Structural basis for endothelial nitric oxide synthase binding to calmodulin

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The enzyme nitric oxide synthase (NOS) is exquisitely regulated *in vivo* by the Ca²⁺ sensor protein calmodulin (CaM) to control production of NO, a key signaling molecule and cytotoxin. The differential activation of NOS isozymes by CaM has remained enigmatic, despite extensive research. Here, the crystallographic structure of Ca²⁺-loaded CaM bound to a 20 residue peptide comprising the endothelial NOS (eNOS) CaM-binding region establishes their individual conformations and intermolecular interactions, and suggests the basis for isozyme-specific differences. The α -helical eNOS peptide binds in an antiparallel orientation to CaM through extensive hydrophobic interactions. Unique NOS interactions occur with: (i) the CaM flexible central linker, explaining its importance in NOS activation; and (ii) the CaM C-terminus, explaining the NOS-specific requirement for a bulky, hydrophobic residue at position 144. This binding mode expands mechanisms for CaM-mediated activation, explains eNOS deactivation by Thr495 phosphorylation, and implicates specific hydrophobic residues in the Ca²⁺ independence of inducible NOS.

Keywords: calcium/calmodulin/crystal structure/intermolecular interaction/nitric oxide synthase

Introduction

Nitric oxide (NO), an important physiological messenger and cellular cytotoxin (Nathan, 1992), is synthesized by three nitric oxide synthase (NOS) isozymes (endothelial NOS, eNOS; neuronal NOS, nNOS; inducible NOS, iNOS) via the five electron oxidation of L-arginine (Marletta, 1994; Griffith and Stuehr, 1995). All NOS isozymes share the same architecture (Hemmings and Mayer, 1998; Stuehr, 1999): (i) the N-terminal catalytic oxygenase module (NOSox) binds Fe-protoporphyrin IX (heme), substrate L-arginine and (6R)-5,6,7,8-tetrahydrobiopterin; (ii) the C-terminal reductase module (NOSred) contains binding sites for NADPH, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN); and (iii) the intervening region interacts with calmodulin (CaM). The three isozymes are regulated differentially. eNOS (Pollock *et al.*, 1991) and nNOS (Bredt and Snyder, 1990), which are constitutively expressed in certain cells, are activated by Ca²⁺-loaded CaM. iNOS (Stuehr *et al.*,

1991) is expressed in response to immunostimulants and, once expressed, is active independently of Ca²⁺; CaM is a tightly bound subunit even at resting basal levels of intracellular Ca²⁺ (Cho *et al.*, 1992; Nathan and Xie, 1994). Regardless of the isozyme differences in Ca²⁺ dependence, electron transfer in all biologically active NOS homodimers is triggered by CaM binding to the central linker region and occurs *in trans* from NADPH via the FAD/FMN on one subunit to the heme iron on the other subunit (Panda *et al.*, 2001). Despite extensive studies to date, the CaM-mediated electron transfer process in NOS and the assembly of the holo enzyme are poorly understood.

CaM is a ubiquitous, small, acidic, Ca²⁺-binding protein that binds to and activates diverse target proteins to transduce the Ca²⁺ signal into many cellular processes (Zhang and Yuan, 1998; Chin and Means, 2000; Hoeflich and Ikura, 2002). Classical high affinity CaM-binding sites on target proteins often are non-homologous in sequence but, in general, have a tendency to form a basic, amphipathic α -helix of 14–26 amino acids in length (O’Neil and DeGrado, 1990; Crivici and Ikura, 1995; Rhoads and Friedberg, 1997). Ca²⁺ binding to CaM induces significant conformational changes exposing methionine-rich hydrophobic surfaces that form critical van der Waals interactions with the hydrophobic face of the target recognition site. In this classical binding mode, Ca²⁺-loaded CaM clamps a single helical target peptide between its two lobes (Ikura *et al.*, 1992; Meador *et al.*, 1992). The target-specific unraveling of the flexible central linker region (residues 76–81), located between the N- and C-terminal lobes, enables CaM to bind classical recognition sites of different lengths, defined by two hydrophobic/aromatic residues that anchor the peptides to the two CaM lobes (Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993; Roth *et al.*, 1992). In addition to the classical CaM–target complexes, recent studies have demonstrated how CaM binds non-classical recognition sites, such as those from small conductance Ca²⁺-activated K⁺ channel (Schumacher *et al.*, 2001) and anthrax adenyl cyclase exotoxin (Drum *et al.*, 2002), which do not form a single α -helix.

The putative CaM-binding region in NOS isozymes, encompassing 20–25 amino acids located between NOSox and NOSred, was first identified based on primary and secondary structure analyses (Bredt *et al.*, 1991; Stuehr *et al.*, 1991; Lamas *et al.*, 1992; Lyons *et al.*, 1992; Xie *et al.*, 1992), and later verified by functional studies using synthetic peptides (Vorherr *et al.*, 1993; Anagli *et al.*, 1995; Zhang *et al.*, 1995b; Venema *et al.*, 1996; Zoche *et al.*, 1996; Matsubara *et al.*, 1997; Yuan *et al.*, 1998). Based on the positions of conserved hydrophobic residues, the NOS CaM-binding regions correspond to the classical Ca²⁺-dependent ‘1-5-8-14’ motif (Rhoads and Friedberg,

Fig. 1. Overall structure of CaM bound to the eNOS-derived peptide. (A) Sequence alignment of the '1-14' CaM recognition sites from eNOS and skeletal (sk) and smooth (sm) muscle MLCK. Key hydrophobic residues predicted or known to be essential in CaM binding are shown in red. (B) Antiparallel binding of the eNOS-derived peptide (blue) to CaM (green), mediated by hydrophobic residues (pink) located at positions '1-5-8-14'. CaM N- and C-termini and helices are labeled, along with the central linker (residues 76-81), which connects the N-terminal lobe (helices I-IV) and the C-terminal lobe (helices VI-VIII). (C) The bound eNOS-derived peptide (blue) shown with its $2F_o - F_c$ electron density map, contoured at 1σ (purple) and 3σ (green). Residues Phe496-Ala507 form an α -helix, leaving the terminal residues in an extended, flexible conformation. (D) Schematic diagram of interactions between CaM (black) and the eNOS-derived peptide (blue) with its key hydrophobic residues (pink). CaM N-terminal lobe residues located within 4 Å of the bound peptide are shown below the peptide, while CaM C-terminal lobe residues, which mainly interact with the N-terminal half of the peptide, are shown above the peptide. Met76 located in the central linker is indicated in a box. Binding of the peptide to CaM is mediated mainly by hydrophobic interactions, except for several hydrogen bonding and ionic interactions, which are indicated with asterisks. Interactions that are only found in one of the four non-crystallographic symmetry-related molecules are shown in parentheses.

1997) (Figure 1A), which includes many well-studied Ca^{2+} /CaM-dependent proteins, such as myosin light chain kinase (MLCK). Synthetic peptides of 20 residues corresponding to the eNOS CaM-binding region (bovine residues 493-512 and human residues 492-511) bind as tightly as the intact enzyme to CaM (10^{-9} M binding affinity) in the presence of Ca^{2+} , but do not bind in the absence of Ca^{2+} (Venema *et al.*, 1996; Matsubara *et al.*, 1997). When substituted into the iNOS CaM-binding region, this 20 amino acid region from eNOS completely abolishes Ca^{2+} /CaM-independent iNOS activity (Venema *et al.*, 1996). Although spectroscopic studies (Zhang *et al.*, 1995b) demonstrated that the nNOS-derived peptide binds in an antiparallel manner (the peptide N-terminus interacts with the CaM C-terminal lobe; the peptide C-terminus interacts with the CaM N-terminal lobe) at methionine-rich hydrophobic patches on CaM, analogously to the MLCK-derived peptides (Ikura *et al.*, 1992; Meador *et al.*, 1992), a detailed molecular basis for CaM-NOS association remains undetermined. Structural characterization of interactions between NOS and CaM is not only the first step toward understanding isozyme-specific CaM binding to the NOS enzymes, but also expands our knowledge of the mechanisms that underlie CaM's ability to recognize diverse targets.

Here, we report a crystallographic structure of Ca^{2+} -loaded CaM in complex with the 20 residue synthetic peptide derived from the human eNOS CaM-binding region. Our structure reveals that binding of CaM antiparallel to the helical eNOS target site: (i) occurs with unexpected relative positioning of the CaM N- and

C-terminal lobes around a shorter peptide helix; (ii) involves novel interactions between the flexible central linker of CaM and the bound peptide; and (iii) provides a structural basis for the importance of the CaM C-terminus in NOS activation. Furthermore, based on the binding mode of the eNOS-derived peptide, we propose that specific sequence features unique to the iNOS CaM-binding region contribute to the absolute requirement of this region for Ca^{2+} -independent iNOS activity.

Results and discussion

Overall structure of the CaM-eNOS peptide complex

We determined the 2.05 Å resolution crystallographic structure (Table I) of Ca^{2+} -bound CaM complexed with the CaM-binding peptide derived from human eNOS ($_{492}\text{RKKT}\text{FKEVANAVKISASLMG}_{511}$; Figure 1). The four non-crystallographic symmetry-related models each include all but four residues (terminal residues 1-2 and 148 for CaM, and 511 for the eNOS peptide). Above average thermal parameters (B -factors ≥ 50) associated with CaM terminal and loop residues, and peptide termini contribute to the relatively high overall B -factor for the complexes (Table I). The four models are structurally similar, having an average C_α distance root mean square deviation (r.m.s.d.) between any two of the four molecules of 0.9 Å. Like the peptides derived from MLCK (Ikura *et al.*, 1992; Meador *et al.*, 1992) and calmodulin-dependent protein kinase IIa (CaMKII) (Meador *et al.*, 1993), the eNOS peptide binds CaM in a classical

Table I. Crystallographic data collection, phasing and refinement statistics

Data collection and phasing			
Space group	$P2_1$		
Unit cell dimensions	$a = 68.5 \text{ \AA}, b = 74.0 \text{ \AA}, c = 71.1 \text{ \AA}, \beta = 111.4^\circ$		
	Se edge	Se peak	Se remote
Wavelength (Å)	0.979214	0.979071	0.911656
Resolution (Å)	20–2.05 (2.12–2.05) ^a	20.0–2.05 (2.12–2.05)	20.0–2.05 (2.12–2.05)
Total reflections	127 631	125 220	129 886
Unique reflections	41 353	41 125	41 626
Completeness (%)	99.4 (98.0)	98.7 (98.6)	99.7 (99.0)
R_{sym}^b	0.065 (0.35)	0.075 (0.34)	0.060 (0.35)
$\langle I/\sigma \rangle^c$	24.28 (3.55)	24.19 (3.78)	24.14 (3.76)
Overall figure of merit		0.72 (0.88) ^d	
Refinement statistics for merged data			
Reflections		41 186	
R^e		0.222	
R_{free}^f		0.248	
No. of non-hydrogen atoms		5478 (four molecules)	
No. of waters		278	
<Overall B > (Å ²)		45.02 ^g	
R.m.s. bond (Å)		0.023	
R.m.s. angle (°)		1.13	

^aHighest resolution shell for compiling statistics.^b $R_{\text{sym}} = \sum_j |I_j - \langle I \rangle| / \sum_j I_j$.^cAverage intensity signal-to-noise ratio.^dAfter solvent flattening.^e $R = \sum |F_o| - |F_c| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.^f5% of the reflections were set aside randomly for R_{free} calculation.^gAverage B -values per residue (all atoms) range from 17 to 92.

antiparallel orientation; the N-terminal (residues 3–75) and C-terminal (residues 82–147) lobes of CaM wrap around the bound peptide, interacting with the C- and N-terminal halves of the peptide, respectively (Figure 1B). Within the individual CaM lobes, secondary and tertiary structures closely resemble those from the Ca^{2+} -CaM–MLCK peptide complex (Meador *et al.*, 1992) with average C_α r.m.s.d. values of 1.4 Å for the N-terminal and 0.9 Å for the C-terminal lobes.

The core of the bound eNOS peptide, encompassing residues Phe496–Ala507, is α -helical, leaving four residues at each end in an extended conformation, as evident from the well-defined electron density observed for almost the entire eNOS peptide (Figure 1C). In common with the CaM complex with the MLCK peptide (Ikura *et al.*, 1992) sharing the classical ‘1-5-8-14’ motif, the α -helical core of the eNOS-derived peptide binds antiparallel to CaM, with each of the four key hydrophobic residues (Phe496, Ala500, Val503 and Leu509 for eNOS) forming critical interactions with CaM (Figure 1B and D). In contrast to other CaM complexes with classical antiparallel-binding peptides (Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993), however, the α -helix formed by the eNOS peptide is the shortest, containing only 12 residues, compared with 14–16 residues. This shorter 12 residue helix leaves the fourth key hydrophobic residue, Leu509, positioned outside the helical core. A cluster of three basic residues (Arg492, Lys493 and Lys494) at the N-terminal end of the eNOS peptide interacts electrostatically with distinct glutamate clusters on both lobes of CaM (Figure 1D),

although this basic cluster adopts different conformations and interactions in each of the four non-crystallographic symmetry-related molecules. Similar electrostatic interactions observed for the peptides derived from MLCK (Ikura *et al.*, 1992; Meador *et al.*, 1992) and CaMKII (Meador *et al.*, 1993) are proposed to play critical roles in determining the directionality of CaM binding with respect to the classical target recognition site (Osawa *et al.*, 1999; Kurokawa *et al.*, 2001).

Target recognition unique to eNOS CaM-binding region

When our structure is compared with those of other CaM complexes with antiparallel binding peptides, significant differences in the overall tertiary structure and quaternary assembly are evident (Figure 2), emphasizing the importance of CaM’s plasticity in the formation of classical high affinity complexes. Alignment of our CaM–eNOS peptide structure with the CaM–MLCK-derived peptide structure (Meador *et al.*, 1992) with respect to backbone atoms of the ‘1–14’ peptide residues (Figure 2, left) reveals shifts in the relative positions of CaM lobes. The N- and C-terminal lobes of CaM clamp around the helical eNOS peptide more tightly by $\sim 15^\circ$ (Figure 2, top), while the CaM latch domains (Meador *et al.*, 1992) (helices II and VI; see Figure 1B) of the two lobes are $\sim 2 \text{ \AA}$ further apart from each other along the helical axis of the eNOS peptide (Figure 2, bottom). In comparison with the CaM–MLCK peptide complex (Meador *et al.*, 1992), the CaM–eNOS peptide complex has both CaM lobes rotated (counter-

Fig. 2. Superpositions of the CaM-eNOS peptide structure (green, CaM; and blue, peptide) and the CaM-MLCK peptide structure (gray, PDB code 1CDL) (Meador *et al.*, 1992) showing significant differences in the relative binding orientation of CaM lobes. The two structures are aligned by superimposing backbone atoms of the '1-14' residues of the bound peptide (left column), the CaM N-terminal lobe (middle column) and the CaM C-terminal lobe (right column). In the top panel, the CaM-peptide complexes are viewed along the bound peptide helix from its C-terminus, while in the bottom panel the complexes are rotated 90° around the horizontal axis, placing the bound peptide vertically with the C-terminus on top. For clarity, the bound peptides are truncated to include only the 14 residues anchored by two key hydrophobic residues.

clockwise in Figure 2, top) around the peptide helix, and bound closer to the peptide C-terminus by approximately one turn (N-terminal lobe) and one-half turn (C-terminal lobe) of the peptide helix (Figure 2, bottom). Both the tertiary and quaternary structural differences were unexpected since the eNOS peptide shares with the MLCK peptides the classical '1-5-8-14' sequence motif (Ikura *et al.*, 1992; Meador *et al.*, 1992), with 12 residues between the two key anchoring residues (Rhoads and Friedberg, 1997). However, these differences in the overall tertiary and quaternary structure explain why our initial attempts to obtain crystallographic phasing information from molecular replacement methods by using available CaM peptide complex structures (Ikura *et al.*, 1992; Meador *et al.*, 1992) as search models were unsuccessful.

Binding of the eNOS-derived peptide with the classical '1-5-8-14' motif to CaM induces an unexpected conformation of the flexible central linker (CaM residues 76–81) (Kuboniwa *et al.*, 1995; Zhang *et al.*, 1995a) connecting the two lobes of CaM (Figure 1B). The central linker exhibits high flexibility in free CaM in solution (Kuboniwa *et al.*, 1995; Zhang *et al.*, 1995a). In the presence of other classical target peptides, helices IV and V adjacent to the central linker unravel, thus elongating the central linker and positioning the CaM lobes to accommodate different target sequences (Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993; Osawa *et al.*, 1999; Kurokawa *et al.*, 2001). Surprisingly, when CaM binds to the eNOS peptide, the helices adjacent to the central linker do not unwind (Figure 3A). Central linker residues 77–81 assume different loop conformations within the crystal, and have weaker than average electron density. However, in all

these conformations, this flexible central linker binds close to the helical eNOS peptide.

Within the central linker, CaM Met76 mediates interactions with the eNOS peptide and appears structurally to prevent unraveling of helix IV (residues 65–75). Met76, which occurs in a single conformer, packs with eNOS Ile505 (position 10) and Ser508 (position 13) (Figures 1D and 3B). Based on multiple sequence alignment of NOS isozymes, the bulky hydrophobic residue at position 10 (Ile505 in eNOS, or phenylalanine in nNOS and iNOS) is a conserved feature of NOS CaM-binding regions, and is likely to be a common critical factor contributing to the observed conformational effects of NOS on the CaM central linker. In contrast, the residue at position 13 varies among NOS isozymes and is not specific to NOS, ranging from a small polar serine in eNOS to a flexible, basic lysine in nNOS or MLCK; thus, eNOS Ser508 (position 13) is probably less important in the central linker binding to the peptide. The spacing of the two key anchoring hydrophobic residues (Meador *et al.*, 1993) in their shared classical '1-14' sequence motif (Rhoads and Friedberg, 1997) is not solely responsible for the degree of central linker elongation, as evident in the distinct conformations of the central linker induced by the eNOS peptide and MLCK peptide (Ikura *et al.*, 1992; Meador *et al.*, 1992).

eNOS Ile505 (position 10) makes NOS-specific interactions with helix I in the CaM N-terminal lobe, as well as the CaM central linker. In particular, interactions between Ile505 and CaM Phe12 and Ala15 (Figure 1D) are unprecedented in the other classical '1-14' peptide CaM complexes (Ikura *et al.*, 1992; Meador *et al.*, 1992), and are coordinated with a shift of the eNOS peptide relative to helix I of CaM (Figure 3B). When superimposed by the

Fig. 3. Binding interactions unique to the eNOS-derived peptide. **(A)** Differences in the central linker conformation when CaM is bound to the eNOS-derived peptide (green, CaM; blue, peptide) and to the MLCK-derived peptide (gray, PDB code 1CDL) (Meador *et al.*, 1992). CaM structures are superimposed on the C_α atoms of the N-terminal lobe, and the MLCK-derived peptide is not shown for clarity. CaM binding to the eNOS-derived peptide does not lead to extension of the central linker (residues 76–81; arrows), possibly due to close contacts formed between Met76 and the C-terminal half of the bound peptide. **(B)** Close-up views of hydrophobic interactions responsible for the unique conformation of the central linker when CaM (green) binds the eNOS peptide (blue). Ile505 and Ser508 of eNOS pack with Met76 of CaM, which has the lowest, average atomic B -value in the central linker. A hydrophobic cluster provided from the N-terminal CaM lobe (Phe12, Ala15, Phe68 and Met72) is also in close contact with Ile505, possibly contributing to the tethering of the central linker to the bound peptide. **(C)** A shift of helix I when CaM is bound to the eNOS-derived peptide (green, CaM; blue, peptide) compared with CaM bound to the MLCK-derived peptide (gray, PDB code 2BBM) (Ikura *et al.*, 1992). Two complex structures were superimposed with respect to backbone atoms of the ‘1–14’ peptide residues. The shift of helix I results in changes in the local environment surrounding eNOS Glu498, an acidic residue unusual in a CaM target site. Ala10 replaces Glu14 in close proximity to Glu498. **(D)** An $F_o - F_c$ omit electron density map (pink, 2.5 σ and cyan, 4.0 σ), calculated by replacing charged residues with alanine, showing the close proximity of neighboring glutamate residues located on CaM helix I (green) in the vicinity of Lys494, Glu498, Lys497 and Asn501 of the bound eNOS peptide (blue).

backbone atoms of the ‘1–14’ peptide residues, helix I in our CaM–eNOS peptide complex is shifted by ~ 4 residues (1.1 turn) relative to that in classical CaM complexes with MLCK peptides (Ikura *et al.*, 1992; Meador *et al.*, 1992) (Figures 2, left and middle, and 3C). This shift in helix I positions CaM Ala10 in place of Glu14, which might otherwise interact unfavorably, either sterically or electrostatically, with Glu498 (position 3) on the eNOS CaM-binding peptide (Figure 3C). Furthermore, the shift in helix I not only moves CaM Glu14 within hydrogen bonding distance of Glu114 from the CaM C-terminal lobe, but also places a counterion, eNOS Lys494, nearby to complement the negative charge(s) (Figure 3D). Similarly, CaM Glu7 and Glu11 are within hydrogen bonding distance, and are positioned near Asn501 and a counterion Lys497 of eNOS (Figure 3D). One member of each glutamate pair is probably protonated under the acidic crystallization conditions, reducing otherwise unfavorable electrostatic interactions with eNOS Glu498. Thus, the positioning of the eNOS peptide with respect to helix I of CaM, within our structure, compensates for the unusual presence of a negatively charged residue (O’Neil

and DeGrado, 1990) (eNOS Glu498) within the classical recognition motif for the highly acidic CaM.

The eNOS-derived peptide not only interacts uniquely with the CaM central linker and the N-terminal lobe, but also binds the CaM C-terminal lobe in a distinct way, compared with the MLCK peptides. The side chain of the key hydrophobic residue Phe496 (position 1) on the eNOS peptide is in a different conformation from the corresponding tryptophan on the MLCK peptides (Ikura *et al.*, 1992; Meador *et al.*, 1992) (Figure 4A and B). The side chain conformation at position 1 determines positioning of CaM helices VII and VIII, which contain Met124 and Met144, respectively; the Met124 and Met144 C_α are 2.9 and 3.6 Å closer to the bound peptide for eNOS, compared with the CaM/MLCK peptide structures (Ikura *et al.*, 1992; Meador *et al.*, 1992) (Figures 2, right, and 4B). As a result of the Met144 position determined by eNOS Phe496 and the space provided by the small size of eNOS Ala500 (position 5), Met145 faces toward and packs with Asn501 (position 6), forming interactions not observed with MLCK (Figure 4A and B). Interestingly, the three residues (Phe496, Ala500 and Asn501) that contact Met144 and

Fig. 4. Interactions between the CaM C-terminal lobe and the bound eNOS peptide. (A) Stereoview of the $2F_o - F_c$ electron density map (blue, 1.6σ ; red, 3.4σ) showing binding of the N-terminal half of the peptide to the C-terminal lobe of CaM. (B) The CaM-eNOS peptide structure (green, CaM; blue, peptide) was superimposed onto the CaM-MLCK-derived peptide structures [gold, PDB code 1CDL (Meador *et al.*, 1992); gray, PDB code 2BBM (Ikura *et al.*, 1992)] with respect to backbone atoms of the '1-14' residues on the bound peptides. The differing conformations of the first anchoring hydrophobic residue (Phe496 in eNOS and Trp800 in MLCK) appear to determine the positions of helix VII (Met124) and helix VIII (Met144 and Met145). The two C-terminal methionine residues at positions 144 and 145 make eNOS-unique interactions with Phe496, Ala500 and Asn501, located at positions 1, 5 and 6. Side chains of some nearby residues are omitted for clarity. (C) The phosphorylation site, Thr495, exposed at the solvent-accessible surface of CaM. Some residue side chains are omitted for clarity. Thr495, which N-caps the peptide helix (blue), is surrounded by acidic residues provided by both lobes of CaM (green).

Met145 are nearly conserved in eNOS and nNOS, except that nNOS has glutamate in place of Asn501. Thus, eNOS-specific interactions described here illustrate how the conformational flexibility of CaM can differentially modulate target recognition of α -helical peptides that share the same classical binding motif.

Correlations with biochemical studies

The unexpected interactions between the eNOS peptide and Met76 (Figure 3A and B) may explain why the CaM central linker is required for maximum NOS activation. CaM tryptic fragments corresponding to each lobe (residues 1-75 and 78-148) induce only 50% of the maximum nNOS activation by intact CaM, compared with 80-85% activation of MLCK (Persechini *et al.*, 1994). Furthermore, the tight association between the CaM central linker and NOS may be the structural basis for differences in Ca^{2+} dissociation and enzyme inactivation kinetics of CaM bound to nNOS- and MLCK-derived peptides (Persechini *et al.*, 1996). For CaM bound to nNOS, rapid Ca^{2+} dissociation from the N-terminal lobe leading to enzyme inactivation is followed by slower Ca^{2+}

dissociation from the C-terminal lobe and dissociation of CaM (Persechini *et al.*, 1996; Weissman *et al.*, 2002). In contrast, three Ca^{2+} ions slowly dissociate from CaM bound to MLCK, suggesting coupled enzyme inactivation and CaM dissociation (Persechini *et al.*, 1996). Additional contacts between CaM and NOS provided by the central linker residue Met76 may also contribute to the tighter association of the C-terminal lobe, and help to maintain the two distinct events, namely NOS inactivation and CaM dissociation.

Based on our structure, we propose that the structural basis of recently reported antagonizing effects of a plant CaM on NOS is specific hydrophobic interactions between CaM C-terminal residues (Met144 and Met145) and the bound eNOS peptide (Figure 4A). A plant CaM isoform, which is ~90% identical to mammalian CaM, inhibits NOS, but activates other CaM-dependent systems, such as MLCK (Lee *et al.*, 2000), calcineurin (Cho *et al.*, 1998) and cyclic nucleotide phosphodiesterase (Lee *et al.*, 1995). Mutation of Met144 to valine (in helix VIII) in mammalian CaM to mimic the plant CaM isoform also leads to NOS inhibition, with a K_i value similar to that of the plant

CaM (15 nM). Thus, Val144 is entirely responsible for the inhibitory effects of the plant CaM on NOS (Kondo *et al.*, 1999). In our structure, Met144 and Met145 pack tightly with the bound eNOS peptide, precisely positioning the CaM C-terminal lobe with respect to the N-terminal residues of the bound peptide. Bulky hydrophobic residues, but not valine, replacing Met144 probably could maintain observed interactions between the CaM C-terminus and eNOS Phe496, Ala500 and Asn501. This is consistent with the earlier report that the CaM mutants M144L and M144F gave >85% NOS activation, while M144V and M144C gave ~20% NOS activation (Kondo *et al.*, 1999). Moreover, based on the sequence and structural alignment (Rhoads and Friedberg, 1997), larger residues, such as isoleucine in calcineurin and phenylalanine in MLCK (Ikura *et al.*, 1992), in place of eNOS Ala500 (position 5), may restrict the Met145 side chain conformation and result in the different positioning of the CaM C-terminus (Figure 4B). Hence, the size of the second key hydrophobic peptide residue (position 5) may also affect the importance of Met144 in enzyme activation.

Implications for eNOS Thr495 phosphorylation

Our structure of this CaM–eNOS peptide complex provides a basis for evaluating the structural consequences of regulatory phosphorylation of Thr495 in the eNOS CaM-binding region. Thr495 has been shown to be phosphorylated both *in vivo* in porcine aortic endothelial cells (Fleming *et al.*, 2001), and *in vitro* by protein kinase C (Matsubara *et al.*, 1996), AMP-activated protein kinase (Chen *et al.*, 1999) and cyclic-nucleotide dependent protein kinases (Butt *et al.*, 2000). Thr495 phosphorylation deactivates eNOS by hindering the binding of CaM (Fleming *et al.*, 2001). In our crystallographic structure, Thr495 (position 0) N-terminally caps the eNOS peptide helix via a hydrogen bond between its side chain OG1 and Glu498 backbone N (Figure 4C), and directs the preceding N-terminal basic cluster (Arg492, Lys493 and Lys494) toward the N-terminal lobe of CaM. Interestingly, N-capping Thr495 resides on the solvent-exposed surface (Figure 4C) opposite from the CaM latch domain (helices II and VI) (Meador *et al.*, 1992), which is proposed to contact other NOS domains outside the CaM-binding region during NOS activation (Su *et al.*, 1995).

Based on our crystallographic structure, several possible structural consequences of eNOS Thr495 phosphorylation could explain the resultant observed weaker CaM binding. First, phosphorylation at Thr495 OG1 would disrupt its hydrogen bond with the Glu498 backbone amide, possibly affecting the α -helical secondary structure of the peptide. Loss of the N-capping of the helix, however, is unlikely to be sufficient to distort the entire helix since a non-capping lysine or arginine residue occupies the equivalent position on MLCK peptides (Ikura *et al.*, 1992; Meador *et al.*, 1992). Although recent studies using synthetic phosphopeptides demonstrated that the phosphate group located within CaM-binding sequences can alter their helical propensity, the effects on the helical content do not correlate with the observed binding affinity for CaM, suggesting the importance of steric and electrostatic contributions by the phosphate (Vetter and Leclerc, 2001). Secondly, a phosphate group could interact with the basic patch nearby on the enzyme, causing conformational changes, resulting in inaccessibility of the CaM recognition site. Although this is the most attractive scenario in which phosphorylation of a single residue is amplified to drastic conformational changes affecting CaM binding, the exact mechanisms by which Thr495 phosphorylation regulates eNOS await structural characterization of other NOS domains in combination with the CaM-binding region. Finally, a negative charge on a phosphate group could cause electrostatic repulsion with nearby glutamate residues of CaM. This repulsion could occur directly between the Thr495 phosphate and proximal CaM Glu7 and Glu127, or indirectly through phosphate-induced movements of nearby eNOS Glu498 (Figure 4C), an unusual acidic residue within the classical CaM-binding region (O'Neil and DeGrado, 1990). The Glu498 carboxylate is surrounded by CaM Glu7, Glu11 and Glu14. The reduced ability of CaM to co-immunoprecipitate with the phosphorylation-mimicking T495D mutant eNOS (Fleming *et al.*, 2001) suggests that the negative charge introduced by Thr495 phosphorylation indeed decreases CaM binding.

tional changes, resulting in inaccessibility of the CaM recognition site. Although this is the most attractive scenario in which phosphorylation of a single residue is amplified to drastic conformational changes affecting CaM binding, the exact mechanisms by which Thr495 phosphorylation regulates eNOS await structural characterization of other NOS domains in combination with the CaM-binding region. Finally, a negative charge on a phosphate group could cause electrostatic repulsion with nearby glutamate residues of CaM. This repulsion could occur directly between the Thr495 phosphate and proximal CaM Glu7 and Glu127, or indirectly through phosphate-induced movements of nearby eNOS Glu498 (Figure 4C), an unusual acidic residue within the classical CaM-binding region (O'Neil and DeGrado, 1990). The Glu498 carboxylate is surrounded by CaM Glu7, Glu11 and Glu14. The reduced ability of CaM to co-immunoprecipitate with the phosphorylation-mimicking T495D mutant eNOS (Fleming *et al.*, 2001) suggests that the negative charge introduced by Thr495 phosphorylation indeed decreases CaM binding.

Implications for isozyme-specific NOS activation by CaM

The crystallographic structure of CaM bound to the eNOS-derived peptide described here not only provides insights into the regulatory mechanisms unique to eNOS, but also is the first step towards elucidating a structural basis for NOS isozyme-specific differences in the Ca^{2+} dependence of CaM binding. Recent studies suggest that the iNOS CaM-binding region is necessary, but not sufficient for Ca^{2+} -independent iNOS activity (Ruan *et al.*, 1996; Venema *et al.*, 1996; Lee and Stull, 1998). Synthetic peptides corresponding to the iNOS CaM-binding region generally have higher affinity ($<10^{-10}$ – 10^{-9} M) for Ca^{2+} -loaded CaM than do those derived from the eNOS or nNOS CaM-binding region (10^{-9} – 10^{-8} M) (Vorherr *et al.*, 1993; Zhang and Vogel, 1994; Anagli *et al.*, 1995; Venema *et al.*, 1996; Zoche *et al.*, 1996; Matsubara *et al.*, 1997; Yuan *et al.*, 1998; Leclerc *et al.*, 1999; Censarek *et al.*, 2002). Chimeric nNOS or eNOS containing the iNOS CaM-binding region shows the decreased Ca^{2+} sensitivity (Ruan *et al.*, 1996; Venema *et al.*, 1996), suggesting that the CaM-binding region itself contributes to the Ca^{2+} -independent iNOS activation by CaM. Furthermore, several studies report that synthetic peptides corresponding to the iNOS CaM-binding region, unlike the eNOS and nNOS counterparts, form complexes with CaM in the absence of Ca^{2+} (Anagli *et al.*, 1995; Zoche *et al.*, 1996; Matsubara *et al.*, 1997; Yuan *et al.*, 1998).

In light of the binding mode and secondary structure of the bound eNOS peptide observed in our crystallographic structure, we propose several sequence features unique to the iNOS CaM-binding region that may be responsible for its high, subnanomolar affinity for Ca^{2+} -loaded CaM and/or for its ability to bind apo-CaM. The sequence alignment indicates that, in addition to the four key hydrophobic residues present among all three isozymes, iNOS contains the largest number of hydrophobic residues within a region corresponding to the eNOS peptide used in this study (Figure 5A). Thus, CaM binding mediated primarily by hydrophobic interactions can be strengthened by the increased number of hydrophobic residues in iNOS.

Fig. 5. Sequence comparisons of CaM-binding domains from the three NOS isoforms. (A) Sequence alignment of the CaM recognition sites of NOS isoforms from different species. Acidic residues are shown in red, and basic residues in blue. Residues corresponding to the four key hydrophobic residues of the '1-5-8-14' motif involved in classical CaM binding are highlighted in pink. Secondary structures of the eNOS peptide used in this study are shown in schematic drawings. Abbreviations used are HUM (human), BOV (bovine) and MUR (murine). GenBank accession numbers for the sequences shown are: human eNOS, D26607; bovine eNOS, M89952; human nNOS, D16408; human iNOS, U31511; murine iNOS, M84373; rat iNOS, D14051. (B) Helical wheel representations of the α -helical portion and N-capping Thr495 from the eNOS-derived peptide and of the corresponding residues from nNOS and iNOS. eNOS and nNOS have similar distribution patterns of charged or polar residues (open circles) and hydrophobic or non-polar residues (filled circles). Among species, sequence variations in iNOS give mixed residue characteristics at some positions (gray circles). Hydrophobic residues in iNOS replace some charged residues in eNOS and nNOS.

When the core α -helical portion of the eNOS CaM-binding region and corresponding residues from nNOS and iNOS are compared using helical wheel analyses, the iNOS sequence shows distinct distribution patterns of hydrophobic and polar/charged residues compared with eNOS and nNOS (Figure 5B). Ca^{2+} -dependent nNOS strikingly resembles eNOS in that it shows a tendency to form an amphipathic helix with hydrophobic and charged/polar residues distributed on different faces of the helix; in contrast, hydrophobic residues are distributed over a larger face of a putative α -helix in iNOS (Figure 5B). In particular, iNOS has hydrophobic residues, such as Val517 and Leu523 (in human), in place of solvent-exposed, charged residues in eNOS (Glu498 and Lys504) (Figures 4C and 5) and nNOS (Lys738 and Lys744) at positions 3 and 9 (Figure 5). Analysis of the structure of the eNOS peptide bound to CaM suggests two possible structural implications from these additional hydrophobic residues in the iNOS CaM-binding region: (i) more favorable van der Waals contacts with CaM; and (ii) structural arrangements to minimize unfavorable solvent exposure of these hydrophobic residues. Both consequences would favor tighter association between CaM and the iNOS CaM-binding region. Furthermore, recent studies reported that mutations of nNOS Lys738 (position 3) and Lys744 (position 9) to hydrophobic residues increased the apparent binding affinity for CaM (Censarek *et al.*, 2002). Interestingly, like the iNOS CaM-binding region, a few other '1-5-8-14' CaM target sites (Crivici and Ikura, 1995) have additional hydrophobic residues at position 3 and 9. Mastoporan, for instance, is a 14 residue wasp venom peptide (INLKALAALAKKIL), which binds tightly to Ca^{2+} -loaded CaM with a 1:1

stoichiometry at 10^{-10} M affinity (Malencik and Anderson, 1983). However, possibilities remain that the binding mode of the eNOS peptide observed in this study may not be equally applicable to other NOS isoforms due to a low sequence homology among NOS isoforms immediately upstream of the eNOS CaM-binding region used in this study (Figure 5A). Whether a longer recognition site is involved in CaM binding to other NOS isoforms and how other regions outside of the CaM-binding region interact with CaM to achieve Ca^{2+} -independent activity (Ruan *et al.*, 1996; Venema *et al.*, 1996; Lee and Stull, 1998) await structural determination of other NOS domains in complex with CaM.

Conclusions

We have determined and analyzed the crystallographic structure of CaM bound to the 20 residue peptide corresponding to the eNOS CaM-binding region. The α -helical eNOS peptide binds in the classical antiparallel orientation with respect to the terminal lobes of CaM through extensive hydrophobic interactions. Unlike other peptides with similar classical sequence motifs, the eNOS peptide forms the shortest α -helix, induces different tertiary conformations of CaM and interacts tightly with the CaM central linker through Met76. The eNOS Phe496 side chain orients the CaM C-terminal lobe, allowing CaM Met144 and Met145 to interact extensively with the N-terminal half of the peptide. Finally, based on the secondary structure and binding mode of the eNOS peptide reported here, we propose that several hydrophobic residues present only in iNOS contribute to the higher affinity of the iNOS CaM-binding region for CaM.

Materials and methods

Protein expression and crystallization

Rat CaM cDNA cloned into the pET9a expression vector (Novagen) was a kind gift from Madeline A. Shea (University of Iowa, IA). Recombinant selenomethionine (SeMet)-substituted CaM was expressed in the *Escherichia coli* methionine auxotroph strain B834(DE3) (Novagen), and was purified as described previously for the native protein (Pedigo and Shea, 1995). Incorporation of nine SeMet residues per molecule in the final homogeneous protein was confirmed by electrospray mass spectrometry analysis. A 20 residue peptide, RKKTFKEVANAVKI-SASLMG, corresponding to the human eNOS CaM-binding region (residues 492–511), previously shown to be highly soluble, and thus suitable for structural studies (Matsubara *et al.*, 1997), was synthesized at the Protein/Nucleic Acids Core Facilities (The Scripps Research Institute, CA). All crystallization experiments were performed using the hanging-drop vapor diffusion method at room temperature. A 20 μ l aliquot of the SeMet CaM protein (23 mg/ml) in 20 mM Tris-HCl pH 7.5, 1 mM dithiothreitol was mixed with 7 μ l of the 20 amino acid eNOS peptide (5 mM) in the presence of 5 mM CaCl₂. An aliquot of 1–2 μ l of the CaM-bound peptide was mixed with an equal volume of reservoir solution containing 25–30% (w/v) polyethylene glycol (PEG) 8000, 0.3–0.4 M ammonium sulfate, giving a final pH of 4.5–5.0.

Data collection and structural determination

Table I summarizes statistics for crystallographic diffraction data collection and structural refinement. The data were collected from a flash-cooled crystal (100 K) at beamline 9-2 (Stanford Synchrotron Radiation Laboratory, CA). The cryoprotectant solution consisted of the equilibrated crystallization solution [30% (w/v) PEG 8000, 0.4 M ammonium sulfate] with 30% (v/v) ethylene glycol. Diffraction data collected at three different wavelengths were processed with DENZO and SCALEPACK (Otwinowski and Minor, 1997) (Table I). Twenty-one Se positions were identified by SOLVE (Terwilliger and Berendzen, 1999) and were refined using SHARP (de La Fortelle and Bricogne, 1997). The experimental multiple wavelength anomalous diffraction electron density map after density modification (CCP4, 1994) was used to build the initial model, which included Thr5–Met76 and Glu82–Ala147 of CaM, and Lys494–Leu509 of the bound eNOS peptide.

Programs CNS (Brünger *et al.*, 1998) and XFIT (McRee, 1999) were used for structure refinement and model building, respectively. The flexible central linker of CaM (residues 76–81) and water molecules were modeled based on standard $2F_o - F_c$ and $F_o - F_c$ maps after cycles of minimization and simulated annealing refinement followed by *B*-factor refinement. Stereochemistry of the final model was verified by PROCHECK (Laskowski *et al.*, 1993). PDBFIT (McRee, 1999) was used to obtain superposition of the crystallographic models and r.m.s.ds among different models. The coordinates for the final structure reported have been deposited to the Protein Data Bank (PDB) with the accession code 1N1W.

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