Characterization of the Ca^{2+}-dependent and -independent interactions between calmodulin and its binding domain of inducible nitric oxide synthase

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Abstract Most interactions of calmodulin (CaM) with its target proteins are Ca^{2+}-dependent, but a few Ca^{2+}-independent CaM-target protein interactions have been identified. One example is the inducible isoform of nitric oxide synthase (iNOS) expressed in macrophages. We describe here the characterization of the Ca^{2+}-independent interaction between CaM and a synthetic peptide corresponding to the CaM-binding domain of murine macrophage iNOS using circular dichroism (CD) spectroscopy. The CD spectrum of free iNOS peptide indicated a β-sheet conformation. The interaction of iNOS peptide with apo-CaM in the absence of Ca^{2+} resulted in the peptide acquiring a type II β-turn structure. This is in contrast to the situation in the presence of Ca^{2+} in which case the peptide acquired an α-helical conformation upon interaction with CaM, i.e. similar to the Ca^{2+}-dependent interactions of CaM with numerous targets such as myosin light chain kinase (MLCK). Consistent with this similar structural change, iNOS peptide inhibited the Ca^{2+}-CaM-dependent activation of smooth muscle MLCK by competing with MLCK for binding to Ca^{2+}-CaM. The K_d of Ca^{2+}-CaM for iNOS peptide was calculated from competition assays to be 0.3 nM. These results indicate that the structure of the CaM-binding domain of iNOS is quite different when bound to apo-CaM than Ca^{2+}-CaM.

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Key words: Calmodulin; Nitric oxide synthase; Myosin light chain kinase; Circular dichroism; Ca^{2+}

1. Introduction

Calmodulin (CaM), the ubiquitous Ca^{2+}-binding regulatory protein, binds to and activates more than 40 different target proteins, including protein kinases, protein phosphatase 2B (calcineurin), nitric oxide synthases, cyclic nucleotide phosphodiesterase, ion channels, and myofilament-associated proteins such as caldesmon and calponin (for reviews, see [1–4]). In the absence or presence of four Ca^{2+} ions, CaM adopts a dumbbell-shaped structure with two lobes connected by a 26-residue central linker region [5–7]. Each lobe of CaM contains two helix-loop-helix Ca^{2+}-binding motifs connected by a small β-sheet. Although the central linker region of Ca^{2+}-CaM is an α-helix in the X-ray structure [5], nuclear magnetic resonance (NMR) relaxation measurements indicated that the central

part of the linker region is unstructured in solution [8]. The majority of hydrophobic residues in apo-CaM are buried inside the protein [6,7]. Upon binding four Ca^{2+} ions, CaM experiences significant movements among the α-helices in each lobe and the interhelix angle changes significantly. As a result, Ca^{2+}-CaM exposes two Met-rich hydrophobic patches, one on each lobe of CaM, which are involved in target protein binding [5]. The CaM-binding domains in target proteins usually comprise a stretch of ~20 amino acid residues which have the potential to form a basic amphipathic α-helix [3,9]. The high resolution structure of Ca^{2+}-CaM-target peptide complexes revealed that the peptides bind to Ca^{2+}-CaM as α-helices and the side-chain hydrophobic interactions between peptide and Ca^{2+}-CaM constitute the dominant force in the binding [10–12].

It is generally believed that the flexible central linker region and the two Met-rich hydrophobic patches are important features for CaM's versatility. The flexible central linker region can unwind to different extents to accommodate various target proteins, while the Met residues provide a highly adjustable hydrophobic surface for distinct target protein binding due to the intrinsic flexibility of the Met residues and the high polarizability of sulfur atoms [13].

While most CaM-target protein interactions are Ca^{2+}-dependent, a few examples of Ca^{2+}-independent binding of CaM to specific proteins have been discovered. For instance, one of the isoforms of nitric oxide synthase (NOS) binds CaM in a Ca^{2+}-independent manner. NOS catalyzes the synthesis of the messenger molecule NO from L-arginine [14,15]. NO has many physiological and pathological effects including smooth muscle relaxation, neurotransmission and cytotoxicity. NOS can be divided into constitutive and inducible forms (cNOS and iNOS, respectively). Neuronal (type I) and endothelial (type III) cNOS are activated by CaM in response to agonist-induced increases in intracellular free Ca^{2+} concentration, i.e. they behave like classical Ca^{2+}-CaM-regulated enzymes. In contrast, macrophage (type II) iNOS, whose expression is induced by cytokines and bacterial endotoxin, remains active independent of Ca^{2+} concentration; furthermore, CaM binds tightly to iNos even in the absence of Ca^{2+} [16]. Both cNOS and iNOS have similar structural features including a cytochrome P-450-like heme protein domain and a cytochrome P-450 reductase-like flavoprotein domain connected by a CaM-binding domain [17]. The CaM-binding domains of cNOS and iNOS were identified by sequence inspection and predicted secondary structure (basic amphipathic α-helix) and correspond to residues 725–754 of rat brain cNOS [18,19] and residues 504–532 of murine macrophage iNOS [20].

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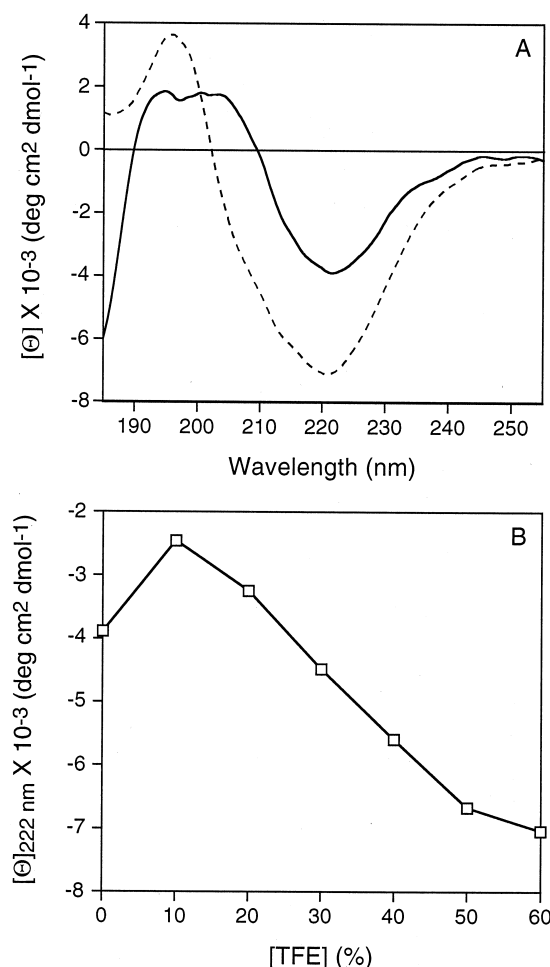E-mail: walsh@acs.ucalgary.ca

Fig. 1. Effect of trifluoroethanol on the CD spectrum of iNOS peptide. A: The CD spectrum of iNOS peptide alone was recorded in aqueous solution (thick line) or in 60% TFE (dashed line). B: The effect of increasing [TFE] on mean residue ellipticity at 222 nm; values were determined from CD spectra of iNOS peptide alone recorded at the indicated concentrations of TFE.

A useful approach to studying the mechanism of activation of target enzymes by CaM involves the use of synthetic peptides corresponding to the CaM-binding domains of the target proteins. To date, relatively little is known about CaM-target protein interactions that occur independent of Ca^{2+}. We report here the characterization of the interaction between a 27-residue synthetic peptide corresponding to the CaM-binding domain of iNOS and apo-CaM or Ca^{2+}-CaM.

2. Materials and methods

2.1. Materials

$[\gamma^{-32}P]ATP$ (>5000 Ci/mmol) was purchased from Amersham Corp. (Oakville, Ontario, Canada). CaM was purified from bovine brain [21] or was expressed in *E. coli* and purified as described by Zhang and Vogel [22]. Myosin light chain kinase (MLCK) was purified from chicken gizzard smooth muscle [23]. iNOS peptide was synthesized by the Protein and DNA Synthesis Facility at Queen's University, Kingston, Ontario, Canada. MLC_{11-23} peptide, used as substrate for MLCK, was synthesized by the Peptide Synthesis Core Facility at the University of Calgary, Calgary, Alberta, Canada. The purity of peptides was confirmed by analytical high performance liquid chromatography, amino acid analysis and mass spectrometry. The sequences of these peptides are (i) MLC_{11-23}: KKRPQRATSNV-

FA, corresponding to residues 11–23 of the 20 kDa light chain subunit of chicken gizzard smooth muscle myosin II, Ser-19 being the site of phosphorylation by MLCK [24]; (ii) iNOS peptide: RRREIRFRVLVKVVFFASMLMRKVMAS, corresponding to the CaM-binding domain of murine macrophage iNOS (residues 503–529) [25,26].

2.2. CD spectroscopy

CD spectra were acquired on a Jasco J-715 spectropolarimeter in the Department of Chemistry, University of Calgary, Calgary, Alberta, Canada. All experiments were performed at room temperature (22°C) using a 1 mm path length cylindrical quartz cuvette. The parameters used were as follows: 0.2 nm step resolution, 50 nm/min speed, 2 s response time, 1 nm bandwidth. The sensitivity is 20 mdeg and spectra were averaged from 10 scans. The CaM or iNOS peptide concentration was 10 μM in 10 mM Tris-HCl, pH 7.2, with 2 mM $CaCl_2$ or 2 mM EDTA, and a total sample volume of 200 μl. The background signals due to the buffer were subtracted from each spectrum, which was then smoothed and converted to molar ellipticity using Jasco software. The CD spectra were reported either as molar ellipticity or as mean residue molar ellipticity.

2.3. MLCK assays

MLCK activity was measured at 30°C under the following conditions: 20 nM Tris-HCl, pH 7.5, 60 mM KCl, 4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM $[\gamma^{-32}P]ATP$ (~300 cpm/pmol), 0.1 mM MLC_{11-23}, 0–400 nM CaM, 10 nM MLCK and varying concentrations of iNOS peptide. reactions (30 μl) were started by addition of ATP and quenched after 5 min by spotting 20 μl onto P81 phosphocellulose paper squares (Whatman) which were washed and counted as previously described [27]. Phosphate incorporation into MLC_{11-23} was shown to be linear for at least 5 min. Maximal MLCK activity observed at 400 nM CaM was 0.44 ± 0.16 μmol P_i incorporated/min mg MLCK (mean $\pm$ S.D. of 28 independent measurements, each carried out in triplicate). Basal MLCK activity in the absence of CaM was 0.08 ± 0.03 μmol P_i incorporated/min mg MLCK (mean $\pm$ S.D. of 30 independent measurements, each carried out in triplicate).

The dissociation constant (K_d) of CaM for iNOS peptide was calculated from the midpoints of the activation curves of MLCK by CaM in the absence and presence of a known concentration of peptide using the following equation [28]:

$$K_d = \frac{[P_t] + K - [CaM]K}{[CaM] - K}$$

where $[P_t]$ is the total concentration of peptide added, K and [CaM] are the concentrations of CaM required to reach half-maximal activation in the absence and presence of peptide, respectively.

3. Results

3.1. CD spectroscopy

The structure of the free iNOS peptide was examined initially by CD spectroscopy, and the effects of increasing concentrations of trifluoroethanol (TFE) on the CD spectrum were analyzed. TFE induces an α-helical conformation in peptides that have a tendency to form this structure, which is the case for many CaM-binding domain peptides [1,3]. The CD spectrum of iNOS peptide in aqueous solution (no TFE) is indicative of a β-sheet conformation in equilibrium with a random coil conformation (Fig. 1A). TFE titration of the iNOS peptide in aqueous solution induced an initial increase followed by a decrease of the ellipticity at 222 nm which was incomplete even at a TFE concentration of 60% (Fig. 1B). The CD spectrum of iNOS peptide in 60% TFE is still similar to a β-sheet conformation with a positive band around 197 nm and a negative band around 220 nm (Fig. 1A). This structure contrasts with classical CaM-binding domain peptides which display an α-helical conformation, particularly in the presence of TFE [1,3,29].

Since CaM binds tightly to iNOS even in the absence of

Ca^{2+} [16], we studied the apo-CaM-iNOS peptide interaction by CD spectroscopy. As shown in Fig. 2A, addition of the iNOS peptide increased the molar ellipticity of apo-CaM at 208 nm and decreased that at 222 nm. The resulting difference spectrum (Fig. 2B) shows a typical type II β-turn CD spectrum with a positive band between 200–210 nm and a weak negative band between 220–230 nm [30,31]; other β-sheet and β-hairpin structures may, however, result in similar spectra so that such structures cannot be totally excluded [30,31]. Since apo-CaM usually maintains its secondary structure in the apo-CaM-peptide complex [32], the difference CD spectrum can be assigned to the bound peptide. As indicated earlier, the free iNOS peptide in aqueous solution probably adopts a β-sheet conformation in equilibrium with a random coil conformation. There is a clear conformational change induced in the iNOS peptide when it binds to apo-CaM (Fig. 2B) and, most significantly, the peptide does not acquire an α-helical conformation as a result of this Ca^{2+}-independent interaction.

The secondary structure changes occurring upon formation of the Ca^{2+}-CaM-iNOS peptide complex were also examined by CD spectroscopy (Fig. 3). Addition of iNOS peptide to

Fig. 3. Far-UV CD spectra of iNOS peptide, Ca^{2+}-CaM and the Ca^{2+}-CaM-iNOS peptide complex. CD spectra were recorded as described in Section 2 for iNOS peptide alone (dash-dot line), Ca^{2+}-CaM alone (thick line) or an equimolar mixture of iNOS peptide and Ca^{2+}-CaM (dashed line).

CaM in the presence of Ca^{2+} decreased the molar ellipticity at both 208 nm and 222 nm. Since Ca^{2+}-CaM usually does not gain any secondary structure upon interaction with a target peptide [10,11], this ellipticity change can be attributed to the iNOS peptide which binds to Ca^{2+}-CaM in an α-helical conformation. The structural changes occurring in the iNOS peptide as it binds to apo-CaM and Ca^{2+}-CaM are, therefore, quite distinct.

3.2. Effect of iNOS peptide on Ca^{2+}-CaM-dependent activation of MLCK

The affinity of Ca^{2+}-CaM for iNOS peptide was determined by a competition assay with MLCK, a target enzyme which is activated by CaM in a Ca^{2+}-dependent manner [33]. Fig. 4 shows the effect of increasing concentrations of iNOS peptide on MLCK activity at a fixed concentration of CaM (100 nM) sufficient for maximal activation of the kinase. Half-maximal inhibition was observed at ∼60 nM iNOS peptide. Inhibition of MLCK by 60 nM iNOS peptide could be overcome by increasing the CaM concentration (Fig. 5). A higher concentration of iNOS peptide (1 μM) completely prevented activation of MLCK over the range 0–400 nM CaM (Fig. 5). The K_d value of Ca^{2+}-CaM for iNOS peptide was determined from the CaM activation curves in the absence and presence of 60 nM iNOS peptide to be 0.3 nM.

4. Discussion

Previous studies of synthetic peptides corresponding to residues 499–532 and 504–532 of murine macrophage iNOS indicated this region to be the CaM-binding domain [20]. We have confirmed this conclusion by CD spectroscopy of a synthetic peptide corresponding to residues 503–529 of iNOS and by the demonstration that this peptide inhibits the Ca^{2+}-CaM-dependent activation of smooth muscle MLCK in a manner which is overcome by Ca^{2+}-CaM. Competition assays with MLCK enabled determination of the K_d of Ca^{2+}-CaM for iNOS peptide to be 0.3 nM, comparable to values reported previously using similar iNOS peptides but different methods: < 0.1 nM [34], < 1.0 nM [20], 1.5 nM [35] and 3.3 nM [36].
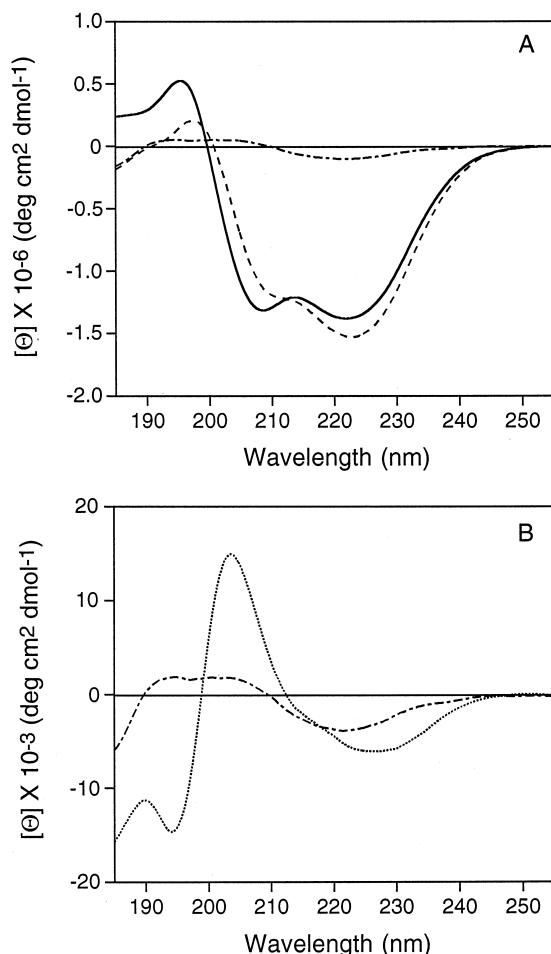

Fig. 2. A. Far-UV CD spectra of iNOS peptide, apo-CaM and the apo-CaM-iNOS peptide complex. CD spectra were recorded as described in Section 2 for iNOS peptide alone (dash-dot line), apo-CaM alone (thick line) or an equimolar mixture of iNOS peptide and apo-CaM (dashed line). B: Far-UV CD spectra of iNOS peptide (dash-dot line) and the difference spectrum between apo-CaM-iNOS peptide complex and apo-CaM (dotted line).
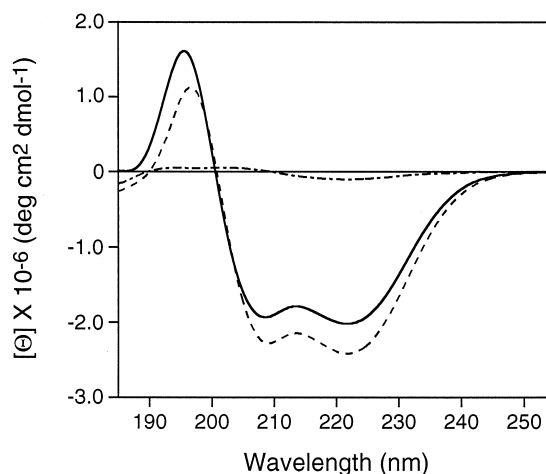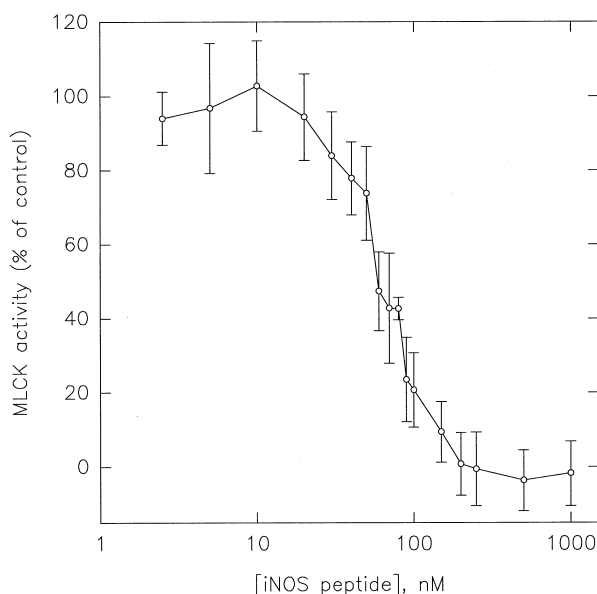
Fig. 4. iNOS peptide-mediated inhibition of Ca^{2+}-CaM-dependent MLCK. MLCK activity was measured in the presence of the indicated concentrations of iNOS peptide. 100% activity refers to the kinase activity in the absence of iNOS peptide.
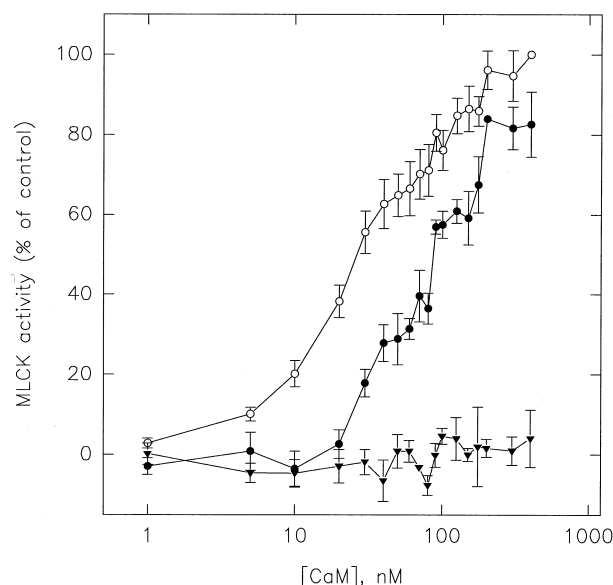

Fig. 5. Activation of MLCK by Ca^{2+}-CaM in the absence and presence of iNOS peptide. MLCK activity was measured in the presence of the indicated concentrations of CaM and in the absence (○) or presence of 60 nM (●) or 1 μM (▼) iNOS peptide. The K_d for iNOS peptide binding to Ca^{2+}-CaM was calculated using the following values: K = 26.7 nM (calculated from the CaM activation curve in the absence of iNOS peptide), [CaM] = 86 nM (calculated from the CaM activation curve in the presence of 60 nM iNOS peptide) and $[P_t] = 60$ nM.

The peptide most closely related to the one used in this study was that of Zoche et al. [34] which lacked the C-terminal Ser.

We have utilized CD spectroscopy to analyze the interaction between the CaM-binding domain of iNOS and either Ca^{2+}-CaM or apo-CaM. Our results show that the iNOS peptide binds to Ca^{2+}-CaM in an α-helical conformation. This is a normal binding mode for the CaM-binding domains of CaM target proteins [1–4]. Since the CaM-binding domain peptide of MLCK also binds to Ca^{2+}-CaM with an α-helical conformation [10,11], iNOS peptide may compete with MLCK binding to Ca^{2+}-CaM, and this is supported by the demonstration that iNOS peptide inhibits MLCK and inhibition is overcome by increasing [CaM]. A similar iNOS peptide inhibited the Ca^{2+}-CaM-activated erythrocyte plasma membrane Ca^{2+} pump [20]. The iNOS CaM-binding domain retains the three aromatic or long-chain hydrophobic side chains that form key contacts with CaM in the Ca^{2+}-CaM-MLCK peptide complex (Fig. 6). Although hydrophobic residues appear to be more important than charged residues in binding to CaM, basic residues are invariably found in CaM-binding domains and many of them contribute to CaM binding via electrostatic interactions between basic residues in the CaM-binding protein and acidic regions in CaM. All seven basic residues in the CaM-binding domain of MLCK make salt bridges with CaM [11]. Although only one of these is conserved in the iNOS CaM-binding domain (as an Arg for Lys substitution; shaded in Fig. 6), the iNOS peptide contains eight basic residues and only one acidic residue for a net charge of +7 (Fig. 6). Interestingly, iNOS peptide is predominantly in a β-sheet conformation in aqueous solution (Fig. 1) and it becomes α-helical when bound to Ca^{2+}-CaM (Fig. 3), i.e. a β-sheet to α-helix transition is induced in iNOS peptide by Ca^{2+}-CaM binding. In contrast, for cNOS the CaM-binding domain has a random structure in aqueous solution, but it forms an α-helix in TFE or upon binding to Ca^{2+}-CaM [37,38]. In contrast to iNOS (see below) the cNOS peptide also does not bind to apo-CaM, showing that the CaM-bind-

ing domains of iNOS and cNOS have markedly distinct conformational properties, which are related to the different activation properties of both enzymes.

CD spectroscopy also indicated that the iNOS peptide binds to apo-CaM (Fig. 2), which is consistent with previous findings that the iNOS peptide interacts with CaM even in the presence of EDTA [34,39]. Most interestingly, the iNOS peptide binds to apo-CaM in a type II β-turn conformation (Fig. 2B), i.e. quite different from the α-helical conformation the peptide acquires upon binding to Ca^{2+}-CaM. This conformation is also quite different from the β-sheet conformation adopted by iNOS peptide in aqueous solution (Fig. 2B). A recent study utilizing CD and NMR spectroscopy showed that a shorter, modified iNOS peptide analog (KRREIPLKVLV-KAVLFACMLMRK) assumes a nascent α-helical structure in aqueous solution and binds to both apo-CaM and Ca^{2+}-CaM in an α-helical conformation [36]. This peptide was used because of its greater solubility and since longer iNOS peptides caused formation of large aggregates when added to CaM, leading to precipitation of the peptide-CaM complex [20,39]. The Agadir secondary structure prediction program

iNOS   R R R E I R [F] R V L V K V [V] F F A S M [E] M R K V M A S

MLCK   Y M A R R K [W] Q K T G H A [V] R A I G R [L] S S M A M I S

Fig. 6. Comparison of the amino acid sequences of the CaM-binding domains of iNOS and MLCK. The iNOS peptide sequence corresponds to residues 503–529 of murine macrophage iNOS [25,26] and the MLCK sequence to residues 794–820 of chicken gizzard MLCK [48]. Boxed residues indicate the three hydrophobic amino acids that have been implicated in the binding of MLCK to CaM and the corresponding conserved residues in the CaM-binding domain of iNOS. Shaded residues indicate the location of the conserved positive charge.

which has been specifically developed for peptides rather than proteins (available on the web site of The European Molecular Biology Laboratory) [40] predicted the modified iNOS peptide analog of Matsubara et al. [36] to have $\sim 12\%$ α-helical content while the unmodified iNOS peptide used in this study is predicted to have an α-helical content of only $\sim 4\%$. Thus, the substitutions in the modified iNOS peptide likely change its structural properties; hence, caution must be exercised when extending the results obtained with the modified peptide to the native protein. We observed no solubility or aggregation problems with the native iNOS peptide at the concentrations used in these studies, but were unable to carry out near-UV CD spectroscopy, infrared or NMR spectroscopy studies due to such problems at the higher concentrations required to successfully apply the latter techniques.

The region outside the putative CaM-binding domain of iNOS (residues 501–532) has been implicated in the Ca^{2+}-independent binding of iNOS to CaM [35,41]. Further studies with longer peptides or intact protein are required to elucidate the detailed interaction between CaM and iNOS. Nevertheless, our studies are insightful in understanding the interaction between CaM and a target protein that binds to CaM in the absence and presence of Ca^{2+}. Other such target enzymes include a cyclic nucleotide phosphodiesterase isoenzyme, PDE1A1 [42], the γ subunit of glycogen phosphorylase kinase [43] and adenylyl cyclase of *Bordetella pertussis* [44].

To date, the only peptide that binds to apo-CaM and has been well characterized is the CaM-binding domain of neuromodulin. The neuromodulin peptide binds to apo-CaM as an α-helix, as demonstrated by NMR spectroscopy [45,46] and CD spectroscopy [47]. It has also been shown that the general secondary structure of apo-CaM is maintained in this complex [32]. Thus, our study with iNOS peptide presents a novel peptide-apo-CaM interaction in which a type II β-turn rather than an α-helix is formed.

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