of either FAD reduction (24) or ferricyanide reduction (24–26) can be relieved without activating electron transfer from FMN to the oxygenase heme. Thus, functions of the FMN module are separable, and may be controlled by different facets of CaM binding. Exactly how does CaM alter FMN module function and its interaction with the FNR module? Our fluorescence data suggest that CaM causes a conformational change exclusively in the FMN module that alters the FMN environment. Perhaps this alters the redox relationship between the flavins, or alters the FAD reduction potential relative to NADPH such that its reduction is thermodynamically more favorable. The mechanism may also involve a ~30 amino acid insert that is present in the FMN module of nNOS but absent in related flavoproteins, and has been proposed to regulate CaM affinity (48). These and other possibilities can now be addressed.

Acknowledgments—We thank Carol Crooks for molecular biology assistance and Pam Clark for protein purification of wild-type nNOS.

REFERENCES

1. Craven, S. E., and Bredt, D. S. (1998) *Cell* **93,** 495–498
2. Garthwaite, J., and Boulton, C. L., (1995) *Annu. Rev. Physiol.* **57,** 683–706
3. Kerwin, J. F., Jr., Lancaster, J. R., Jr., and Feldman, P. L. (1995) *J. Med. Chem.* **38,** 4343–4362
4. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H., (1991) *Nature* **351,** 714–718
5. Richards, M. K., Clague, M. J., and Marletta, M. A. (1996) *Biochemistry* **35,** 7772–7780
6. Sheta, E. A., McMillan, K., and Masters, B. S. S. (1994) *J. Biol. Chem.* **269,** 15147–15153
7. Klatt, P., Schmidt, K., Brunner, F., and Mayer, B. (1994) *J. Biol. Chem.* **269,** 1674–1680
8. McMillan, K., and Masters, B. S. S. (1995) *Biochemistry* **34,** 3686–3693
9. Gachhui, R., Presta, A., Bentley, D. F., Abu-Soud, H. M., McArthur, R., Brudvig, G., Ghosh, D. K., and Stuehr, D. J. (1996) *J. Biol. Chem.* **271,** 20594–20602
10. Vorher, T., Knopfel, L., Hofmann, F., Mollner, S., Pfeuffer, T., and Carafoli, E. (1993) *Biochemistry* **32,** 6081–6088
11. Mayer, B., and Werner, E. R. (1995) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **351,** 453–463
12. Marletta, M. A. (1994) *Cell* **78,** 927–930
13. Abu-Soud, H. M., and Stuehr, D. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10769–10772
14. Abu-soud, H. M., Yoho, L. L., and Stuehr, D. J. (1994) *J. Biol. Chem.* **269,** 32047–32050
15. Siddhanta, U., Presta, A., Fan, B., Wolan, D., Rousseau, D. L., and Stuehr, D. J. (1998) *J. Biol. Chem.* **273,** 18950–18958
16. Porter, T. D. (1991) *Trends Biochem. Sci.* **16,** 154–158
17. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. S., and Kim, J. P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8411–8416
18. Sevrioukova, I. F., and Peterson, J. A. (1995) *Biochimie* (*Paris*) **77,** 562–572
19. Schmidt, H. H. H. W., Smith, R. M., Nakane, M., and Murad, F. (1992) *Biochemistry* **31,** 3243–3248
20. Vermilion, J. L., Ballou, D. P., Massey, V., and Coon, M. J. (1981) *J. Biol. Chem.* **256,** 266–277
21. Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. M., and Kredich, N. M. (1989) *J. Biol. Chem.* **264,** 15796–15808
22. Sevrioukova, I. F., Li, H., Zhang, H., Peterson, J. A., and Poulos, T. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1863–1868
23. Narayanasami, R., Nishimura, J. S., McMillan, K., Roman, L. J., Shea, T. M., Robida, A. M., Horowitz, P. M., and Masters, B. S. S. (1997) *Nitric Oxide* **1,** 39–49
24. Gachhui, R., Abu-Soud, H. M., Ghosh, D. K., Presta, A., Blazing, M. A., Mayer, B., George, S. E., and Stuehr, D. J. (1998) *J. Biol. Chem.* **273,** 5451–5454
25. Stevens-Truss, R., Beckingham, K., and Marletta, M. A. (1997) *Biochemistry* **36,** 12337–12345
26. Cho, M. J., Vaghy, P. L., Kondo, R., Lee, S. H., Davis, J. P., Rehl, R., Heo, W. D., and Johnson, J. D. (1998) *Biochemistry* **37,** 15593–15597
27. Shen, A. L., and Kasper, C. B. (1995) *J. Biol. Chem.* **270,** 27475–27480
28. Nisimoto, Y. (1986) *J. Biol. Chem.* **261,** 14232–14239
29. Jenkins, C. M., Genzor, C. G., Fillat, M. F., Waterman, M, R., and Gomez-Moreno, C. (1997) *J. Biol. Chem.* **272,** 22509–22513
30. Burkhart, B. M., Ramakrishnan, B., Yan, H., Reedstrom, R. J., Markley, J. L., Straus, N. A., and Sundara lingam, M. (1995) *Acta Crystallogr. Sect. D* **51,** 318–330
31. Huang, L., Abu-Soud, H. M., Hille, R., and Stuehr, D. J. (1999) *Biochemistry* **38,** 1912–1920
32. Wu, C., Zhang, J., Abu-Soud, H. M., Ghosh, D. K., and Stuehr, D. J. (1996) *Biochem. Biophys. Res. Commun.* **22,** 439–444
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY
34. Stuehr, D. J., and Ikeda-Saito, M. (1992) *J. Biol. Chem.* **267,** 20547–20550
35. Abu-Soud, H. M., Feldman, P. L., Clark, P., and Stuehr, D. J. (1994) *J. Biol. Chem.* **269,** 32318–32326
36. Shen, A. L., Porter, T. D., Wilson, T. E., and Kasper, C. B. (1989) *J. Biol. Chem.* **264,** 7584–7589
37. Klein, M. L., and Fulco, A. J., (1993) *J. Biol. Chem.* **268,** 7553–7561
38. Sevrioukova, I. F., Shaffer, C., Ballou, D. P., and Peterson, J. A. (1996) *Biochemistry* **35,** 7058–7068
39. Beaty, N. B., and Ballou, D. P. (1981) *J. Biol. Chem.* **256,** 4611–4618
40. Shen, A. L., Sem, D. S., and Kasper, C. B. (1999) *J. Biol. Chem.* **274,** 5391–5398
41. Aliverti, A., Bruns, C. M., Pandini, V. E., Karplus, A., Vanoni, M. A., Curti, B., and Zanetti, G. (1995) *Biochemistry* **34,** 8371–8379
42. Vermilion, J. L., and Coon, M. J. (1978) *J. Biol. Chem.* **253,** 8812–8819
43. Eschenbrenner, M., Coves, J., and Fontecave, M. (1995) *J. Biol. Chem.* **270,** 20550–20555
44. Nishida, R. C., and Ortiz de Montellano, P. R. (1998) *J. Biol. Chem.* **273,** 5566–5571
45. Smith, G. C. M., Tew, D. G., and Wolf, C. R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8710–8714
46. Govindaraj, S., and Poulos, T. L. (1997) *J. Biol. Chem.* **272,** 7915–7921
47. Sevrioukova, I. F., Truan, G., and Peterson, J. A. (1996) *Biochemistry* **35,** 7528–7535
48. Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M. E., Martasek, P., Roman, L. J., Masters, B. S. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997) *J. Biol. Chem.* **272,** 29769–29777