Identification, Characterization, and Comparison of the Calmodulin-binding Domains of the Endothelial and Inducible Nitric Oxide Synthases*

(Rceived for publication, September 26, 1995, and in revised form, December 5, 1995)

Richard C. Venema§, Hassan S. Sayeghv, Jonathan D. Kents, and David G. Harrison
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From tVascular Biology Center, Medical College of Georgia, Augusta, Georgia 30912 and tDivision of Cardiology,
Emory University School of Medicine, Atlanta, Georgia 30322

The calmodulin (CaM)-binding regions in bovine endo-
thelial nitric oxide synthase (eNOS) and murine indu-
cible nitric oxide synthase (iNOS) are identified in
this study as eNOS residues 493-512 and iNOS residues
501-532. Peptides corresponding to eNOS 493-512 and
iNOS 501-532 produce a Ca2+-dependent, electro-
phoretic mobility shift of CaM on 4M urea gels. The two
peptides are also potent inhibitors of the CaM-mediated
activation of neuronal nitric oxide synthase and have
dissociation constants for CaM binding of 4.0 and 1.5 nm,
respectively. Substitution of eNOS and iNOS CaM-bind-
ing domains in eNOS/iNOS chimeric proteins produces
major alterations in the Ca2+ and CaM dependence of
the intact enzymes expressed and purified from a bacu-
lovirus/Sf9 insect cell system. Replacement of aligned
iNOS sequence with eNOS 493-512 creates a functional,
chimeric iNOS that is both Ca2+- and CaM-dependent.
Replacement of aligned eNOS sequence with iNOS 501-
532 creates a functional, chimeric eNOS that is CaM-
dependent but that remains Ca2+-dependent. Specific
amino acid residues critical for CaM binding by eNOS
are also identified in this study as Phe-498, Lys-499, and
Leu-511 in the bovine eNOS sequence.

Nitric oxide (NO)1 is produced in various tissues by the
oxidation of l-arginine in a reaction catalyzed by the
enzyme NO synthase (NOS). Three distinct isoforms of NOS have
been identified by cDNA cloning and sequencing. NOS cDNAs for
the three isoforms derived from brain (1), macrophage (2–4),
liver (5), and endothelial cells (6–10) show about 50% identity
in deduced amino acid sequence. Each of the sequences con-
tains conserved regions that correspond to functional domains
for binding of heme, calmodulin (CaM), FMN, FAD, and
NADPH (11). Type I neuronal NOS (nNOS) and Type III en-
dothehial NOS (eNOS) are distinguished from Type II inducible
NOS (iNOS) in that both are Ca2+-dependent, constitutive
enzymes. nNOS and eNOS are activated in neurons, endothe-

dial cells, and certain other cell types by agonist-stimulated

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§ To whom correspondence should be addressed: Vascular Biology
Center, Medical College of Georgia, Augusta, Georgia 30912-2500. Tel.: 706-721-2576; Fax: 706-721-9799.
1 The abbreviations used are: NO, nitric oxide; NOS, nitric oxide
synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal
nitric oxide synthase; iNOS, inducible nitric oxide synthase; CaM, cal-
modulin; 1, inducible.
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by Dr. C. J. Lowenstein (Johns Hopkins University), cDNA for human nNOS (20) was a generous gift of Dr. J. S. Pollock (Abbott Laboratories).

Gel Mobility Shift Assays—High affinity binding of peptides to CaM was demonstrated by gel mobility shifts of CaM on 4 M urea-15% polyacrylamide gels. Gels were run in the presence of either 0.1 mM CaCl2 or 2 mM EGTA in order to determine the Ca2+ dependence and stoichiometry of binding according to the method described by Erickson-Viitanen and DeGrado (21).

Quantitation of Binding Affinities of Peptide Calmodulin Complexes—Dissociation constants for CaM binding of the eNOS 493–512 peptide and the iNOS 501–532 peptide were determined by competition assays with nNOS according to the procedure described by Erickson-Viitanen and DeGrado (21) for competition with myosin light chain kinase. Dissociation constants were estimated from the midpoints of the activation curves where the following equation is valid:

\[
K_D = \frac{[P]_t + K}{[CaM]}K - K \quad (\text{Eq. 1})
\]

where \([P]_t\) is the total concentration of added peptide (20 nM) and [CaM] and K are concentrations of calmodulin required to reach half-maximal activation in the presence and the absence of peptide, respectively.

Preparation of Recombinant DNA Constructs for Baculovirus Expression of Recombinant Proteins—cDNA sequences encoding wild-type bovine eNOS (8), wild-type murine iNOS (3), and wild-type human nNOS (20) were subcloned into the EcoRI site of the plasmid transfer vector pVL1393. Chimeric cDNA sequences were created from the wild-type sequences in the transfer vector by insertional mutation using the polymerase chain reaction-based splicing-by-overlap-extension technique described by Ho et al. (22). For the inducible (I) 501–523 eNOS protein, wild-type bovine eNOS sequence encoding residues 493–512 (TRKKTFKEVANAVKISASLM) was replaced by sequence encoding murine iNOS residues 501–523 (RPRREIRFRVLVVFFASMLM). For the I 501–532 eNOS protein, wild-type bovine eNOS sequence encoding residues 493–521 (TRKKTFKEVANAVKISASLMGVKMRKV) was replaced by sequence encoding murine iNOS residues 501–532 (RPRREIRFRVLVVFFASMLMGVKMRKV). For the endothelial 493–512 INOS protein, wild-type murine iNOS sequence encoding residues 501–523 was replaced with bovine eNOS sequence encoding residues 493–512. The precise, in-frame exchange of DNA sequences in the chimeric constructs was confirmed by digestion of the molecules with restriction enzymes.

Expression, Purification, and Characterization of NOS Proteins—Recombinant pVL1393 transfer vectors encoding either wild-type eNOS, I 501–523 eNOS, I 501–532 eNOS, wild-type iNOS, endothelial 493–512 iNOS, or wild-type nNOS were separately cotransfected with Baculoviral DNA into Sf9 insect cells in order to produce a high titer recombinant viral stock for use in subsequent infections of Sf9 cells. Infected cells and purification of the six overexpressed proteins was carried out as described previously for wild-type eNOS (23). High level expression of wild-type iNOS in Sf9 cells was achieved by addition of 1 mM N-nitro-arginine methyl ester to the culture medium to inhibit the overexpressed enzyme and thereby reduce the toxic effects of excessive NO production. Proteins were purified by 2′,5′-ADP-Sepharose chromatography and were >90% pure as determined by Coomassie staining of purified proteins separated on SDS-polyacrylamide gels. Enzyme activities were determined by measuring the rate of conversion of l-arginine to l-citrulline under conditions described previously (23). Native, wild-type iNOS was also purified from RAW 264.7 macrophages by 2′,5′-ADP-Sepharose chromatography as described by Steuher et al. (24) following activation of the cells (16 h) with Escherichia coli lipopolysaccharide (2 μg/ml) and recombinant murine interferon-γ (100 units/ml).

RESULTS AND DISCUSSION

Identification of eNOS and iNOS CaM-binding Sequences by Gel Mobility Shift Assays—Based on computer algorithms that search for sequences of appropriate length, charge, hydrophobicity, and helical hydrophobic moment (25) the CaM-binding domain of bovine eNOS has been variously identified as either residues 493–512 (6), residues 496–512 (8), or residues 496–519 (7). The CaM-binding region of human eNOS has been predicted as residues 489–512 (10). Sequence analysis has also been used to predict the iNOS CaM-binding domain as murine residues 503–532 (2), murine residues 504–522 (3), or human residues 507–525 (5). Identification of the eNOS CaM-binding domain as residues 493–512 is likely correct because we have shown that deletion of this region results in loss of CaM-binding capacity of the purified mutant enzyme (23). It is conceivable, however, that loss of binding by the deletion mutant is due to secondary effects of deletion on overall three-dimensional structure of the protein. Furthermore, no experimental evidence currently exists with regard to identification of the iNOS CaM-binding domain, and it is known that prediction of CaM-binding domains solely on the basis of computer analysis can result in misidentification, as has occurred previously with CaM-dependent adenylate cyclase (16) and caldesmon (26). Therefore, in order to definitively establish the location of the CaM-binding sequences in eNOS and in iNOS, synthetic peptides representing the putative CaM-binding domains of these proteins were prepared and tested for their abilities to produce an electrophoretic mobility shift of CaM during polyacrylamide gel electrophoresis in 4 M urea (21).

Depending on the charge and hydrophobicity of the peptide, high affinity binding of the peptide to CaM is detected by this method as a band with increased or decreased mobility relative to the unbound CaM band. In the presence of Ca2+, the electrophoretic mobility of CaM was retarded by a peptide corresponding to bovine eNOS residues 493–512 (Fig. 1A), suggesting that this sequence (identified previously by Lamas et al. (6) as the eNOS CaM-binding domain) contains all of the residues necessary for high affinity binding of Ca2+ by CaM. At a 2:1 ratio of CaM to peptide, half of the CaM formed a CaM-peptide complex. At a 1:1 ratio of CaM to peptide, all of the
CaM was gel shifted, indicating a 1:1 stoichiometry of peptide-CaM complex formation. The eNOS 493–512 peptide, however, did not form a complex with CaM in the presence of the Ca²⁺ chelator, EGTA, indicating that complex formation is Ca²⁺-dependent. Similar results were obtained when electrophoretic mobility shift assays were performed under non-denaturing conditions, indicating that 4 M urea is not required to demonstrate peptide-CaM complex formation.

A synthetic peptide from iNOS that represents those residues in the murine iNOS sequence (501–523) that are aligned with bovine eNOS residues 493–512 (11) was also tested for its ability to bind either Ca²⁺/CaM or the Ca²⁺-deficient conformation of CaM (apoCaM). No complex formation was detected for the peptide and apoCaM. Furthermore, only trace amounts of Ca²⁺/CaM were retarded on the 4 M urea gel even at a 2:1 molar ratio of peptide to CaM (Fig. 1B). Thus, it appears that residues 501–523 of murine iNOS (which completely encompasses the 504–522 sequence identified by Lowenstein et al. (3) as the iNOS CaM-binding domain) does not represent the entire sequence necessary for either Ca²⁺-dependent or Ca²⁺-independent binding of CaM by iNOS. A longer 501–532 peptide sequence from murine iNOS (which encompasses the longer 503–532 sequence suggested by Xie et al. (2) as the iNOS CaM-binding domain) was also examined for its ability to bind either Ca²⁺/CaM or apoCaM. In contrast to the shorter sequence (501–523), the longer sequence (501–532) formed a 1:1 complex with CaM in the presence of Ca²⁺ (Fig. 1C). Residues 524–532 in iNOS, therefore, which are located outside of the iNOS sequence aligned with the complete eNOS CaM-binding sequence, appear to be necessary for high affinity binding of CaM by the enzyme. Interestingly, no peptide-CaM complex formation occurred with the longer peptide in the presence of 2 mM EGTA, indicating that CaM binding (even by the longer iNOS sequence) is Ca²⁺-dependent.

The Ca²⁺-dependence of CaM binding by the iNOS 501–532 sequence suggests at least three alternative possibilities with regard to the interaction of CaM with the intact iNOS enzyme. One possibility is that binding of CaM by iNOS (which occurs in many cell types under resting cell Ca²⁺ levels) is not a truly Ca²⁺-independent event but requires low concentrations of Ca²⁺. A second possibility is that binding of CaM by iNOS involves two closely adjacent subdomains, as described previously for the γ subunit of phosphorylase kinase (18). Binding of CaM to each individual subdomain in phosphorylase kinase is Ca²⁺-dependent. However, synergism between the two adjacent subdomains in the intact protein produces extremely high affinity for CaM even in the absence of Ca²⁺. If this type of interaction were to occur between iNOS and CaM, a second unrecognized CaM-binding domain may exist in iNOS adjacent in the primary structure to the one predicted by computer algorithm (25). A third possible explanation of the gel shift data is that regions or domains in iNOS that are not adjacent to the CaM-binding domain in the protein primary structure (and that may be physically far removed in the tertiary structure of the macromolecule) are required to produce a binding interaction that is not dependent on the presence of Ca²⁺.

Inhibition of a CaM-dependent Target Enzyme by eNOS and iNOS Peptides—The putative CaM-binding domains of eNOS and iNOS were further characterized by using synthetic peptides to inhibit the Ca²⁺/CaM-dependent activation of human nNOS, purified from a baculovirus/Sf9 insect cell system. nNOS obtained by this method was >90% pure and completely Ca²⁺/CaM-dependent and had a specific activity comparable with that reported for the enzyme purified from brain (1 μmol of L-citrulline produced/mg/min) (27). Dose-dependent effects of various peptides on nNOS activity were examined. The eNOS 493–512 and iNOS 501–532 peptides were both potent inhibitors of nNOS activity with IC₅₀ values of 6 ± 2 nM and 2 ± 2 nM, respectively (mean ± S.D., n = 3). The shorter iNOS peptide (501–523) also inhibited nNOS but with a lower potency. The IC₅₀ of the iNOS 501–523 peptide for nNOS was 9 ± 5 nM (mean ± S.D., n = 3). A peptide corresponding to residues 524–544 in the iNOS sequence, which overlaps with the 9 most carboxyl residues in the iNOS 501–532 peptide, was also inhibitory at 100-fold higher concentrations. This result confirms that iNOS residues 524–532 make important contributions to iNOS CaM binding, because these 9 residues by themselves appear to have a low but significant affinity for Ca²⁺/CaM. However, because the iNOS 501–523 peptide had a greater inhibitory potency for nNOS than did the 524–544 peptide, it appears that most of the residues that contribute to iNOS CaM binding are amino-terminal to residue 524.

The possibility that the murine iNOS sequence contains a second CaM-binding domain immediately adjacent to that contained between amino acids 501–532 was also investigated. Two 26-residue peptides representing murine iNOS sequence immediately carboxy-terminal (residues 475–500) and immediately amino-terminal (residues 533–558) to the 501–532 sequence were prepared and tested for their abilities to inhibit nNOS. No inhibition was observed with either peptide even at peptide concentrations as high as 1000 nM. Therefore, if a second CaM-binding domain does exist in iNOS, it appears that it is not located adjacent to the first domain as in the case, for example, of the two subdomains of the γ subunit of phosphorylase kinase (18).

Quantitation of Binding Affinities of eNOS and iNOS Peptides—The eNOS and iNOS CaM-binding domains were also compared in terms of their dissociation constants for Ca²⁺/CaM. Competition assays employing the target enzymes, phosphorylase subunit (28) and myosin light chain kinase (29), have been used in previous studies to determine CaM-binding domain dissociation constants. In the present study, we have used competition assays with nNOS to determine the KD values of the eNOS 493–512 and iNOS 501–532 peptides for Ca²⁺/CaM binding. KD values were estimated by the method of Erickson-Viitanen and DeGrado (21) as 4.0 ± 1.2 nM and 1.5 ± 0.8 nM for the eNOS and iNOS sequences, respectively (mean ± S.D., n = 3). CaM-binding domains in many proteins have been narrowed down to sequences about 20 residues in length that bind CaM at least as tightly as the intact proteins themselves (15). These domains typically have dissociation constants for reversible binding of Ca²⁺/CaM in the range of 1–10 nM, similar to the values determined in this study for the eNOS 493–512 and iNOS 501–532 sequences. The intact iNOS protein, however, is expected to have a much lower KD for CaM because CaM does not dissociate from intact iNOS even in the presence of high concentrations of EGTA or strongly denaturing conditions (14). Thus, although iNOS does not appear to possess two closely adjacent CaM-binding subdomains like those found in phosphorylase kinase, it is likely that other portions of the iNOS molecule (other than residues 501–532 or adjacent sequences) also contribute to the irreversible association of CaM with iNOS.
mented insect cells at the level of approximately 3% of total insect cell protein as described previously (23). Similar high levels of expression of murine iNOS required that infected cells be treated with the NOS inhibitor, Nω-nitro-L-arginine to reduce the toxic effects of NO production by the tonically active enzyme. NO toxicity to Sf9 cells overexpressing the "high output" iNOS may explain the lower than expected levels of iNOS expression in a baculovirus system previously observed by other investigators (30). In addition to the two wild-type enzymes, three different eNOS/iNOS chimeric proteins were also expressed and purified from the baculovirus system previously observed by other investigators (30). In addition to the two wild-type enzymes, three different eNOS/iNOS chimeric proteins were also expressed and purified from the baculovirus system. A chimeric protein designated as I 501–523 eNOS was expressed in which the wild-type 493–512 sequence in eNOS was replaced by the iNOS 501–523 sequence. A second chimeric protein was expressed, designated as I 501–532 eNOS, in which the 493–521 sequence in eNOS was replaced by the iNOS 501–532 sequence. A final chimeric enzyme was expressed, designated as endothelial 493–512 iNOS, in which the 501–523 sequence in iNOS was replaced by the eNOS 501–532 sequence. A final chimeric enzyme was expressed, designated as endothelial 493–512 iNOS, in which the 501–523 sequence in iNOS was replaced by the eNOS 501–532 sequence. Each of the five expressed proteins were purified to >90% homogeneity by affinity chromatography on 2',5'-ADP-Sepharose. Purifications were carried out in buffer containing 2 mM EGTA in order to dissociate any CaM bound to the enzymes in a Ca<sup>2+</sup>-dependent manner. Interactions between mammalian NOS proteins and the CaM endogenously produced in insect cells are expected to be essentially identical to those that would occur with mammalian CaM, because CaM has been highly conserved throughout eukaryotic evolution. CaM primary sequences, for example, from bovine brain and from jellyfish are identical at 141 out of 148 of residues (31). Furthermore, the purified NOS enzymes obtained from the baculovirus expression system are expected to be uncontaminated with any endogenous NOS expressed in insect cells because uninfected Sf9 cells were found to contain no detectable NOS activity.

Catalytic activity of each of the five purified NOS enzymes was measured in the presence of varying concentrations of exogenously added CaM (Fig. 2). Assays were carried out in the presence of either 2.5 mM CaCl<sub>2</sub> or 10 mM EGTA. Similar results were obtained in three different experiments.

**FIG. 2.** Ca<sup>2+</sup> and CaM dependence of wild-type and chimeric NOS enzymes. NOS activity was determined by estimating the rate of conversion of L-arginine to L-citrulline. Assays were performed in duplicate in the presence of varying concentrations of CaM and in the presence of either 2.5 mM CaCl<sub>2</sub> or 10 mM EGTA. Similar results were obtained in three different experiments.
mained dependent on exogenously added CaM for activity. In contrast, the longer iNOS 501-532 sequence was sufficient to confer CaM independence on eNOS. I 501-532 eNOS (like wild-type iNOS) was completely independent of added CaM (Fig. 2D). An additional conclusion that can be drawn from the CaM-independence observed for I 501-532 eNOS is that regions in iNOS that are required for irreversible CaM binding (but that are located outside of residues 501-532) are also conserved in the eNOS enzyme. If these other putative domains did not exist in eNOS, I 501-532 eNOS would be expected to remain CaM-dependent (like wild-type eNOS). Interestingly, irreversible association of CaM with eNOS (observed with I 501-532 eNOS) was not sufficient to activate the enzyme in the absence of Ca2+ (Fig. 2D). It appears that bound CaM must be in the Ca2+/CaM conformation in order to interact with eNOS in a manner that activates the enzyme. A final conclusion apparent from analysis of the chimeric enzymes is that the 501-523 region of iNOS (which was not sufficient to produce irreversible CaM binding in eNOS) is necessary for the Ca2+ and CaM independence of iNOS. This is illustrated by the endothelial 493-512 iNOS chimeric enzyme, which was completely dependent on both Ca2+ and CaM for catalytic activity (Fig. 2E).

Identification of Amino Acids Essential for CaM Binding by the eNOS CaM-binding Domain—The 493-512 sequence of bovine eNOS contains a number of hydrophobic and basic residues that may be involved in Ca2+/CaM binding by the enzyme. For example, as noted previously by Ikura et al. (35), CaM-binding domains often share a common feature of containing either aromatic residues or long chain hydrophobic residues that are 14 residues apart in sequence and that form key contacts with CaM in CaM-enzyme complexes. Two such residues are found in eNOS at Phe-498 and Leu-511. These residues are also conserved in the sequences of iNOS, nNOS, smooth muscle myosin light chain kinase, and skeletal muscle myosin light chain kinase (Table I). A third hydrophobic residue in eNOS at Val-505 is also conserved in iNOS, nNOS, smooth muscle myosin light chain kinase, and skeletal muscle myosin light chain kinase. This residue has also been shown to be important for CaM binding in CaM-myosin light chain kinase peptide complexes (35, 36). In general, hydrophobic amino acids seem to play a more important role in CaM binding than do charged residues (37). However, basic residues are invariably found in CaM-binding domains and one or more of these residues appear to contribute to CaM binding via electrostatic interactions between basic residues in the enzyme and acidic residues in the highly acidic CaM molecule (15). Bagchi et al. (37) have noted previously that these basic residues are frequently located within CaM-binding domains at a position 13 residues carboxyl to the first hydrophobic residue. Basic residues are found in this position in the CaM-binding domains of smooth muscle myosin light chain kinase, skeletal muscle myosin light chain kinase, and nNOS. The eNOS enzyme, however, contains a serine at this position (Table I). Other basic residues within eNOS that might be important determinants of CaM-binding include Arg-494, Lys-495, Lys-496, Lys-499, and Lys-506.

The importance of specific hydrophobic and basic residues to eNOS CaM binding was assessed using two different approaches. Peptides were prepared in which Phe-498, Val-505, Leu-511, Arg-494, Lys-495, Lys-496, Lys-499, and Lys-506 were each individually replaced by alanine residues. Mutated peptides were then compared with the wild-type peptide for their ability to gel shift CaM on 4 M urea gels as well as for their inhibitory potency for the CaM-mediated activation of nNOS. Peptides that were mutated at either Phe-498, Leu-511, or Lys-499 lost their capacity for high affinity binding of CaM, as evidenced by loss of CaM mobility shift on 4 M urea gels (Table II). Furthermore, these same three peptides showed significantly lower potencies for inhibition of nNOS than did the wild-type peptide. The hydrophobic residues, Phe-498 and Leu-511, as well as the basic residue, Lys-499, thus appear to represent essential determinants of the eNOS/CaM interaction. Phe-498 appears to be especially important because mutation at this residue results in a 150-fold increase in the IC50 of the mutant peptide for inhibition of nNOS.

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J. Biol. Chem. 1996, 271:6435-6440.
doi: 10.1074/jbc.271.11.6435

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