Nitric oxide synthases (NOSs) are classified functionally, based on whether calmodulin binding is Ca\(^{2+}\)-dependent (cNOS) or Ca\(^{2+}\)-independent (iNOS). This key dichotomy has not been defined at the molecular level. Here we show that cNOS isoforms contain a unique polypeptide insert in their FMN binding domains which is not shared with iNOS or other related flavoproteins. Previously identified autoinhibitory domains in calmodulin-regulated enzymes raise the possibility that the polypeptide insert is the autoinhibitory domain of cNOSs. Consistent with this possibility, three-dimensional molecular modeling suggested that the insert originates from a site immediately adjacent to the calmodulin binding sequence. Synthetic peptides derived from the 45-amino acid insert of endothelial NOS were found to potently inhibit binding of calmodulin and activation of cNOS isoforms. This inhibition was associated with peptide binding to NOS, rather than free calmodulin, and inhibition could be reversed by increasing calmodulin concentration. In contrast, insert-derived peptides did not interfere with the arginine site of cNOS, as assessed from \(^{3}H\)-nitro-\(\cdot\)-arginine binding, nor did they potently effect iNOS activity. Limited proteolysis studies showed that calmodulin’s ability to gate electron flow through cNOSs is associated with displacement of the insert polypeptide; this is the first specific calmodulin-induced change in NOS conformation to be identified. Together, our findings strongly suggest that the insert is an autoinhibitory control element, docked with a site on cNOSs which impedes calmodulin binding and enzymatic activation. The autoinhibitory control element molecularly defines cNOSs and offers a unique target for developing novel NOS activators and inhibitors.

Nitric oxide is a ubiquitous cell-signaling molecule, with protein roles in physiology and pathophysiology (1–3). Encoded by distinct genes, mammalian NO synthases (NOSs)\(^1\) comprise a family of three calmodulin-dependent biotrophrohemoflavoproteins that are functionally distinguished by their modes of regulation (4). The two constitutively expressed isoforms of NOS (cNOSs), first identified in neuronal cells (nNOS) and endothelial cells (eNOS), remain dormant until calcium/calmodulin (Ca\(^{2+}\)/CaM) binding is actuated by transient elevations in intracellular Ca\(^{2+}\). This Ca\(^{2+}\)-dependent mode of regulation provides pulses of NO for moment-to-moment modulation of vascular tone and neurosignaling. In contrast, activity of the immunostimulant-induced isoform of NOS (iNOS) is Ca\(^{2+}\)-independent, providing continuous high output NO generation for host defense. A remarkably high affinity for CaM, even at basally low levels of intracellular calcium, is responsible for the Ca\(^{2+}\)-independence of iNOS (5).

Whether a given NOS isoform binds CaM in a Ca\(^{2+}\)-dependent or -independent manner has been assumed to be a property solely of the amino acid sequence specified by a 20–25-amino acid CaM binding site. However, this restrictive view is challenged by findings that chimeric eNOS and nNOS, which have had their CaM binding sequences replaced with the corresponding sequence from iNOS, still require Ca\(^{2+}\) for full activity (6, 7). Because regulation of enzyme systems by Ca\(^{2+}\)/CaM typically involves displacement of an intrinsic autoinhibitory polypeptide (8, 9), we hypothesized that the binding of Ca\(^{2+}\)/CaM to cNOSs may similarly trigger activation by displacing a control element. Here we identify a multiple amino acid insertion which serves as a control element unique to cNOSs and which molecularly defines Ca\(^{2+}\)-dependent isoforms of NOS.

**EXPERIMENTAL PROCEDURES**

**Protein Modeling**—Molecular modeling of the FMN binding module of nitric oxide synthase isoforms was done using the Insight and Homology programs from Biosym (Biosym/Molecular Simulations, San Diego, CA) running on a Silicon Graphics Indigo2 workstation. After alignment of NOS sequences with homologous FMN binding proteins of known structure (see “Results”), structurally conserved region (SCR) boxes were created corresponding to conserved regions of secondary structure and regions involved directly in FMN binding. These regions were characterized by high positive scores as evaluated by Dayhoff’s mutation matrix (10). After assignment of coordinates in the SCR regions, the loop regions between the SCR boxes were modeled by searching the Brookhaven protein data base. The crude model structure was relaxed to a sterically and energetically reasonable state using the Discover program (Biosym/Molecular Simulations) for molecular me-
chances and dynamics calculations. This includes splice repair to remove unrealistic structural features at SCR-loop junctions, end repair to assign reasonable structures to C-terminal and N-terminal extensions, and structural optimization to remove steric overlaps and to reduce the structure to a energetic minimum. Energy minimizations were performed until the steepest descent calculated by conjugate gradient method as convergence was approached.

**Purification of NOS Isoforms**—Bacterial nNOS (nNOS) and bovine endothelial NOS (eNOS) were purified from E. coli expressing the protein. nNOS was purified from bacteria using a Ni-affinity chromatography column. eNOS was purified from mammalian cells using a DEAE-deoxycellobiose chromatography column. Both proteins were purified to homogeneity and stored at -80°C.

**Cell Culture**—Rat neuronal (nNOS) and bovine smooth muscle cells (eNOS) were grown in a 100-mm dish. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO2. Media were changed every 2 days.

**NOS Activity Measurement**—The activity of NOS was measured using [3H]NGNitro-L-arginine binding assay. The binding reaction mixture contained [3H]NGNitro-L-arginine (3 nM, 3000 cpm), 10 μM calcium, 50 μM EGTA, 1 mM DTT, and the indicated concentration of peptide in a final volume of 100 μl. Reactions were initiated by the addition of 20 pmol of nNOS, 10 pmol of eNOS, or 15 μg of rat iNOS-rich cytosol. NOS activity was determined from the rate of decrease in [3H]NGNitro-L-arginine binding to NOS complexes as monitored continually at 405 nm for 60 min at 15-s intervals. Assay blanks were additionally contained 10 μM EGTA, resulting in >90% inhibition of activity.

**Proteolysis of NOS Isoforms**—Limited proteolysis was performed on incubates containing 40 pmol of recombinant nNOS purified from stably transfected (eNOS) or HEK-293 cells (nNOS) or 50 pmol of recombinant eNOS purified from E. coli (eNOS). Samples were preincubated at room temperature for 15 min in a 100-μl volume containing: 50 mM Tris, 7.6, 1 mM DTT, 10 μM CaM, 100 μM CaCl2, with or without 10 μM EGTA. Proteolysis was initiated by the addition of 20 micromolar of 1-152-glutaryl-2-phénylthionyl chloromethyl ketone-immobilized trypsin (Sigma) per pmol of NOS. Samples (25 μl) were collected after 0, 5, 10, and 20 min, and proteolysis was terminated by boiling with an equal volume of 2 × SDS gel-loading buffer. Peptide products were resolved on a 8–16% gradient SDS-polyacrylamide gel electrophoresis and visualized by staining with Coomassie Blue. Accurate molecular mass determination of tryptic fragments was performed by mass spectrometry at the Rockefeller University Protein/DNA Biotechnology Center, using trypsin-assisted laser desorption and time of flight detection (Perseptive Biosystems Inc., Framingham, MA). For N-terminal sequence analysis, tryptic digests were prepared as above and subject to SDS-polyacrylamide gel electrophoresis, but then electrotransferred to polyvinylidene difluoride membranes. Amino acid sequencing was performed on an Applied Biosystems 477A protein sequencer.

**Peptides**—Synthetic peptides were obtained from SynPep (Dublin, CA) and other commercial suppliers. Purity was evaluated by high performance liquid chromatography and mass spectroscopy and exceeded 80% in all cases with typical purity >90%. Predominant impurities differed from the desired products by one amino acid residue, resulting from incompletely coupled synthesis.

**Chemicals**—Rat recombinant interferon-γ, RPMI culture medium, and cell culture reagents were from Life Technologies, Inc. Radioisotopes were from Dupont NEN, lipopolysaccharide (E. coli serotype 011:B4), chemicals, and 1-152-glutaryl-2-phénylthionyl chloromethyl ketone-immobilized trypsin were obtained from Sigma, calmodulin was from Calbiochem, and tetrahydrobiopterin was from Schirks Laboratories (Bonna, Switzerland). Enzymes were purchased from Pharmacia LKB Biotechnology, Promega, or New England Biolabs.

**RESULTS AND DISCUSSION**

Nitric oxide synthase is a large multidomain enzyme in which a series of gene fusion events has resulted in the incorporation of modules showing significant homology to smaller ancestral proteins (18, 19). NOSs can be coarsely dissected into an N-terminal oxygenase domain and a C-terminal reductase domain, bridged by a canonical binding sequence for CaM (20). Calmodulin binding initiates electron transfer between the reductase and catalytic domains, thereby activating catalysis (21). The oxygenase domains have binding sites for substrate (arginine), heme, and tetrahydrobiopterin, whereas the reductase domain has binding sites for FAD, FMN, and NADPH.

Bredt et al. (22) were first to reveal the homology between the C-terminal half of NOS and NADPH-cytochrome P450 reductase (CPR), noting conserved regions corresponding to FMN, FAD, and NADPH binding domains. The CPR-binding modules of NOS isoforms and CPR are in turn highly homologous to the flavodoxins, which are small FMN-binding proteins that function as electron carriers in bacteria (23). The FAD and NADPH binding domains are closely related to chloroplast ferredoxin-NADP + reductases and other related proteins.

**Sequence Alignments**—Five flavodoxins have been crystallized and solved by x-ray diffraction (24). Three regions in these flavodoxins are involved in binding the FMN prosthetic group; the first of these is close to the N terminus, and is immediately preceded by the initial strand of the structure. Although only one of these FMN binding regions was identified in nNOS by Bredt et al. (22), each of them has a corresponding homolog
in NOSs. The first step in alignment of the NOS FMN binding domain with the flavodoxins was the identification of these regions in each NOS isoform. This was followed by the identification of conserved secondary structural elements in NOS, primarily by their homology to the corresponding elements in flavodoxin crystal structures as evaluated with Bio-sym's Homology software. The C-terminal end of the domain (~35 residues for Desulfovibrio sequence) is omitted to conserve space.

As shown in Fig. 2 (upper left), the backbone structure of iNOS and CPR are virtually superimposable on the backbone of Desulfovibrio vulgaris flavodoxin, the closest solved structural homolog of the FMN binding modules of the NOS isofoms.

The structure, a Rossmann fold motif (28), is a five stranded parallel β-sheet with the FMN binding site along one edge. Homology predicts that two aromatic residues in murine iNOS, Phe369 and Tyr370, are in contact with the FMN ring system; Tyr370 serves as a shielding residue.

Fig. 2 (upper right) shows the corresponding backbone structure of enoS; nNOS is extremely similar but not shown. Most of the eNOS backbone can be superimposed on homologs shown in Fig. 2 (upper left), with the insertion projecting from the upper edge of the sheet opposite the FMN binding site. Structurally, it corresponds to the replacement of a tight 5–10 residue loop with an ~50-residue structure about one-third the size of the entire FMN binding module. We are unable to propose a conformation for the insertion because we lack a solved homolog; the structure shown is merely intended to convey relative position and size.

The CaM binding site is immediately adjacent to the N-terminal edge of the FMN binding domain (painted white in Fig. 2, upper left). With CaM bound, the CaM recognition site would predictably be in a helical conformation (29, 30); steric constraints suggest that it extends almost directly away from the FMN binding domain. The lower panels of Fig. 2 show models of the FMN binding domains of iNOS (left) and eNOS (right) with CaM (yellow ribbon; based on Vorherr et al. (30)) positioned above the N-terminal strand of the FMN domain. There are ~8 residues between the end of the CaM recognition site and the start of the initial strand of the β-sheet; 2–3 residues at each end of this short linker are needed to clear the van der Waals surfaces of CaM and the FMN domain. This leaves 2–3 residues that are conformationally unrestricted, and hence, there is uncertainty about the exact position above the β-sheet of CaM and the orientation of the axis of the CaM recognition site. The position of CaM relative to the FMN domain is unspecified with respect to rotations about the y axis of Fig. 2 by available information (corresponding to the axis of

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2 Recently, the crystal structure of NADPH-cytochrome P450 oxidoreductase has been reported at 3.0 Å resolution (27). The FMN-binding module bears striking homology to D. vulgaris flavodoxin.
the CaM-binding helix of lower panels). It is notable that calmodulins (molecular mass ~ 17 kDa) are larger than the entire FMN-binding module. Although the insertion is midway through the sequence of the FMN-binding module, in three dimensions the model predicts it to be directly adjacent to the CaM binding site. The model predicts also that CaM binding would be sterically hindered by the insertion, suggesting that the insertion can exist in more than one physiologically relevant conformation.

Two aspects of this model strongly suggest that the insert functions as a control element, 1) the correlation between Ca$^{2+}$/CaM control and the presence of the insertion and 2) the proximity of the CaM binding site to the insertion and the probable steric interactions which would ensue. An attractive potential role for the insert is that of an inhibitory polypeptide which is displaced by CaM binding. It differs from inhibitory polypeptides common to other CaM-dependent enzymes, and CaM itself, in its lack of acidic and hydrophobic amino acids; this makes direct binding of the insertion to CaM sites in NOS isoforms unlikely. Nonetheless, CaM could conceivably displace the polypeptide insert from a neighboring site by binding domain overlap or through allosteric effects.

**Synthetic Polypeptide Effects on CaM Binding—Overlap of the cNOS polypeptide insert and the CaM recognition site, suggested from molecular modeling, implies that the insert may obstruct CaM binding. If this involves “docking” of the insert within cNOSs, synthetic homologs of the insert might similarly bind and interfere with CaM binding. As shown in Table II, potent inhibition of $^{125}$I-CaM binding to nNOS was observed with insert-derived polypeptide fragments; relative peptide potency for inhibiting CaM binding mirrored that for blocking nNOS activation. IC$\text{_{50}}$ values for eNOS-derived peptide fragments ranged from 1 to 10 μM, and potency increased as the RRKRK motif was progressively lengthened to include up to 33 amino acids (Fig. 3B). Inhibition of nNOS activity and CaM binding by insert-derived peptides was fully reversed by excess CaM (see Fig. 3, C and D, for findings with eNOS$^{607-634}$), indicative of a competitive mode of inhibition. Thus, the greater apparent potency of peptides for inhibiting CaM binding *versus* activity, indicated in Table II, is explained by differences in assay conditions; lower CaM concentrations...
were used to assess binding (1 nM) versus activity (100 nM). Inhibition of CaM binding by peptide could not be overcome by excess Ca\(^{2+}\) (Fig. 3E).

Conceivably, the synthetic peptides could interfere with CaM binding to NOS by interacting with either NOS or CaM itself. That NOS is the actual binding target for eNOS-derived insert peptides is indicated by several findings. First, direct binding of peptide to \(^{125}\)I-CaM, quantified in the absence of NOS, was undetectable at concentrations that inhibited >90% of CaM binding to nNOS (data not shown). Second, the CaM-dependent phosophatase caseinunir, which resembles eNOSs in having a \(K_i\) for CaM of 5 nM (31), was not inhibited by concentrations of insert-derived peptides that potently inhibit nNOS activity (see Fig. 4). Third, eNOS-derived peptides markedly enhanced the dissociation rate of \(^{125}\)I-CaM from preformed complexes with nNOS (Fig. 3D). In this experimental setting, dissociated \(^{125}\)I-CaM is prevented from reassociating with NOS by addition of a 3,000-fold molar excess of unlabeled CaM. Thus, in order for a synthetic eNOS-derived peptide to eject CaM from its binding site on nNOS, it must at least transiently form a ternary CaM-containing complex with NOS. Conceivably, this transient ternary complex could involve interactions of peptide with CaM as well as NOS. These findings suggest that the binding domain of the putative eNOS autoinhibitory element on nNOS either overlaps or allosterically perturbs the CaM binding domain.

Previously described inhibitors with demonstrated selectivity for NOS influence the arginine site in a manner that can be detected as a loss in sites or binding affinity for the arginine site on nNOS, it must at least transiently form a ternary ternary complex could involve interactions of peptide with CaM as well as NOS. These findings suggest that the binding domain of the putative eNOS autoinhibitory element on nNOS either overlaps or allosterically perturbs the CaM binding domain.

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TABLE I

| Designation | Derivation | Sequence |
|-------------|------------|----------|
| eNOS628–633 | h          | WRRKK    |
| eNOS626–636 | h          | SSRWRRKRESS |
| nNOS835–845 | h          | QERKSYKVF |
| eNOS604–615 | h          | RFEQKSYKIRF |
| eNOS601–615 | h          | SSPRFEQKSYKIRF |
| eNOS607–634 | b          | OKHSYKIRFNSVCSVDPLVSSWRKKK |
| nNOS851–864 | h          | QERKSYKVRFNSVSSYSDQSSGGDFDL |
| nNOS853–864 | h          | QERKSYKVRFNSVSSYSDQSSGGDFDL |

a h, human; b, bovine, r, rat.

TABLE II

| Peptide   | NOS activity* | Ligand Binding to nNOS° |
|-----------|---------------|------------------------|
|           | µg (ml) (µs) | µg (ml) (µs) | % of control | µg (ml) (µs) |
| eNOS628–633 | 100 (170.0)  | 11.0 ± 3.3 | 24.0 ± 2.8 | 91.1 ± 5.7 |
| eNOS626–636 | 100 (71.2)   | 19.1 ± 0.9 | 27.7 ± 2.0 | 92.1 ± 1.3 |
| nNOS835–845 | 100 (68.1)   | 102.0 ± 2.1 | 93.6 ± 2.3 | 101.1 ± 3.7 |
| eNOS604–615 | 100 (63.0)   | 54.5 ± 1.8 | 80.5 ± 2.0 | 99.7 ± 5.7 |
| eNOS601–615 | 300 (76.0)   | 30.4 ± 1.7 | 57.2 ± 7.5 | 62.2 ± 2.9 |
| eNOS607–634 | 300 (87.7)   | 28.2 ± 0.9 | 40.2 ± 4.0 | 64.3 ± 3.2 |
| nNOS851–864 | 100 (72.0)   | 98.4 ± 1.9 | 102.6 ± 4.1 | 99.7 ± 2.8 |
| nNOS853–864 | 300 (84.8)   | 103.0 ± 1.3 | 80.2 ± 3.8 | 84.1 ± 3.5 |

a NOS activity measurements were performed using purified recombinant nNOS and eNOS, or native iNOS. Values are means ± S.E. of triplicate determinations.

° Radioligand binding was performed after incubation of 1–2 pmol of NOS for 15 min at 23°C with either \(^{125}\)I-calmodulin (1 nM) or \(^{3}H\)N\(^{-}\)-nitro-L-arginine (NNA) (200 pm) and the indicated peptides. Values are means ± S.E. of triplicate determinations.
binding site (eNOS 1–518 56,877 Da, eNOS 519–1204 76,308 Da) and at a second site, likely to be Lys 545, which resides between the CaM binding site and insert peptide (eNOS1–545 59,916; eNOS546–1204 73,270). Exposure of Lys 545 and proximity to the CaM binding site is predicted in the model shown in Fig. 2; this site appears to be within a helix-turn transition at the edge of the β-sheet distant from the FMN binding site. Lack of cleavage in nNOS at the site homologous to eNOS Lys545 may be explained by the presence of a single basic residue, while eNOS contains paired basic residues (RK).

**Fig. 3.** Influence of eNOS-insert peptides on NOS activity and CaM binding. Panel A, NOS activity is inhibited by eNOS<sup>601–633</sup>. nNOS activity was measured kinetically, based on the rate of NADPH consumption; iNOS activity was measured as the rate of NO formation, based on Fe<sup>2+</sup>-myoglobin oxidation. Activities are expressed as percent of control samples in which eNOS601–633 was omitted. Panel B, <sup>125</sup>I-CaM binding to nNOS was inhibited by eNOS insert-derived peptides. Specific inhibition of <sup>125</sup>I-CaM binding to nNOS was assessed as a function of concentration of added peptide in a 96-well microfiltration plate assay. Panel C, Inhibition of nNOS activity by eNOS<sup>601–633</sup> is reversed by excess CaM. Activity was measured as percent NADPH consumption rate in the presence of a maximally effective concentration of CaM (0.1 mM), prior to addition of peptide. Note that inhibition of nNOS activity was greater than 80% after addition of 100 μM eNOS<sup>601–633</sup>, but restored to the control level by addition of 10-fold more CaM (solid bar). Panel D, Inhibition of <sup>125</sup>I-CaM binding to nNOS by eNOS<sup>607–634</sup> is competitive with [CaM]. Panel E, Inhibition of CaM binding to nNOS by eNOS<sup>607–634</sup> is not reversed by Ca<sup>2+</sup> excess. Panel F, Dissociation of <sup>125</sup>I-CaM from nNOS is accelerated by eNOS<sup>607–634</sup>.
any event, binding of CaM simplifies this cleavage pattern by providing a single dominant cut site. Neglecting the intact proteins and the 10-kDa band from small unresolved fragments of eNOS, only two strong bands are visible at 60 and 65 kDa. These are predicted by cleavage of the molecule within the pentabasic RRRKKR motif in the insert peptide at residues Lys625. Thus, cleavage at Lys625 (with additional cleavage of the N-terminal fragment at Lys3045) produces fragments of 59,916 Da (eNOS1-545) and 63,251 Da (eNOS633-1024). Alignment reveals close correspondence between Lys625 of eNOS and Lys3046 of nNOS (see Fig. 1), suggesting that CaM binding similarly displaces the insert peptide in each cNOS isoform.

To summarize, CaM binding not only protects the CaM binding site from degradation by trypsin, but exposes cleavage sites on both nNOS and eNOS, which are otherwise inaccessible. A preponderance of evidence points to the clusters of basic residues in the FMN domain insert as the trypsin cleavage sites which are exposed by CaM binding. Exposure of cryptic sites by CaM binding could occur by an allosteric mechanism, or by displacement through binding domain overlap. CaM-driven movement of the insert strongly suggests a switch function for activation of NO synthesis.

Mechanism of NOS Control—Herein we have shown that cNOSs possess a polypeptide insert in their FMN binding modules that is 1) unique to NOS isoforms which are regulated by transient CaM binding; 2) positioned adjacent to the CaM binding domain; 3) an impediment to CaM binding and hence, NOS activation; and 4) displaced when CaM binding does occur. Together, these results strongly imply that the insertion in cNOSs is an autoinhibitory control element. We propose that inhibition of NOS by the insert requires occupancy of key sites on cNOS. CaM binding displaces the insert, thus activating cNOS catalysis by "dissinhbition." Close proximity of the inhibitory polypeptide to its cognate binding site(s) on cNOSs would result in an exceedingly high local concentration, thus favoring the bound/inhibited state in the absence of CaM. The detection of basal activity with either purified eNOS or nNOS, in the simultaneous presence of EGTA and absence of CaM (~5% of maximal),3,4 may arise from a low steady-state concentration of the disinhibited cNOS conformer.

The control mechanism requires that CaM displace the insert upon binding to cNOS; this should translate into a reduced affinity for CaM. Reciprocally, absence of the insert from iNOS would preclude the otherwise expected steric hindrance to CaM binding, contributing to the much tighter binding of CaM at low levels of Ca2+. Studies of polypeptides, corresponding to the putative CaM binding sites on eNOS and iNOS, and of chimeras in which the putative CaM binding sequence of one NOS isoform is substituted with the corresponding portion of another, have indicated that affinity and calcium-dependence of CaM binding is provided by elements on NOS in addition to the recognized CaM binding sequence itself (6, 7). These results have been interpreted as indicating the presence of an auxiliary CaM binding region on iNOS that augments binding. An alternative explanation, raised by our findings, is that the absence of the autoinhibitory polypeptide from iNOS contributes to enhanced CaM affinity at low Ca2+ levels. We hypothesize that iNOS evolved from an ancestral cNOS-like protein by loss of the inhibitory peptide; nonetheless, vestigial regulatory sites are suggested by a weak inhibition of activity in the presence of synthetic fragments of the eNOS inhibitory peptide. The CaM binding sites on iNOS and cNOSs are apparently related to a similar basic region near the N terminus of CPR, and may have evolved from such a region in a common ancestral protein.

Our data suggest that binding of the inhibitory peptide may involve at least two regions. At least one recognition site binds the RRRKKR motif. A second possible site might recognize sequences such as EERKSYKRF and EQHKSYKIRF that occur in the N-terminal half of the eNOS and nNOS insertions; peptides that lack RRRKR but contain these sequences weakly inhibit NOS activity and CaM binding. Some similarity between the first and second halves of the insertion can be readily noted by comparing the sequences of peptide eNOS628-633 and eNOS626-636 with those of nNOS835-845 and eNOS604-615. The insert peptide also contains an abundance of serine and threonine residues which provide potential sites for phosphorylation (12/45 residues in the bovine eNOS insert). We speculate that phosphorylation/dephosphorylation may influence the affinity of insert peptides for binding cognate sites on cNOSs and hence, impact on parameters of NOS activation and/or deactivation. In this regard, it is notable that skeletal muscle possesses an nNOS splice variant in which the insert peptide is expanded by 36 residues (33), providing additional sites for possible cell-type specific modification.

Many important questions remain to be answered. The location and identity of the sites of interaction with the inhibitory polypeptide on the surface of the enzyme are not known. Regions of interaction could include the flanking surface loops of...
the FMN domain, bound CaM, and additional more distant sites. In particular, a site on the oxygenase domain consisting of an array of acidic groups could serve as a binding site for the basic regions on the inhibitory polypeptide insert which stabilizes the inhibited conformation of cNOS.

While it seems clear that CaM binding and activation of cNOS is associated with displacement of the inhibitory polypeptide, it is not known how the presence of the polypeptide in its initial conformation inhibits electron transfer. An obvious mechanism would involve interference by the inhibitory polypeptide with interactions between the oxygenase and reductase domains or flavin subdomains, stabilizing a conformation which does not support rapid electron transfer. Such interference could involve changes in either heme/FMN or FMN/FAD distances driven by domain realignment. Intramolecular electron transfer rates are often determined by the ability of electrons to tunnel, and therefore fall off exponentially with distance at roughly an order of magnitude per bond length (34); thus, a small increase in interdomain distance could produce a large reduction in electron flux. Displacement of the inhibitory peptide may not be the only mechanism by which CaM binding stabilizes the activate conformation of cNOSs, inasmuch as CaM removal results in inactivation of iNOS.

In conclusion, we have identified a novel control element in cNOSs which will serve as a prototype for the development of potent peptide inhibitors. The exposure of tryptic cleavage sites in this element represents the first demonstration of a specific CaM-induced conformational change in NOS and may be a hallmark of the active conformer of NOS. Abu-Soud and Stuehr (21) pointed out that the use of CaM to control electron transfer is unique to cNOS. A more fundamental difference between cNOS and other CaM-regulated proteins is the lack of a CaM analog within the cNOS inhibitory peptide. Ultimately, cNOS may not be unique in this regard; it may presage the identification of other CaM regulated systems in which the CaM/inhibitor interaction is mediated through binding domain overlap or allosteric effects, rather than competition for a common recognition site.

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The Autoinhibitory Domain of cNOSs

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