The neuronal and endothelial nitric-oxide synthases (nNOS and eNOS) differ from inducible NOS in their dependence on the intracellular Ca^{2+} concentration. Both nNOS and eNOS are activated by the reversible binding of calmodulin (CaM) in the presence of Ca^{2+}, whereas inducible NOS binds CaM irreversibly. One major divergence in the close sequence similarity between the NOS isoforms is a 40–50-amino acid insert in the middle of the FMN-binding domains of nNOS and eNOS. It has previously been proposed that this insert forms an autoinhibitory domain designed to destabilize CaM binding and increase its Ca^{2+} dependence. To examine the importance of the insert we constructed two deletion mutants designed to remove the bulk of it from nNOS. Both mutants (Δ40 and Δ42) retained maximal NO synthesis activity at lower concentrations of free Ca^{2+} than the wild type enzyme. They were also found to retain 30% of their activity in the absence of Ca^{2+}/CaM, indicating that the insert plays an important role in disabling the enzyme when the physiological Ca^{2+} concentration is low. Reduction of nNOS heme by NADPH under rigorous anaerobic conditions was found to occur in the wild type enzyme only in the presence of Ca^{2+}/CaM. However, reduction of heme in the Δ40 mutant occurred spontaneously on addition of NADPH in the absence of Ca^{2+}/CaM. This suggests that the insert regulates activity by inhibiting electron transfer from FMN to heme in the absence of Ca^{2+}/CaM and by destabilizing CaM binding at low Ca^{2+} concentrations, consistent with its role as an autoinhibitory domain.

The nitric-oxide synthases (NOSs) are a family of dimeric enzymes found in a variety of organisms and cell types. They generate nitric oxide through the five-electron oxidation of L-arginine, consuming two equivalents of molecular oxygen and three electron equivalents derived from NADPH (1, 2). All the NOSs bind heme and 6R-5,6,7,8-tetrahydro-L-biopterin within a structurally unique N-terminal oxygenase domain (3) and bind FMN and FAD within a C-terminal reductase domain that is related to microsomal cytochrome P-450 reductase (4–6). NOS dimerization occurs largely through contacts between oxygenase domains and is augmented by the binding of (6R)-5,6,7,8-tetrahydro-L-biopterin (7). Evidence suggests that the reductase domains may supply electrons to the heme domain of the alternate subunit, making dimerization essential for NO synthase activity (8). The many different NOS sequences currently available for mammalian enzyme variants show all to be closely related. However, they can be broadly grouped into three different isoforms. Neuronal NOS (nNOS) and endothelial NOS (eNOS) were found to be constitutively expressed in their respective cell types, whereas inducible NOS (iNOS) was first found to be induced by cytokines in macrophages (9). The three isoforms can be categorized by both amino acid sequence and functionality (10), because they exhibit different rates of catalysis and sensitivity to regulatory ligands. The constitutive NOSs are regulated by the reversible binding of calmodulin (CaM) through changes in the intracellular Ca^{2+} concentration. iNOS, however, is expressed with CaM bound permanently and is insensitive to changes in Ca^{2+} concentration (11). The CaM binding site lies within a linker region between the N-terminal oxygenase and C-terminal reductase domains of the NOSs and consists of 20–25 amino acids. As Fig. 1 shows, the sequence of this region is not well conserved between the three NOS isoforms, and it has been found through the study of chimeric enzymes that it plays a major role in determining the CaM binding affinity (12, 13). Regulation of NO synthase activity by CaM has been shown to occur via the control of FMN to heme electron transfer across the domain-domain interface (14). Additionally, CaM has been shown to activate the nNOS reductase domain both within the native dimer and in a mutant consisting of the reductase domain only (15). The iNOS reductase domain, on the other hand, appears to be influenced little by CaM binding (16). As well as having different CaM binding sites to iNOS, the constitutive NOSs contain an additional 40–50-amino acid insert in the middle of a conserved region of the FMN-binding subdomain (see Fig. 1). The FMN-binding subdomain is related by amino acid sequence to the large, diverse family of flavodoxins, which generally function as small electron transfer proteins and to the FMN-binding subdomain of cytochrome P-450 reductase, which behaves similarly. However, the presence of such an insert is highly unusual. Furthermore, the insert lies between the two strands of β-sheet that form key binding interactions with the FMN, by analogy with the structure of cytochrome P-450 reductase (17). It has been proposed that the region acts as an autoinhibitory domain, competing with CaM for space on the interdomain linker (18, 19). Salerno et al. (19) showed that peptides based on the sequence of the eNOS insert are able to inhibit CaM binding to eNOS and nNOS. However, peptides based on the nNOS insert were less effective. This indicates that in the case of eNOS at least, the insert may make contact with a sequence-specific binding site in the vicinity of the CaM-binding site. To investigate the role of the nNOS insert, we constructed two mutants in which 40 and 42 amino acids have been deleted (Δ40 and Δ42), removing the bulk of the insert region (see Fig. 1).
Role of the Autoinhibitory Loop of nNOS

CaM binding region

nNOS
NGTPTKRBA1 GFKLAEYVK FSKALGQGAM AKRRKTALI TETGKSOAY AKTLCIE1F 779
eNOS
GI1TRKTK---- GVEQVAYK IASLGNOTY AKRKTATLLS GTGRTGQQS AGQLGRLFK 543
iNOS
KRRPKRRE-I PLKVLKAYL FACMLNRKTM ASRVRVT1LF ATETGKSAW ADWLGFSC 563
redu
GTTAPPK-- ----FS-- EVKMYTGRNN IFYGVSSGT AEAEFLRSLA DHARYGMG 109

Fig. 1. Amino acid sequence alignment of the FMN-binding subdomains of rat nNOS, human eNOS, human iNOS, and rat cytochrome P-450 reductase (redu). Sequences were taken from the SWISS-PROT data base and aligned using ClustalX (20). The nNOS calmodulin-binding site (725–754), the nNOS region deleted in the Δ40 mutant (Δ40 deletion), and two additional amino acids deleted in the Δ42 mutant (Δ42) are indicated. The two aromatic residues involved in FNM binding (Phe879 and Tyr889) for nNOS are marked by a down arrow. Conserved residues are marked by an asterisk.

1. We also constructed a Y889A mutant of nNOS designed to destabilize the binding of FNM to examine the effect of CaM binding on the properties of the FAD-binding subdomain.

EXPERIMENTAL PROCEDURES

Materials—(6R)-5,6,7,8-Tetrahydro-L-biopterin was purchased from Schircks Laboratories (Jona, Switzerland). Other reagents were obtained from Sigma or Wako Pure Chemicals (Osaka, Japan).

Molecular Biology—Rat nNOS cDNA was a kind gift of Dr. S. H. Snyder (Johns Hopkins School of Medicine). The Δ40 nNOS mutant plasmid (pSD1Δ40) was generated by ligating the Sal I site at Thr724 in the protein sequence to the Ban I site at Arg429 after blunt-end ligation with the Klenow fragment of DNA polymerase I. This was conducted on the plasmid pSD1 consisting of the 622-kilobase EcoRI fragment of nNOS (Gly724–Arg296) in the plasmid pUC19. The mutation site was transferred within the BglII–SpaI fragment of nNOS (Ile776–His897) to the corresponding region of the plasmid pBS-nNOS (21). The following mutations were generated using the oligonucleotide-directed dual amber, long and accurate polymerase chain reaction kit for site-directed mutations were generated using the oligonucleotide-directed dual amber

Cytochrome c reduction was monitored as an absorbance decrease at 420 nm using an extinction coefficient of 1.01 mM cm−1 (29), with 10 μM oxyhemoglobin, 0.1 mM NADPH, and 1 mM l-arginine. The NADPH oxidation rate was determined spectrophotometrically as an absorbance decrease at 340 nm using an extinction coefficient of 0.22 mM cm−1, with the concentration of NADPH at 0.1 mM. The ferricyanide reduction rate was determined by monitoring the decrease at 420 nm using an extinction coefficient of 1.01 mM cm−1, with concentrations of ferri-cyanide at 1 mM and NADPH at 0.5 mM unless otherwise indicated. Cytochrome c was modulated by CaM, the concentrations were 1 mM and 10 mM CaM, and 1 μM CaM, the concentrations were 1 mM and 10 μM CaM. All assays were carried out at 25°C in 50 mM Tris-HCl (pH 7.5) buffer containing 10 units/ml superoxide dismutase and 100 units/ml catalase. For assays containing Ca2+ and CaM, the concentrations were 1 mM and 10 μg/ml, respectively.

EGTA Titrations—In the above assays for NO synthesis and ferricyanide reduction, the concentration of free Ca2+ was modulated by introducing variable quantities of EGTA into the assay mixture. For these assays, the concentration of Ca2+ was kept at precisely 50 μM, and CaM was kept at 10 μM. The final concentration of enzyme in the assay mixture was 0.10–0.01 μM for both ferricyanide reduction and NO synthesis experiments.

Aneuratic Hemodilution—Concentrated, purified enzyme (e.g. 2 ml x 20 μl) was passed through an aneuratic Sephadex G25 column (1.5 x 10 cm) contained within a Hirasawa Works (Japan) glove box under an 80% N2/10% H2/10% CO2 atmosphere with O2 at less than 50 ppm. Spectra were recorded on a Shimadzu 1201 scanning UV/Vis spectrophotometer contained within the anaerobic environment. Aliquots of anaerobic solutions of NADPH, l-arginine, Ca2+/CaM, and CO-saturated buffer were added as described. The temperature of the glove box was maintained at 15°C throughout.

RESULTS

Construction of the Mutants—The alignment shown in Fig. 1 compares the amino acid sequences of the FMN-binding subdomains of the three NOS isoforms and that of cytochrome P-450 reductase, the most similar protein outside the NOS family. According to the crystal structure of cytochrome P-450 reductase (17), the FMN is bound between two aromatic resi-
dyes, Tyr\textsuperscript{139} and Tyr\textsuperscript{177}. In nNOS these align with Phe\textsuperscript{609} and Tyr\textsuperscript{688}, which likely perform a similar function (structures for NOS isoforms in this region are not available). The sequence similarity in the vicinity of these aromatic residues is striking. However, in both nNOS and eNOS, inserts of 42 and 45 amino acids, respectively, lie between the two homologous regions. To test the functional role of the nNOS insert, the deletion mutants \(\Delta 40\) and \(\Delta 42\) were constructed. The \(\Delta 40\) mutation was constructed by ligating the ends of nNOS DNA generated at the supply of electrons to nNOS heme does not limit the rate of catalytic turnover.

All mutants were expressed and purified in the same way as the wild type enzyme. Initially, the \(\Delta 40\) and \(\Delta 42\) mutants were found to lose activity during purification; however, this was partially recovered on incubation with excess FMN. Inclusion of FMN in the purification buffer and completion of the procedure within 1 day prevented any loss of activity. The \(\Delta 40\) mutant was found to be more stable than the \(\Delta 42\) mutant and is therefore the subject of more detailed analysis. Unlike nNOS, the native form of iNOS is permanently complexed with CaM. However, despite the similarity between the nNOS deletion mutants and iNOS, addition of excess CaM did not stabilize the mutants, which were readily purified on CaM-Sepharose. The Y889A mutant was found to be inactive and lacking the stable blue semiquinone observed during the purification of the other enzymes, consistent with an absence of FMN in this mutant. This was confirmed by measuring the FMN and FAD content relative to heme using the different fluorescence response of the two flavins in different buffers. The Y889A mutant was found to contain 0.06 (± 0.10) FMN and 0.92 (± 0.15) FAD per heme, compared with 0.97 (± 0.10) FMN and 1.01 (± 0.10) FAD per heme in the case of the wild type enzyme. Following purification and removal of free flavin, the FMN content of the \(\Delta 40\) and \(\Delta 42\) mutants was found to be 0.60 (± 0.10) and 0.67 (± 0.10), respectively, per heme with 0.96 (± 0.15) and 1.13 (± 0.15) FAD, respectively.

**Steady-state Kinetics**—Rate constants for the steady-state turnover of wild type and mutant forms of nNOS for NO synthesis, aerobic oxidation of NADPH, reduction of ferricyanide, and reduction of cytochrome \(c\) are collated in Table I. For the wild type enzyme, Ca\(^{2+}/\text{CaM}\) is required for NO synthesis or ferricyanide reduction, and reduction of cytochrome \(c\) is CaM-dependent. Both \(\Delta 40\) and \(\Delta 42\) mutant forms of nNOS are affected less by CaM binding as the rates observed in the absence of CaM are both higher than for the wild type enzyme. For the \(\Delta 42\) mutant, the ferricyanide reductase activity is CaM-independent. The Y889A mutation results in a severe loss of cytochrome \(c\) reductase activity, indicating that in the wild type enzyme, FMN is the chief electron donor to cytochrome \(c\). The ferricyanide reductase activity of this mutant is retained, but the effect of CaM is reversed, such that CaM binding causes a

| Ca\(^{2+}/\text{CaM}\) | NO synthesis \(^{\text{a, b}}\) | NADPH oxidation \(^{\text{c, d}}\) | Cytochrome \(c\) reduction \(^{\text{e, f}}\) | Ferricyanide reduction \(^{\text{g, h}}\) |
|----------------------|-----------------|-----------------|------------------|-----------------|
|                      | \(\min^{-1}\)    | \(\min^{-1}\) | \(\min^{-1}\) | \(\min^{-1}\) |
| Wild type            | +               | 45 ± 4          | 217 ± 20        | 58 ± 15         | 3550 ± 250       | 3270 ± 100       |
|                      | −               | 0               | 0.74 ± 0.15     | 2.9 ± 0.3       | 360 ± 20         | 1120 ± 500       |
| \(\Delta 40\)        | +               | 14 ± 3          | 293 ± 10        | 48 ± 1          | 3100 ± 180       | 3950 ± 30        |
|                      | −               | 4.0 ± 0.9       | 4.8 ± 1.0       | 15 ± 2          | 1340 ± 20        | 2980 ± 100       |
| \(\Delta 42\)        | +               | 9.9 ± 2.0       | 252 ± 30        | ND              | 2140 ± 20        | 3600 ± 100       |
|                      | −               | 4.2 ± 0.4       | 5 ± 1.0         | ND              | 1420 ± 20        | 3600 ± 100       |
| Y889A                | +               | 0               | 1.6 ± 2.0       | ND              | 86 ± 20          | 3950 ± 60        |
|                      | −               | 0               | 2.0 ± 2.0       | ND              | 56 ± 12          | 6450 ± 100       |

\(^{a}\) In the presence (+) or absence (−) of 1 mM Ca\(^{2+}\) and 10 \(\mu\)g/ml CaM.

\(^{b}\) Rate of conversion of oxhemoglobin (10 \(\mu\)M) to methemoglobin on NO synthesis in the presence of 0.1 mM NADPH and 1 mM L-arginine.

\(^{c}\) Rate of NADPH (0.1 mM) oxidation in the presence (+) or absence (−) of 1 mM L-arginine.

\(^{d}\) Rate of reduction of cytochrome \(c\) (100 \(\mu\)M) in the presence of 0.1 mM NADPH.

\(^{e}\) Rate of reduction of ferricyanide (1 mM) in the presence of 0.5 mM NADPH.

\(^{f}\) ND, not determined.
enzyme, the NO synthase activity rapidly drops to zero after CaM binding, and the enzymes lose activity. For the wild type m

mNOS (Y889A mutant in the presence and absence of Ca2+

the concentration of ferricyanide for wild type nNOS and the

EGTA Titrations—Fig. 3 plots the normalized NO synthase activity of wild type nNOS (●) and the Δ40 (□) and Δ42 (■) mutant enzymes on EGTA concentration in the presence of 50 μM Ca2+. The concentration of Ca2+ has dropped below the threshold value necessary to stimulate the enzyme. Both the Δ40 and Δ42 mutants retain maximal activity at higher EGTA concentrations and lose activity more slowly as the EGTA concentration increases. The mutants therefore require lower concentrations of free Ca2+ for stimulation. The mutants also retain 30–40% of their maximal activity even at high EGTA concentrations and turnover at a similar rate as in the absence of CaM (Table I).

Fig. 4 plots the normalized ferricyanide reductase activity against the EGTA concentration under similar conditions as those described above. Because the concentrations of Ca2+, CaM, and enzyme are the same, the plots can be compared directly with those in Fig. 3. The wild type enzyme loses activity rapidly as the concentration of free Ca2+ is depleted, reaching a value consistent with the loss of bound CaM. The Δ40 mutant retains maximal activity for longer than the wild type enzyme as with the NO synthase activity. The ferricyanide reductase activity of the Δ42 mutant shows no Ca2+ dependence, which is consistent with its lack of CaM dependence (Table I). For the Y889A mutant, the ferricyanide reductase activity increases as the free Ca2+ is depleted, indicating that the FAD-binding subdomain is itself Ca2+/CaM-dependent. The resultant curve is similar in shape to that of the wild type enzyme, indicating similar Ca2+ sensitivity.

Heme Reduction—Fig. 5 shows the anaerobic reduction of wild type nNOS on addition of NADPH. The first addition of NADPH (Fig. 5a) causes a broad decrease in absorbance between 400 and 500 nm consistent with the reduction of the two bound flavins of the enzyme. The position of the Soret peak is unchanged at around 398 nm. A further addition of NADPH and incubation for 20 min led to no significant change. Addition of CO up to 10% causes the formation of the reduced heme-CO complex characterized by the appearance of a peak at 444 nm.

Fig. 6 shows the anaerobic reduction of the Δ40 mutant upon addition of NADPH. As with the wild type enzyme, the first addition of NADPH causes a decrease in absorbance between 400 and 500 nm associated with the reduction of the two enzyme-bound flavins. However, in the case of the mutant, incubation over 20 min results in a shift in the position of the heme Soret peak to 409 nm, consistent with direct heme reduction (33, 34) in the absence of Ca2+/CaM. Addition of 0.1 mM L-arginine at this stage had no apparent effect. Addition of CO
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Fig. 5. Visible absorbance spectra showing the reduction of wild type nNOS heme by NADPH. a, spectrum i, after anaerobic gel filtration; spectrum ii, after addition of 300 μM NADPH and 20 min of incubation; spectrum iii, after addition of 100 μM L-arginine and 20 min of incubation. b, spectrum iv, same as spectrum iii; spectrum v, after addition of 1 mM Ca²⁺/10 μg/ml CaM and 20 min; spectrum vi, after addition of CO to 10% saturation and incubation for 25 min; spectrum vii, after addition of excess dithionite.

Addition of NADPH to the Y889A mutant under similar conditions failed to result in heme reduction even in the presence of Ca²⁺/CaM and CO. The heme reduced fully, however, on addition of dithionite, producing a peak at 444 nm in the presence of CO (not shown).

DISCUSSION

All three NOS isoforms consist of a diflavin-binding reductase domain coupled directly to a heme-binding monooxygenase domain via a CaM-binding linker region. CaM is bound by all functional forms of NOS. However, it is bound irreversibly by iNOS, whereas in nNOS and eNOS its binding is Ca²⁺-sensitive. CaM binding enables NADPH dehydrogenation to be coupled to the monooxygenation of L-arginine via interdomain electron transfer (34). At low Ca²⁺ concentrations, nNOS and eNOS are unable to function and have negligible activity. In neuronal and endothelial cells, Ca²⁺-sensitive CaM binding is used to regulate NO synthesis directly and therefore regulates important physiological functions. It may also be involved in a regulation mechanism involving isoforms of caveolin, which are reported to inhibit CaM binding and NO synthesis (35, 36).

Although the structure of the dimeric iNOS heme domain was recently reported (3), the nature of its interaction with the reductase domain is not known. The structure of the reductase domain itself is probably related to that of cytochrome P-450 reductase (17), which has a similar amino acid sequence. The route of electron transfer through the NOSs has therefore been assumed to be the same as in the cytochrome P-450 system, i.e., NADPH to FAD to FMN to heme. This is confirmed by our observation that the Y889A mutant of nNOS remains an efficient NADPH dehydrogenase-ferricyanide reductase (Table I) but is unable to transfer electrons to the nNOS heme. This is almost certainly because the mutant no longer binds FMN. The concentration dependence of the ferricyanide reductase activity of the Y889A mutant (Fig. 2) shows approximately second order behavior, indicating weak ferricyanide binding at the FAD site. In contrast, the plot for wild type in the presence of Ca²⁺/CaM indicates that the rate of ferricyanide reduction is largely independent of ferricyanide concentration. This suggests that ferricyanide binds much more tightly to the electron transfer site when FMN is present, resulting in the line intersecting the x axis at around 2200 min⁻¹, which would correspond to the rate of electron transfer from FAD to FMN. This value is similar to that reported for pre-steady-state flavin reduction by NADPH using stopped flow spectrophotometry (15). In the absence of Ca²⁺/CaM, the intercept value is decreased considerably (as is the pre-steady-state rate constant). If the y intercept does correspond to the rate of ferricyanide reduction at the FMN site, the slopes of the lines indicate the concentration dependence of ferricyanide reduction at the FAD site. However, the gradients of the lines plotted for the wild type enzyme are much less than for the Y889A mutant. This indicates that the mutation and consequent loss of FMN has also disrupted the FAD site, making it more accessible to ferricyanide binding. This is not surprising given the proximity of FAD to FMN reported in the x-ray crystal structure of cytochrome P-450 reductase (17).

To find the structural basis for the difference between the CaM sensitivity of the inducible (iNOS) and constitutive (nNOS and eNOS) isoforms, a series of chimeric enzymes have been studied. Because the amino acid sequences of the NOS isoforms consist of mostly homologous regions, it is reasonable to expect individual domains and subdomains to be interchangeable. This led Ruan et al. (12) to swap the CaM-binding
sites of iNOS and nNOS. Both chimeric enzymes required lower concentrations of Ca\(^{2+}\) than nNOS for stimulation but were not active in the absence of Ca\(^{2+}\), indicating that the sequence of the CaM-binding site affects the Ca\(^{2+}\) sensitivity but does not determine it entirely. Nishida and Montellano (37) generated chimeras in which the nNOS reductase domain was coupled with the iNOS and eNOS heme domains, including the CaM-binding sites. The iNOS chimera, which was coexpressed with CaM for stability, was found to be partially Ca\(^{2+}\)-dependent, retaining 40% of its activity in the presence of 2.5 mM EGTA. Lee and Stull (38) studied a similar chimera and found that the CaM was dissociable but retained activity in the absence of Ca\(^{2+}\). They also showed that a chimera consisting of the reductase domain and CaM-binding site of iNOS and the heme domain of nNOS bound CaM irreversibly and retained 60% of its activity in the absence of Ca\(^{2+}\). These studies all show that the Ca\(^{2+}\)-independent activity of iNOS is brought about by a range of structural features including elements in the reductase and oxygenase domains and in the CaM-binding site.

The 40–50-amino acid insert found only in the nNOS and eNOS isoforms has been speculatively proposed to be an autoinhibitory domain (18, 19), but direct evidence for this was not presented in the previous studies. Therefore, to determine the role of the insert in nNOS, we generated two deletion mutants (Δ40 and Δ42) lacking the bulk of this region. As expected, both of the mutants behaved similarly, with the more stable one, Δ40, being studied in most detail. Steady-state analysis indicated that the deletion mutants supported NO synthesis in the absence of CaM at 30% of the rate in its presence. NO synthesis in the absence of CaM has not previously been demonstrated for any intact NOS form, although was thought to occur in a chimeric mutant consisting of the nNOS oxygenase domain and iNOS reductase domain (38). In our case, it is unlikely that CaM generated by the yeast could have co-purified with the enzymes following chromatography on both ADP-Sepharose in the presence of 1 mM EGTA and CaM-Sepharose. This is confirmed by the fact that the mutants were not activated by Ca\(^{2+}\) alone but required addition of CaM to reach maximal activity. Furthermore, the EGTA titrations shown in Fig. 3 indicate that the catalytic effects of CaM binding are lost at high concentrations of EGTA, consistent with CaM dissociation. Addition of NADPH to the Δ40 mutant under anaerobic conditions (Fig. 6) led to spontaneous heme reduction in the absence of CaM, an effect that was manifested in the bulk of the enzyme (80–90%). This could not be repeated for the wild type. Therefore, it is unlikely that the Ca\(^{2+}\)/CaM-independent NO synthase activity exhibited by the deletion mutants could be caused by a minority contaminant of strongly bound CaM.

The wild type enzyme and the deletion mutants all catalyze NADPH oxidation at similar rates in the presence of CaM, indicating that electron transfer to the heme is not impeded by deletion of the insert. However, the Δ40 mutant catalyzes NO synthesis at only 30% of the rate of the wild type enzyme in the presence of CaM. For the wild type enzyme NO synthesis is closely coupled to NADPH oxidation in the presence of L-arginine, but the Δ40 mutant is only 50% efficient. The heme active site may have been affected directly by the mutation or indirectly via the disruption of precise interactions formed between the reductase and oxygenase domains on CaM binding.

The rate of NADPH oxidation is low for Δ40 in the absence of CaM and increases on addition of L-arginine. This is opposite to the effect of L-arginine in the presence of CaM. L-Arginine binding is known to shift the spin state of the NOS heme from mixed to high spin, which facilitates heme reduction by electron transfer. This is consistent with heme reduction being rate-determining for the Δ40 mutant in the absence of CaM. However, in the presence of CaM and L-arginine accumulation of NO at the heme active site is reported to slow the enzyme down (39). For the wild type enzyme, CaM binding is prerequisite for electron transfer to occur to the heme; this is demonstrated by the anaerobic heme reduction experiments (Fig. 5). For the Δ40 mutant, CaM binding is not essential but increases the rate by at least 60-fold (which is the effect on the NADPH oxidation rate). In wild type nNOS, therefore, the insert appears to ensure that the rate of electron transfer to the heme is zero in the absence of CaM, which would enable NO synthesis to be switched off completely during Ca\(^{2+}\)/CaM-dependent regulation.

CaM binds to the interdomain linker of NOS and is thought to transform this region into an encapsulated helix, causing a change in the interface between the reductase and oxygenase domains. The insert appears to impede this process. It is possible, therefore, that by inhibiting the structural rearrangement the insert might simultaneously inhibit CaM binding, i.e., by destabilizing the active (CaM-bound) configuration.

As well as activating FMN to heme electron transfer, CaM binding increases the cytochrome c and ferricyanide reductase activities of nNOS. This has been shown to occur even in a construct lacking the heme domain (15). It was proposed that cytochrome c reduction occurred primarily at the FMN site and that CaM binding increased the rate of electron transfer from FAD to FMN. This is confirmed by the low cytochrome c reductase activity observed for the Y889A mutant (Table I), which is deficient in FMN. This mutant retains ferricyanide reductase activity, indicating that the FAD domain of the enzyme is still functioning as an effective NADPH dehydrogenase. Mutants consisting of the reductase domains of human and mouse iNOS were recently reported to show CaM-independent reductase activity and appeared to bind CaM irreversibly (16). CaM binding was also found to have less effect on the reductase activity of the deletion mutants than the wild type enzyme (Table I). This suggests that the insert, not present in iNOS and removed from the deletion mutants, also plays a role in regulating the reductase activity of nNOS. It is possible, therefore, that CaM binding induces a repositioning of the FMN with respect to both the FAD and the heme in nNOS to create an optimal electron transfer chain.

During the review of this manuscript, a similar study involving the deletion of the corresponding region of eNOS was reported (40). It was found that the eNOS loop deletion mutant was activated at a lower concentration of Ca\(^{2+}\) than wild type eNOS but did not function as an NO synthase in the absence of Ca\(^{2+}\). These results are consistent with the proposal of Salerno et al. (19) that the eNOS loop competes directly with CaM binding by interacting with a sequence-specific binding site, based on the observation that peptides derived from the sequence of the eNOS loop could act as potent inhibitors. Peptides based on the nNOS loop were not effective inhibitors, however, suggesting a different mode of action for the loop of this isoform. Our results suggest that the nNOS loop not only affects the Ca\(^{2+}\) sensitivity of the enzyme but also directly inhibits FMN to heme electron transfer in the absence of Ca\(^{2+}\)/CaM, ensuring that the wild type enzyme can be completely deactivated at low Ca\(^{2+}\) concentrations. Both effects help to define the response of nNOS to changes in Ca\(^{2+}\) concentration and therefore have physiological relevance.

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