Removal of a Putative Inhibitory Element Reduces the Calcium-dependent Calmodulin Activation of Neuronal Nitric-oxide Synthase*

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Neuronal nitric-oxide synthase (NOS) and endothelial NOS are constitutive NOS isoforms that are activated by binding calmodulin in response to elevated intracellular calcium. In contrast, the inducible NOS isoform binds calmodulin at low basal levels of calcium in resting cells. Primary sequence comparisons show that each constitutive NOS isoform contains a polypeptide segment within its reductase domain, which is absent in the inducible NOS enzyme. To study a possible link between the presence of these additional polypeptide segments and constitutive NOS enzymes and their calcium-dependent calmodulin activation, three deletion mutants were created. The putative inhibitory insert was removed from the FMN binding regions of the neuronal NOS holoenzyme and from two truncated neuronal NOS reductase enzymes in which the calmodulin binding region was either included or deleted. All three mutant enzymes showed reduced incorporation of FMN and required reconstitution with exogenous FMN for activity. The combined removal of both the calmodulin binding domain and the putative inhibitory insert did not result in a calmodulin-independent neuronal NOS reductase. Thus, although the putative inhibitory element has an effect on the calcium-dependent calmodulin activation of neuronal NOS, it does not have the properties of the typical autoinhibitory domain found in calmodulin-activated enzymes.

The mammalian nitric-oxide synthases (NOSs, EC 1.14.13.39) all produce nitric oxide (NO) and the by-product l-citrulline from the substrates l-arginine, NADPH, and molecular oxygen (1, 2). The constitutively expressed neuronal NOS (nNOS, NOS-1) and endothelial NOS (eNOS, NOS-3) produce NO as an intercellular signal within the nervous and endothelial systems, respectively. These two constitutive NOS (eNOS) isoforms are controlled by the calcium-dependent binding of the regulatory protein calmodulin (CaM). The inducible NOS isoform (iNOS, NOS-2) binds CaM at basal levels of calcium (Ca$^{2+}$) (3) and is expressed in macrophages where its production is transcriptionally regulated and induced by inflammatory mediators such as tumor necrosis factor α, interleukin 1, and bacterial lipopolysaccharides (1, 2, 4).

The structure of all three isoforms of NOS can be divided into two domains, an NH$_2$-terminal oxygenase (heme) domain and a COOH-terminal reductase (flavin) domain (5). Separating these two domains is the CaM binding region (6–8). Heme and tetrahydrobiopterin are essential cofactors found in the oxygene-nase domain, which also contains the l-arginine binding site. FAD and FMN are essential cofactors found in the reductase domain, which also possesses a site for NADPH binding. Both the oxygenase and reductase domains of NOSs are functionally independent and can be separated (either by enzymatic digestion or by recombinant means) from one another (7, 9–11). The Ca$^{2+}$-dependent CaM activation of cNOS enzymes and the Ca$^{2+}$-independent activation of the iNOS isoform are maintained by their respective reductase domains (7, 12, 13). Although the CaM response is complete within the reductase domains of these enzymes, the Ca$^{2+}$-independent activity of iNOS apparently depends on the interactions of CaM with the reductase domain and oxygenase domain, as well as the CaM binding region (6, 8, 14).

Sequence alignments of the flavin binding regions of flavoproteins and the NOS isoforms revealed that the cNOSs contain an extra 43–46 amino acids within their FMN binding domains (Fig. 1) which are absent in iNOS, cytochrome P450 reductase, and flavodoxin (15, 16). The additional amino acids in cNOS enzymes were proposed to be autoinhibitory elements that interfere with CaM binding (15). Autoinhibitory domains have been reported in a number of CaM-dependent enzymes, including smooth muscle myosin light chain kinase, CaM-dependent protein kinase II, and calcineurin (17–19). Peptides derived from the putative autoinhibitory domain of eNOS, but not nNOS, inhibited CaM binding and Ca$^{2+}$ activation of both nNOS and eNOS (15).

To investigate the possibility that the 43-amino acid insert affects the Ca$^{2+}$-dependent CaM activation of nNOS, this element was removed from the rat nNOS reductase domain and holoenzyme using site-directed mutagenesis. Three deletion mutants were constructed, the reductase mutants ΔnNOS CaM$^-$ (which does not possess the CaM binding region) and ΔnNOS CaM$^+$ (which does possess the CaM binding region), as well as a mutant ΔnNOS holoenzyme. The mutant and native enzymes were used in a comparative study of the effect(s) of deleting this element on the enzyme flavin composition and the Ca$^{2+}$-dependent CaM activation of nNOS. The results from these mutant enzymes are compared with the wild-type nNOS and the iNOS reductases and holoenzymes. Our findings establish that the 43-amino acid element of nNOS affects but
Sequence alignment of the FMN binding region of NOS enzymes and other flavoproteins. A structure-based sequence alignment of the FMN binding region shows the additional amino acid elements found only in nNOS and eNOS isoforms as reported previously (14). The FMN binding regions are identified from the three-dimensional structure of the rat cytochrome P450 reductase enzyme (15). The proteins represented are: CPR, rat cytochrome P450 reductase; nNOS, rat neuronal NOS; eNOS, bovine endothelial NOS; iNOS, mouse inducible NOS; FDX, Desulfovibrio vulgaris flavodoxin. The eNOS E1 peptide (WRRKRK) as well as the nNOS N1 (SRKSSG) and 2 reductases were identified for the human iNOS and rat nNOS CaM.

A structure-based sequence alignment of the FMN binding region shows the additional amino acid elements found only in nNOS and eNOS isoforms as reported previously. The FMN binding regions are identified from the three-dimensional structure of the rat cytochrome P450 reductase enzyme. The proteins represented are: CPR, rat cytochrome P450 reductase; nNOS, rat neuronal NOS; eNOS, bovine endothelial NOS; iNOS, mouse inducible NOS; FDX, Desulfovibrio vulgaris flavodoxin. The eNOS E1 peptide (WRRKRK) as well as the nNOS N1 (SRKSSG) and 2 reductases were identified for the human iNOS and rat nNOS CaM.

**Fig. 1.** Sequence alignment of the FMN binding region of NOS enzymes and other flavoproteins. A structure-based sequence alignment of the FMN binding region shows the additional amino acid elements found only in nNOS and eNOS isoforms as reported previously (14). The FMN binding regions are identified from the three-dimensional structure of the rat cytochrome P450 reductase enzyme (15). The proteins represented are: CPR, rat cytochrome P450 reductase; nNOS, rat neuronal NOS; eNOS, bovine endothelial NOS; iNOS, mouse inducible NOS; FDX, Desulfovibrio vulgaris flavodoxin. The eNOS E1 peptide (WRRKRK) as well as the nNOS N1 (SRKSSG) and N2 (IRSKYKRF) peptides, shown as underlined amino acids in the above sequences, are composed of sequences found within the amino acid insertions of the respective NOS isoforms.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—The expression plasmids for the human iNOS and rat nNOS CaM+ reductases coexpressed with CaM and the plasmids for the human iNOS and rat nNOS CaM− reductases were identical to those described previously (7). Expression plasmids containing the rat nNOS holoenzyme and the mouse iNOS holoenzyme were generously provided by Dr. B. S. S. Masters (20, 21). Overlap extension mutagenesis (22), using the primers NNRDEL1 5′-GGG GAG AAA TTC-3′ and NNRDEL2 5′-CCT CAC ATT GGC CAG GGG TCC AGT-3′, was used to loop out the 126 base pairs that coded for the putative autoinhibitory region Motex-Ser770 (Fig. 1) in the rat nNOS CaM+, CaM−, and holoenzyme plasmids. The resulting expression plasmids were labeled pRPNRD, pRPNRD, and nNOSdpCW, respectively. All polymerase chain reaction experiments were performed using PWO polymerase and the buffers supplied by the manufacturer (Roche). The coding sequence of each plasmid carrying a mutant gene was fully sequenced at the MobiX facility at McMaster University to ensure that no spontaneous mutations had resulted from the polymerase chain reaction.

**Protein Expression and Purification**—The NOS reductases and nNOS and iNOS holoenzymes were expressed and purified as described previously (7, 20, 21). A modification of the method was required for the expression of the ΔnNOS holoenzyme because cotransfection of the nNOSdpCW vector with the pGroESL vector expressing groEL and groES did not result in any appreciable yield of protein. The nNOSDpCW vector with the pGroESL vector expressing groEL and groES was cotransfected into B21 cells. The resulting expression vectors were labeled pCRNNRD, pCRNNRD, and pCRNNRD, respectively. The protein was performed as described previously (20). In the purification of ΔnNOS, all of the buffers and procedures used were the same as for the nNOS holoenzyme except that the resuspension buffer also contained 0.3 mM Ca2+. To remove the bound CaM, the wash buffer for the 2.5-ADP column contained 1 mM EDTA.

**Immunoblot—SDS-polyacrylamide gel electrophoresis and Western blots** were performed using 25-pmol samples of purified protein, as described previously (Fig. 2) (7). Western blots were detected using a polyclonal anti-rabbit secondary antibody conjugated with alkaline phosphatase phosphatase diluted 1:10,000 (Sigma) and the alkaline phosphatase detection system (Sigma).

**Spectroscopy—Visible spectroscopy** was performed on a Cary 1 Bio UV-visible spectrophotometer. The ΔnNOS CaM+ and CaM− mutant reductases each exhibited typical maxima at 390 nm and 455 nm (Fig. 3) and were quantified using a colorimetric protein concentration assay (Bio-Rad) in addition to the extinction coefficient of 15.4 mm−1 cm−1 (7).

For the holoenzymes, formation of the hemoprotein ferrous-CO adduct, with a Soret band at 445 nm, was used to quantitate the heme-proteins. Samples were bubbled with CO for 5–10 s and divided between the sample and the reference cuvettes. The absorbance difference was adjusted to zero, and the contents of the sample cuvette were reduced by the addition of a few grains of dithionite. The difference spectrum was recorded from 500 nm to 400 nm, and the concentration of the target hemoprotein was estimated using the extinction coefficient of 78 mm−1 cm−1 (Amax-A500) (13). Measurements were performed in 10 mM Tris-HCl, pH 7.4, at 25 °C. Enzymes were also quantified using a colorimetric protein concentration assay (Bio-Rad).

**Quantiﬁcation of FAD and FMN released from nNOS CaM− reductase domain by guanine hydrochloride treatment** was performed as described previously (23). The nNOS CaM− reductase domain (53 μM) was incubated in 10 mM Tris-HCl, pH 7.4, in the presence of various concentrations of guanine hydrochloride at 4 °C for 1 h before scans were taken. Ultrafiltration for 15 min was performed at 4 °C using a CentriSart C4 concentrator (Sartorius), and flavins released from the enzyme after the guanine hydrochloride incubation were monitored using the fluorescence assay described below.

**Enzyme Kinetics**—Ferricyanide (FeCN) and dichlorophenolpho- nophenyl (DCPIP) assays on the NOS reductase enzymes were performed on a Cary 1 Bio UV-visible spectrophotometer. Reaction mixtures contained 50 mM Tris-HCl buffer, pH 7.5, 20 units each of superoxide dismutase and catalase, 0.1 mM DCPIP or 1 mM FeCN (extinction coefficients of 20.6 mm−1 cm−1 at 600 nm and 1.02 mm−1 cm−1 at 420 nm, respectively), and 0.2 mM (final concentration) of NADPH (9). Reactions were initiated by the addition of a 31.5 mM concentration of the appropriate probe.

Electron transfer catalyzed by the NOS reductase domains was monitored as NADPH-dependent cytochrome c reduction as described previously (7). Assays were performed on a Varian-Cary Bio 1 spectrophotometer by recording the increase in absorbance changes at 550 nm with time using an extinction coefficient of 21 mm−1 cm−1 for cytochrome c (24). The concentration of cytochrome c was varied between 10 and 150 μM. Reaction mixtures with a total volume of 750 μl contained 20 units Tris-HCl buffer, pH 7.5, 20 units each of superoxide dismutase and catalase to remove any superoxide that may have been produced by the NOS flavoproteins or free flavins. The FMN dependence of the mutant nNOS reductases was determined by preincubation of the enzymes in 25–1000 μM CaM, and 100 μM FMN for 30 min at 4 °C before diluting the flavin content by 150-fold in the reaction mixture. The dependence of these enzymes on FAD and riboflavin was determined in a similar manner.

The initial rate of NADPH oxidation by NOS was determined spectroscopically by monitoring the decrease in absorbance at 340 nm with time (extinction coefficient of 6.22 mm−1 cm−1) at 37 °C (25). The reaction mixture contained 50 mM Tris-HCl, pH 7.6, 3 mM dithiothreitol, 1 mM L-arginine, 4 mM FAD and FMN, 10 units/ml catalase, 0.1 mM NADPH, 4 μM tetrahydrobiopterin, 1 μM CaM, and 100 μM CaCl2 in a final volume of 1 ml. Reactions were initiated by the addition of 150 nM enzyme.

NO synthesis was measured by both the formation of l-arginine from l-arginine (25) and the hemoglobin capture assay (26). For the l-arginine assay, reaction mixtures contained 50 mM Tris-HCl, pH 7.6, 0.5 mM L-arginine, 20 μM l-arginine (0.045 μM), 10 μg/ml CaM, 1 mM CaCl2, 10 μM tetrahydrobiopterin, 100 μg/ml bovine serum albumin, and 1.0 mM NADPH in a final volume of 50 μl. Reactions were started by the addition of 1.5 μg of enzyme. Reactions were incubated at
Visible absorption spectra of wild-type and mutant rat nNOS reductase domains. Semi-quinone spectra were obtained by adding NADPH (15 μM final concentration) and recorded after equilibrium had been reached (>15 min). Panel A, 20 μM wild-type nNOS CaM; panel B, 15 μM ΔnNOS CaM; panel C, 15 μM wild-type nNOS CaM--; panel D, 15 μM ΔnNOS CaM--. Thick lines, semi-quinone form of the proteins.

25 °C for 2 min and then stopped by the addition of 200 μl of stopping buffer (5 mM EGTA and 100 mM Tris-HCl, pH 5.5). The activities of the NOS holoenzymes were measured using the hemoglobin assay (extinction coefficient of 60 mM⁻¹ cm⁻¹ at 401 nm) as described previously (26). Reaction mixtures contained 50 mM Tris-HCl buffer, pH 7.5, 20 units each of superoxide dismutase and catalase, 8 μM oxyhemoglobin, 1 mM arginine, 1 mM CaCl₂, 1 μM CaM, 6 μM tetrahydrobiopterin, and 0.2 mM NADPH in a total volume of 1 ml. Assays were initiated by the addition of 150 nM nNOS, ΔnNOS, or iNOS holoenzyme.

CO Difference Spectroscopy—The samples were equilibrated at 25 °C in Tris-HCl buffer at pH 7.5 saturated with CO as described previously (27). The spectra were obtained using sealed cuvettes containing 0.5 μM enzyme, 0.5 μM CaM, 400 μM CaCl₂, 100 μM L-arginine, and 5 μM BH₄. Analysis was initiated by the addition of concentrated NADPH to a final concentration of 200 μM.

Flavin Analysis—The FAD and FMN contents of the reductases and holoenzymes were determined using a fluorescence method developed by Faeder and Siegel (28). Pure FAD and FMN (greater than 95% pure) were purchased from Sigma. Fluorescence measurements were performed in triplicate on a 4800S SLM spectrophotometer with SMC 210 monochromator control at 27 °C using an excitation slit width of 8 nm and an emission slit width of 16 nm.

Calcium-dependent Measurements—Activity assays for the reductase enzymes and the holoenzymes were performed in triplicate and as described above except that all cofactor solutions and buffers were treated with Chelex 100 (Bio-Rad) before use. The recombinant bovine brain CaM used in our experiments was a generous gift from Dr. Merentes. The CaM used was depleted of bound Ca²⁺ by adjusting the pH to 2.0, desalting in 10 mM HCl on a PD10 column (Amersham Pharmacia Biotech), followed by adjusting the pH to 7.5 with 0.5 M Tris-HCl (21). KCl was added to 100 mM concentration, and the sample was concentrated by a Centricon 10 concentrator (Amicon). The CaM concentration was determined spectrophotometrically at 277 nm using an extinction coefficient of 3029 M⁻¹ cm⁻¹ (29) and by a colorimetric protein concentration assay (Bio-Rad). Assay buffers with exact Ca²⁺-free concentrations were prepared as described previously (30) with an ionic strength of 100 mM KCl.

Peptide Assays—The E1 (WRKRKK), N1 (SRKSSG), and N2 (RKSYYKRF) peptides (Fig. 1) were prepared by the peptide synthesis facility at the University of Waterloo. Purity, evaluated by reverse phase high performance liquid chromatography and electrospray mass spectrometry, exceeded 90% in all cases. Peptide concentrations of 100 μM were used in all NOS activity assays under standard conditions unless stated otherwise. Peptide studies were performed in the presence of up to 100 μg/ml of peptide as indicated. When the CaM–free enzymes were assayed, the assays were supplemented with 12.5 pmol of CaM and 0.3 mM CaCl₂. All assays were initiated by the addition of either 25 pmol of the nNOS reductase enzymes, 30 pmol of iNOS reductase enzymes, or 150 nM NOS holoenzymes.

RESULTS AND DISCUSSION

Expression and Purification of the NOS Enzymes—An explanation has yet to be found for the inherent differences in Ca²⁺-dependent CaM activation of the NOS isoforms. It has long been known that the constitutively expressed neuronal and endothelial NOS enzymes, collectively referred to as eNOS isoforms, contain an additional loop of 40–55 amino acids in the reductase domain which is absent in the iNOS enzyme (2). It has been proposed that these additional amino acids may act as a CaM autoinhibitory domain. The removal of a typical CaM autoinhibitory domain would be expected to decrease significantly the Ca²⁺-dependent CaM activation of the enzyme (17).

We decided to investigate the function of the 43-amino acid insert in both the reductase domain and holoenzyme of nNOS because both enzymes show Ca²⁺-dependent CaM activation. Site-directed mutagenesis was used to remove a 43-amino acid loop found within the FMN binding domain of the nNOS isoform which is not found within the FMN binding domain of the iNOS enzyme (Fig. 1). Removal of this element from the nNOS reductase domains and holoenzyme created the ΔnNOS CaM--; (possessing the CaM binding region) and the ΔnNOS CaM-- (without the CaM binding region) reductase domains as well as the ΔnNOS holoenzyme. Fig. 2A shows the Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis of the mutant and wild-type reductase domains. Using this method of analysis the final purity of the proteins was judged to be greater than 90%. Calmodulin was found to copurify with the enzymes when the gene was coexpressed using a polycistronic plasmid. An immunoblot of the wild-type and mutant reductase domains shows the decrease in the size of the mutants caused by the deletion of the loop (Fig. 2B). The ΔnNOS holoenzyme showed the same reduction in size compared with the wild-type nNOS holoenzyme (data not shown).

The 43-amino acid element appears to have a stabilizing effect on the CaM binding region of the rat nNOS reductase domains. Although both the nNOS CaM+ and ΔnNOS CaM-- reductase enzymes can be expressed and purified without CaM coexpression, we were not able to purify the ΔnNOS CaM+ protein in the absence of coexpression with CaM because of extensive protein degradation (results not shown). A similar degradation problem was found for the CaM binding domain in the human iNOS CaM+ reductase in the absence of CaM.
coexpression (7). Although good quantities of pure enzyme were obtained for the wild-type nNOS holoenzyme, when coexpressed with the chaperonens groEL and groES, under similar conditions, we were again unable to isolate the ΔnNOS holoenzyme because of significant protein degradation. To obtain purified ΔnNOS holoenzyme for our investigation, the enzyme also had to be coexpressed with CaM. This suggests a protein stabilizing role for the 43-amino acid element which may protect the nNOS CaM binding region from proteases as proposed previously by Salerno et al. (15).

Spectral Properties—The native and mutant oxidized reductase domains gave typical flavoprotein visible spectra with peak maxima at 390 nm and 455 nm (Fig. 3). A further investigation of these flavoproteins was performed by recording their spectra after partial reduction by NADPH under aerobic conditions. The partial reduction of the wild-type nNOS CaM+ reductase enzyme produced a spectrum characteristic of the air-stable semiquinone (FAD/FMNH+) with a decrease in absorbance at 455 nm and a broad band at 600 nm (Fig. 3A), as shown for NADPH-cytochrome P450 reductase (31, 32). This characteristic broad band at 600 nm is apparent in both the wild-type nNOS CaM+ and nNOS CaM- reductases and only slightly reduced in size in the ΔnNOS CaM- reductase spectrum. In contrast, the broad band at 600 nm for the ΔnNOS CaM+ mutant is greatly reduced in size (Fig. 3B). Because the air-stable semiquinone is known to be FMNH+, these results suggest that the ΔnNOS CaM+ reductase is significantly depleted of FMN compared with the wild-type enzyme. This is consistent with a reported sequence alignment of the NOS isoforms, flavodoxin, and cytochrome P450 reductases (15), which suggests that the aforementioned 43-amino acid insert, only found in the constitutive NOS enzymes, is located within the FMN binding domain (Fig. 1). The nNOS and iNOS holoenzymes gave characteristic cytochrome P450-like CO difference spectra with a peak at 444 nm. The visible spectra of these enzymes showed a peak maximum at 395 nm and a minimum at 420 nm (results not shown) (20, 21, 23).

Because the spectroscopic studies indicated a depletion in flavin content, a fluorescence assay (28) was used to determine the FAD and FMN content in the NOS reductase enzymes and holoenzymes. Both the wild-type rat nNOS CaM+ and human iNOS CaM+ reductases had full complements of FAD and ratios of protein to FMN of 1:0.8. The rat ΔnNOS CaM+ reductase had a protein to FAD ratio of 1:1 and, as expected from our spectral studies, a protein to FMN ratio of 1:0.2. The wild-type rat nNOS CaM-, human iNOS CaM-, and rat ΔnNOS CaM- reductase enzymes showed a smaller decrease in flavin content, with protein to FAD and protein to FMN ratios of 1:0.8 and 1:0.6, respectively. The only difference between the CaM+ and CaM- reductase domain proteins is the presence or absence of the CaM binding region. This suggests that the presence of the CaM binding region in the wild-type nNOS and iNOS reductase domains helps to stabilize the binding of FMN. These results also indicate that this stabilizing effect is decreased significantly upon the removal of the 43-amino acid loop from within the FMN binding region. Because the ΔnNOS CaM- mutant was able to retain significantly more FMN than the ΔnNOS CaM+, our results indicate that there may be some type of interaction between this CaM binding region and the FMN binding regions of the enzyme.

The wild-type rat nNOS holoenzyme had a full complement of FAD and a protein to FMN ratio of 1:0.4, whereas the human iNOS holoenzyme had a protein to FAD ratio of 1:0.9 and a protein to FMN ratio of 1:0.4. The ΔnNOS holoenzyme had a full complement of FAD and no detectable FMN.

The structure of the eukaryotic cytochrome P450 reductase shows that the FAD and FMN cofactors are in close proximity, and the direct electron transfer between the two flavins is more probable than via a protein pathway (16). In contrast, an investigation on the redox properties of the flavins in P450BM3 suggests that these cofactors are further apart in the P450BM3 reductase than in the eukaryotic P450 reductase (34). Because the structures of the reductase domains of NOS enzymes have yet to be determined, care must be taken when interpreting results based on the known structure of the reductase domain of another protein. The anisotropic environment of the flavins allowed us to monitor further the surroundings of these prosthetic groups in the reductase enzymes by spectropolarimetry (23). Although the wild-type CaM+ and both CaM- forms of the reductase enzyme gave similar visible CD spectra, there was a significant difference observed in the case of the ΔnNOS CaM+ reductase enzyme (results not shown). These results again indicate that some type of interaction exists between the CaM binding region and the 43-amino acid element in the FMN binding region possibly resulting in some form of intrasteric inhibition (35).

Enzymatic Activity of NOS Reductase Domains—NOS reductase domains, as well as mammalian P450 reductases, can use NADPH to reduce artificial electron acceptors such as cytochrome c, FeCN, and DCPIP (12). The NADPH-dependent reductase activities were measured for the CaM+ and CaM- forms of rat ΔnNOS and nNOS reductases as well as for the human iNOS reductase enzymes. All enzymes were able to reduce both FeCN and DCPIP even in the absence of exogenous flavins. The requirement of Ca2+-dependent CaM binding leading to increased electron transfer to these artificial acceptors was investigated by comparing the reaction rates when CaM is bound or with CaM present but not bound due to the addition of the Ca2+-chelator EGTA. The results given in Table I are for experiments conducted in the presence of exogenous flavins as performed by other investigators (9). When measuring cyto-
chrome c reductase activity, the chelation of Ca\(^{2+}\) by EGTA resulted in a major decrease in CaM activation of the nNOS enzymes (Table 1). Both FeCN and DCPIP reduction by wild-type nNOS CaM\(^{+}\) were also significantly affected by the addition of EGTA (Table 1). Our results are consistent with those published by Gachhui et al. (9) who reported CaM-dependent increased electron transfer toward both DCPIP and FeCN. In contrast to the wild-type reductase enzymes, the turnover numbers for the \(\Delta nNOS \ CaM\(^{+}\) mutant did not decrease significantly in the presence of EGTA (Table 1). Therefore, the removal of the amino acid element from the nNOS reductase enzyme apparently affects its Ca\(^{2+}\)-dependent CaM activation. As expected, iNOS isoforms were not affected by the presence or absence of bound CaM.

The reduction of cytochrome c by the NOS isoforms occurs by electron transfer from NADPH to FAD and then FMN prior to being transferred to cytochrome c (12). Thus, cytochrome c reductase activity is dependent on the presence of both FMN and FAD (19). Because there is a deficiency in the FMN content of the mutant proteins, these enzymes would be expected to show reduced activity. Assays of the \(\Delta\) mutants with cytochrome c as the electron acceptor showed no catalytic activity in the absence of added FMN. This effect was specific for FMN as the addition of FAD or riboflavin did not cause any significant increase in electron transfer activity. It is not surprising to find that the removal of a 43-amino acid element from within the FMN binding domain of nNOS leads to a disruption of the flavin binding to this enzyme. A more appropriate experiment would be to replace the element by a small linker that would diminish disruption of the FMN binding consensus sequence. Unfortunately, the design of such a linker will first require solving the three-dimensional structure of the flavin domain of this enzyme.

Subsequent activity assays were performed on the reductase domains of the nNOS and iNOS isoforms in which the proteins were preincubated with excess FMN before testing their cytochrome c reductase activity. To rule out the possibility of superoxide production by exogenous flavins, all experiments were performed in the presence of superoxide dismutase and catalase. The cytochrome c reductase activity of our reductase enzymes was monitored as a function of added FMN (results not shown). From the results of these reconstitution assays, we decided to include a preincubation step, in which 600 \(\mu M\) FMN was added to the enzymes before the start of any kinetic investigations. This gave a concentration of 4 \(\mu M\) FMN in the final reaction mixture which is typically used for this assay (9). The cytochrome c reductase activity of the wild-type nNOS CaM\(^{+}\) reductase domain was increased by about 30% in the presence of exogenous flavins that must compensate the FMN deficiency of the purified enzyme. The preincubation of the mutant \(\Delta\)CaM\(^{+}\) reductase with FMN resulted in cytochrome c reductase activities of more than 70% of the level of the wild-type enzyme (Table 1). This may be caused by the inability of the \(\Delta nNOS \ CaM\(^{+}\) to become fully saturated with FMN. Previous studies on FMN-binding mutants of nNOS and cytochrome P450BM3 have shown the incubation of the mutants enzymes with FMN does not fully recover wild-type levels of activity (19, 36).

For the reductase domains and holoenzymes of the constitutive isoforms of NOS, the rate of electron transfer has been shown to be dependent upon Ca\(^{2+}\)-dependent CaM binding, whereas electron transfer through the inducible NOS isoform is Ca\(^{2+}\)-independent (6–8, 12). We see these same results with our nNOS and iNOS reductase enzymes (Table 1). The iNOS CaM\(^{+}\) reductase is highly active, even in the presence of EGTA; the iNOS CaM\(^{-}\) reductase is active as well, even though the CaM binding domain is not present. The nNOS CaM\(^{+}\) reductase is active only in the presence of Ca\(^{2+}\), and the addition of EGTA decreases its activity by greater than 13-fold. Consistent with the need of the nNOS isoforms for CaM binding to be active, the nNOS CaM\(^{-}\) reductase had little electron transfer activity (Table 1). The \(\Delta nNOS \ CaM\(^{+}\) enzyme was active in the presence of Ca\(^{2+}\), and its activity decreased in the presence of EGTA. This decrease, however, was only by 5-fold, suggesting that either CaM binds more tightly to the mutant or that the mutation modified the Ca\(^{2+}\)-dependent CaM activation of the enzyme. Thus, relative to the nNOS CaM\(^{+}\) reductase enzyme, the loop deletion mutants showed a greater cytochrome c reductase activity in the absence of bound CaM than the wild-type protein. The mutation appears to have reduced the CaM dependence of the enzyme.

Enzymatic Activity of the Holoenzymes—The rate of NADPH oxidation was reduced by approximately half in the mutant holoenzyme (28 nmol/min/nmol) compared with the wild-type enzyme (56 nmol/min/nmol). The radioactive assay that monitors the formation of L-[\(^{3}\)H]citrulline from L-[\(^{3}\)H]arginine and the hemoglobin capture assay for NO synthesis were in very good agreement. The citrulline assay for the wild-type enzyme gave an activity of 254 nmol/min/mg, and the hemoglobin assay gave a value of 259 nmol/min/mg of enzyme. For the \(\Delta nNOS\) holoenzyme, the same assays gave values of 49 nmol/min/mg and 41 nmol/min/mg, respectively. Our results show a greater than 5-fold reduction in activity because of the mutation. This decrease in activity occurs even in the presence of exogenous FMN. In buffers depleted of Ca\(^{2+}\) by the addition of EGTA, the mutant showed a smaller relative decrease in activity than the wild-type enzyme assayed under the same conditions.

Because the rate of NO synthesis was decreased significantly in the mutant enzyme, we decided to monitor the rate of electron transfer between the reductase and heme domains of nNOS. The reduction of the heme domain can be monitored spectrally using a CO-saturated buffer. Reduction of the heme leads to the production of a ferrous-CO complex that can be monitored spectrally by the formation of a 443–445 nm absorption peak (37). The NADPH-dependent heme reduction of the enzyme provides a good indication of the rate of electron transfer between the two domains. Although the NADPH-dependent rate of heme reduction of the wild-type enzyme was very fast, we observed no significant heme reduction for the \(\Delta\) mutant (results not shown). This is consistent with the loss of FMN in the mutant enzyme. Similar results were reported recently for FMN-free neuronal NOS in which the slow CaM-induced NO synthesis and NADPH oxidation in the mutants were attributed to the loss of FMN (36).

\(Ca^{2+}\) Dependence Studies—Because the loop deletion mutation appears to affect the relative Ca\(^{2+}\)-dependent binding of CaM to nNOS, we decided to examine the free Ca\(^{2+}\) concentration dependence of cytochrome c reduction and NO synthesizing activity of the reductase domains and holoenzymes, respectively. The required free Ca\(^{2+}\) concentrations were obtained by adding EGTA and Ca\(^{2+}\)-EGTA based on the Ca\(^{2+}\)-EGTA equilibrium dissociation constant at an exact temperature and ionic strength (30). Our investigation of the Ca\(^{2+}\) concentration dependence of the wild-type nNOS CaM\(^{+}\) and the \(\Delta nNOS \ CaM\(^{+}\) reductase enzymes (Fig. 4A) showed that the mutant required a lower apparent concentration of free Ca\(^{2+}\) to become active (\(K_{D\text{app}}\) = 175 \(\pm\) 16 nm) compared with the wild-type (\(K_{D\text{app}}\) = 242 \(\pm\) 34 nm). Our nNOS holoenzyme gave a \(K_{D\text{app}}\) of 302 \(\pm\) 20 nm similar to the previously reported value for this enzyme (6). The \(\Delta nNOS\) holoenzyme (Fig. 4B) gave a \(K_{D\text{app}}\) of 157 \(\pm\) 3 nm, consistent with the lower value obtained for the \(\Delta nNOS \ CaM\(^{+}\) reductase mutant. The activity of iNOS was not dependent on the free Ca\(^{2+}\) concentration (results not shown). Our results...
indicate that the deletion of the 43-amino acid element has a small but significant effect on the Ca\textsuperscript{2+}-dependent CaM activation of nNOS.

The removal of the 43-amino acid element reduces the Ca\textsuperscript{2+} requirement for NO synthesis by the holo nNOS or cytochrome c reduction by the nNOS reductase domain and reduces the activity of the enzymes. Although the change in Ca\textsuperscript{2+} dependence is significant, the enzyme remains Ca\textsuperscript{2+}-dependent for full activity. The decrease in enzyme activity is likely a result of removing such a large 43-amino acid segment of the enzyme, residues Met\textsuperscript{828}-Ser\textsuperscript{870}, without knowing the juxtaposition of the ends of the element. Incorporation of an appropriate short linker region may lead to higher enzyme activity. During the review of this manuscript, an investigation was reported on the effect of removing 40 amino acid residues (Pro\textsuperscript{801}-Ser\textsuperscript{870}) from the same region of the rat nNOS holoenzyme (38). Their mutant holoenzyme (\(\Delta 40\)) showed a depletion of FMN content as well as kinetic properties and a decreased Ca\textsuperscript{2+} requirement similar to those reported in the present investigation. Both our reductase and holoenzyme \(\Delta\) mutants were less stable than their mutant holoenzyme because our enzymes could only be obtained when coexpressed with CaM. The additional 3 amino acids missing in our mutant enzyme, Met\textsuperscript{828}-Arg\textsuperscript{829}-His\textsuperscript{830}, may account for the difference in stability. Daff et al. (38) do report a decrease in stability when 2 additional amino acids are removed from their mutant enzyme. In the absence of a three-dimensional structure for the reductase domain, it is difficult to ascertain how the differences in these loop out mutants may affect the structure and function of the enzymes.

**Peptide Inhibition Studies**—A synthetic peptide (E1-WRRKKR) derived from the 46-amino acid insert of eNOS (Fig. 1) was reported to inhibit binding of CaM and activation of cNOS isoforms (15). We decided to investigate whether the inhibitor would affect the reductase domain on its own or whether it only inhibits the holoenzyme. The nNOS holoenzyme and the nNOS CaM\textsuperscript{-} reductase enzyme were assayed under the same conditions as reported previously (15). The inhibition of both the nNOS holoenzyme or the nNOS CaM\textsuperscript{-} reductase domain increased with increasing E1 peptide concentration (results not shown). The inhibition was fully reversible in the presence of an equal concentration of enzyme and CaM. The E1 peptide apparently could only inhibit enzymes that were not already bound to CaM. This may explain why the \(\Delta\) nNOS holoenzyme showed no inhibition when incubated with the E1 peptide. The \(\Delta\) nNOS holoenzyme was highly susceptible to proteolysis when coexpressed with groES and groEL. To obtain adequate quantities of enzyme for our investigation, the mutant enzyme was coexpressed with CaM and subsequently stripped of CaM by the addition of EDTA to the buffers used for purification (see "Experimental Procedures"). Although no CaM was present in the sample based upon SDS-polyacrylamide gel electrophoresis, the possibility exists that some CaM was still bound because of the decreased Ca\textsuperscript{2+} dependence of the mutant protein. No apparent inhibition was observed if the E1 peptide was added to either nNOS reductase enzyme or holoenzyme coexpressed with CaM (results not shown). Our results are again consistent with the observation that the CaM-dependent activation of constitutive NOS enzymes is complete within the reductase domain alone (7, 9).

Two other peptides, N1 (SRKSSG) and N2 (RKSYKVRF), derived from the so-called nNOS autoinhibitory element (15), were also synthesized. N1 is a serine-rich, positively charged polypeptide found near the carboxy end of the amino acid element in nNOS. N2 was chosen because of its similarities to the E1 peptide. It consists of a stretch of positively charged residues with a large aromatic residue at one end. This peptide was used in our investigation to see whether the inhibition by E1 was dependent on its primary sequence or simply required a basic polypeptide with an aromatic amino acid. Neither of the peptides derived from the amino acid insert found in nNOS was able to inhibit significantly the activity of the enzymes used in our investigation (Table II). The lone exception was the inhibition by the N2 peptide of the \(\Delta\) nNOS CaM\textsuperscript{-} reductase enzyme. We are not able to explain this result without further knowledge of how these peptides interact with the enzymes. The difference in Ca\textsuperscript{2+}-dependent CaM regulation of the NOS enzymes could be a good target for isoform specific inhibition. The isoform-specific inhibition by the E1 peptide is interesting, but one must question the practicality of an inhibitor requiring such a large excess of peptide to enzyme (500:1) and the need for less than stoichiometric amounts of CaM. In addition, why does the E1 peptide significantly inhibit nNOS, but none of the peptides derived from the nNOS insert show any significant inhibitory properties?

| Enzyme          | E1 Peptide | N1 Peptide | N2 Peptide |
|-----------------|------------|------------|------------|
| nNOS CaM\textsuperscript{-} reductase\textsuperscript{a} | 73 ± 1     | 95 ± 2     | 96 ± 2     |
| nNOS holoenzyme\textsuperscript{b} | 71 ± 2     | 87 ± 6     | 78 ± 1     |
| \(\Delta\) nNOS holoenzyme\textsuperscript{b} | 45 ± 3     | 89 ± 15    | 97 ± 11    |
| iNOS holoenzyme\textsuperscript{b} | 116 ± 1    | 82 ± 9     | 97 ± 3     |
| 108 ± 9         | 95 ± 3     | 96 ± 4     |

\textsuperscript{a} Activity of the reductase enzymes monitored by cytochrome c reduction assay.
\textsuperscript{b} Activity of holoenzymes monitored by hemoglobin assay.

Fig. 4. Calcium dependence of the rat nNOS proteins. Panel A, activity of the wild-type nNOS CaM\textsuperscript{+} (●) and \(\Delta\) nNOS CaM\textsuperscript{-} (○) reductases was measured by cytochrome c reduction. Panel B, activity of the wild-type nNOS holoenzyme (●) and \(\Delta\) nNOS holoenzyme (○) was measured by Met-hemoglobin formation. The results shown represent those of assays performed in triplicate. The rates used to normalize values to 100% were: nNOS CaM\textsuperscript{-} reductase, 3600 min\textsuperscript{-1}; \(\Delta\) nNOS CaM\textsuperscript{-} reductase, 2600 min\textsuperscript{-1}; nNOS holoenzyme, 254 nmol min\textsuperscript{-1} mg\textsuperscript{-1}; \(\Delta\) nNOS holoenzyme, 49 nmol min\textsuperscript{-1} mg\textsuperscript{-1}.
about by CaM (39). The conformational changes resulting from the deletion mutations in our enzymes were not enough to remove the requirement of CaM binding for full activation of both the reductase domain and the holoenzyme.

In the CaM-dependent enzymes calcineurin and the plasma membrane Ca\(^{2+}\) pump, the combined removal of the autoinhibitory and CaM binding regions results in the loss of CaM binding and a basal rate approaching that of the respective CaM-stimulated enzyme (18, 40). Because mutant human and mouse iNOS reductase enzymes with deleted CaM binding regions are fully active (7), the combined removal of the CaM binding region and proposed autoinhibitory element in the nNOS reductase enzyme may result in basal rates similar to the CaM-stimulated enzyme. When the amino acid element was deleted from the nNOS CaM– enzyme to form the ΔnNOS CaM– reductase, the resulting mutant showed a higher activity than nNOS CaM– but was not nearly as active as the CaM-bound nNOS CaM+ enzyme. Thus, enzyme inhibition in the absence of CaM is not reversed by the combined removal of the CaM binding region and the loop insert of nNOS. The 43-amino acid insert in nNOS does not act like a typical CaM autoinhibitory domain. Our results are consistent with a study using chimeric enzymes made from nNOS and iNOS which showed that the NOS enzyme is not regulated by a unique autoinhibitory element in its reductase domain (14). During the preparation of this manuscript, a report was published on the role of the same putative inhibitory domain in eNOS by Nishida and Ortiz de Montellano (41). These authors found that the deletion of the insert in eNOS led to a decrease in the Ca\(^{2+}\) concentration required to activate the enzyme and greatly enhanced the maximal activity of wild-type eNOS, which is the least active of the three NOS isoforms. Although both enzymes consist of an insert in their FMN domain, these authors proposed the low activity of eNOS, when compared with nNOS, is caused by the eNOS autoinhibitory loop being a more efficient inhibitor to electron transfer. Their findings were consistent with the peptide inhibition results (15). Our results and those reported by Daff et al. (38) using nNOS deletion mutants were not as dramatic as those reported for the eNOS investigation. This may be because of the differences in the size and sequence of the deleted elements, in the different expression systems used in the investigations, and/or the different roles and activities of the parent enzymes.

We found that the deleted amino acids protect the CaM binding region of nNOS from proteolysis. This may be of physiological significance because the constitutively expressed NOS enzymes that contain this element do not bind CaM in vivo at basal levels of Ca\(^{2+}\). In contrast, the iNOS enzyme is bound to CaM under similar conditions. These elements may protect the exposed CaM binding region and act as a tether to control the activity of the two cNOS enzymes differentially. This is seen in the higher inhibition of NOS activity by peptides derived from the eNOS insert (15) and the enhancement in the maximal activity of wild-type eNOS when the element is deleted (41).

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