Characterization of the Calmodulin-binding Domain of Rat Cerebellar Nitric Oxide Synthase*

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Nitrergic oxide (NO) has recently been identified as an intercellular messenger which is involved in the regulation of neurotransmission, vasorelaxation, and cytokinesis. In cerebellum and endothelium this compound is synthesized by "constitutive" nitric oxide synthases (NOS); these are Ca2+-calmodulin (CaM)-dependent enzymes. A potential CaM-binding domain for the CaM-dependent NOS has previously been identified in the gene sequence. In this work, a synthetic 23 residue peptide encompassing the putative CaM-binding domain of rat cerebellar NOS was studied. The constitutive NOS peptide binds to CaM in a calcium-dependent manner with 1:1 stoichiometry as determined by polyacrylamide gel electrophoresis of the peptide-CaM complex in 4 M urea. Circular dichroism studies showed that the peptide binds to CaM in an α-helical conformation. Binding of the constitutive NOS peptide inhibits the stimulatory effect of CaM on cyclic nucleotide phosphodiesterase. From competition experiments between the peptide and phosphodiesterase we have determined a Kd of 2.2 μM for the peptide-CaM complex. Two-dimensional NMR and circular dichroism studies were used to determine the structure of the peptide in aqueous solution. In addition, the effect of increasing amounts of trifluoroethanol on the peptide structure was investigated. It was found that the peptide can adopt an α-helical structure which bears close resemblance to the structure of the CaM-bound form of the CaM-binding domains of myosin light chain kinases.

The enzyme nitric oxide synthase (NOS) catalyzes the formation of one of the major intercellular messengers, nitric oxide (NO), from L-arginine (for reviews, see Nathan, 1992; Lowenstein and Snyder, 1992). There appear to be two principal isoforms of this enzyme which differ in the way their activity is regulated. The activity of the constitutive NOS which is localized in neurons (Schmidt et al., 1989; Breit et al., 1991) and endothelium (Pollock et al., 1991) is regulated by calcium through the ubiquitous regulatory Ca2+-binding protein calmodulin (CaM). The inducible NOS is a Ca2+-independent enzyme and it is present in immune cells such as macrophages and neutrophils (Stuehr et al., 1991; Xie et al., 1992); the quantity of this isozyme massively increases after the cells are exposed to a stimulus such as lipopolysaccharide and γ-interferon (Nathan and Hibs, 1991). Thus the activity of the inducible NOS appears to be mainly regulated by gene transcription, while the noninducible enzyme is mainly regulated via Ca2+-CaM.

The absolute requirement of the constitutive NOS for Ca2+-CaM (Bredt and Snyder, 1990) links two major messengers, notably Ca2+ and NO. The amino acid sequence of a number of cNOS enzymes has been determined from their gene sequences (Bredt et al., 1991; Janssens et al., 1992; Nishida et al., 1992; Sessa et al., 1992). All the constitutive NOS enzymes have a conserved stretch of basic and hydrophobic amino acids as seen in other CaM-binding domains, and hence this region has been proposed to be the CaM-binding domain of cNOS. Further characterization of this proposed CaM-binding domain in cNOS would help us to understand how the activity of the constitutive NOS is regulated by Ca2+-CaM.

In this work, we have studied a synthetic 23-residue peptide encompassing the proposed CaM-binding domain of constitutive NOS from rat cerebellum (Breit et al., 1991). We have shown that the peptide forms a 1:1 complex with CaM in a calcium-dependent manner. We have also determined the affinity of the peptide for CaM. Furthermore, using 2D 1H NMR and CD spectroscopy, we have found that the peptide is capable of forming an α-helical structure. This structure will be compared with the CaM-binding domains of myosin light chain kinases (Ikura et al., 1992; Meador et al., 1992).

MATERIALS AND METHODS

The 23-residue cNOS peptide, KRRAIGFKKLAEAVFPSKLMGQ, which corresponds to the amino acid sequence of residues 725–747 in rat cerebellar NOS (Breit et al., 1991) was obtained commercially from the protein and DNA synthesis facility at Queen’s University, Kingston, Ontario, Canada. The peptide was judged to be >95% pure by high performance liquid chromatography and amino acid analysis and was used without further purification. Bovine brain cyclic nucleotide phosphodiesterase (PDE) was a generous gift from Dr. Jerry Wang, University of Calgary. Bovine CaM was expressed and purified from a synthetic gene in Escherichia coli as described elsewhere (Zhang and Vogel, 1993a). All other chemicals were purchased from Sigma.

Urea-polyacrylamide gel electrophoresis was performed following the procedure described by Erickson-Vitanen and Degrado (1987). Inhibition of the CaM-dependent activation of PDE by the peptide was assayed colorimetrically by determining the amount of phosphate which was released from cyclic AMP as described by Sharma and Wang (1978). The reaction mixture was incubated at pH 8.0 for 30 min at 30 °C in 4 ml of reaction buffer containing 20 mM Tris, 2 mM imidazole, 3 mM MgCl2, 0.1 mM CaCl2, 18 mM of CaM, 0.4 nmol of PDE, with variable quantities of the peptide. Then, CAMP (final concentration of 1 μM) was added to initiate the reaction. At 3-min intervals, 450-μl aliquots were withdrawn and mixed with 50 μl of 5% trichloroacetic acid to stop the reaction. The PDE activity was calculated from the slope of the curve giving the time course of the amount of phosphate released.

CD spectra of the peptide were recorded at 15 °C on a Jasco J-500C spectrometer.
CD spectropolarimeter using a cell pathlength of 0.1 cm. The concentration of the peptide was 20 μM in 5 mM citric acid buffer, pH 5.0. Several samples with different ratios (v/v) of citric acid buffer and TFE were studied. CD spectra of CaM complexed with the cNOS peptide were measured at 20 °C in 5 mM Tris buffer, 0.5 mM CaCl₂, pH 7.2.

Two peptide samples, one in 90% H₂O, 10% D₂O and the other in 99.9% D₂O, were studied. CD spectra of CaM complexed with the cNOS peptide were measured at 20 °C in 5 mM Tris buffer, 0.5 mM CaCl₂, pH 7.2. Two peptide samples, one in 90% H₂O, 10% D₂O and the other in 99.9% D₂O, were prepared for NMR studies. The pH value for both samples was 5.0 unless otherwise indicated (direct meter reading), and the concentration of the samples was 4 mM. All NMR spectra were acquired on a Bruker AMX500 spectrometer equipped with an X32 computer. The sequence-specific assignment of the resonances was obtained from TOCSY (Bax and Davis, 1985) and NOESY (Bodenhausen et al., 1984) spectra which were acquired at 5 °C in the phase-sensitive mode using the time proportional phase increment technique. For the NOESY experiments, 512 free induction decays were collected with a sweep width of 5500 Hz and a mixing time of 250 ms. All spectra were processed using the Bruker UXnmr software package. The data were zero filled once in the F1 dimension and a sine-square window function was used in both dimensions before Fourier transformation. In addition, NOESY and TOCSY spectra were also obtained at 15 °C for a sample containing 3 mM peptide in 30% TFE, 70% H₂O, pH 5.0.

RESULTS

The CaM-binding Domain of cNOS Binds to CaM in a Calcium-dependent Manner with 1:1 Stoichiometry—The binding of the synthetic cNOS peptide encompassing amino acid residues 725-747 of rat cerebellar NOS was studied using polyacrylamide gel electrophoresis in 4 M urea. In this method CaM-peptide complexes are loaded at different ratios into the gel slots and subjected to electrophoresis. A band shift is observed.

Fig. 1. Polyacrylamide gel electrophoresis of CaM/cNOS peptide mixtures at different ratios. The peptide to CaM ratio from left to right is 0:1 (lane A); 0.5:1 (lane B); 0.8:1 (lane C); 2:1 (lane D); 3:1 (lane E); 0:1 (lane F); and 3:1 (lane G). The gel with lanes A–E was obtained in the presence of 0.1 mM Ca²⁺, while lanes F and G were obtained from a separate electrophoresis experiment which was performed in the presence of 2 mM EGTA.

![Fig. 1. Polyacrylamide gel electrophoresis of CaM/cNOS peptide mixtures at different ratios.](image)

![Fig. 2. Inhibition of CaM-dependent cyclic nucleotide phosphodiesterase by the cNOS peptide.](image)

![Fig. 3. A, sequential assignment of the cNOS peptide in aqueous solution at pH 5.0, 5 °C. The intraresidue NOE cross-peaks are indicated by their residue number. B, the amide region of the NOESY spectrum of the peptide. The interresidue amide NOE cross-peaks are labeled.](image)
peptide binds to CaM at a 1:1 ratio. We have also studied complex formation of the cNOS peptide with apoCaM. No band shift is observed under these conditions (Fig. 1, lanes F and G), suggesting that calcium is required for complex formation.

The cNOS Peptide Binds CaM with High Affinity and Inhibits the Ability of CaM to Stimulate the CaM-dependent Phosphodiesterase—To obtain further information about the affinity of the peptide for CaM, a competition experiment was carried out. The data presented in Fig. 2 show that the peptide can competitively inhibit the ability of CaM to stimulate the CaM-dependent PDE activity. This result also suggests that the cNOS peptide and the CaM-binding domain of PDE bind to the same regions on CaM. The competition experiment also allowed us to determine that the peptide has a dissociation constant, Kd, of ~2.2 nM, which was obtained by comparison with the known Kd (1 nm) for the CaM-PDE complex (Hansen and Beavo, 1986).

The CaM-binding Domain from cNOS Can Adopt an a-Helical Conformation in Aqueous Solution—2D 1H NMR spectroscopic techniques were used to analyze the structure of the peptide. To reduce the amide exchange rate and simultaneously increase the correlation time of the peptide, a pH value of 5.0 and a temperature of 5 °C were chosen for the NMR studies. Under these conditions, the peptide was soluble; since the appearance of the NMR spectra was not dependent on the concentration of the cNOS peptide, it remained a monomer in aqueous solution. We also observed that the peptide at pH 6.0 gave qualitatively the same spectra as at pH 5.0 (data not shown).

The assignment of the NMR resonances was obtained by following standard procedures for 2D 1H NMR of proteins (Wuthrich, 1986). TOCSY experiments of the peptide in 90% H2O, 10% D2O were used to identify the spin systems of the amino acid residues; and a NOESY experiment of the same sample served to trace out the sequential connectivities between them. The sequential assignment of the peptide is highlighted in Fig. 3a. The chemical shifts of the assigned protons are listed in Table I, a complete assignment could be obtained. A substantial number of NH/NH(i,i+1) NOE cross-peaks are observed for the peptide in aqueous solution (Fig. 3b), suggesting that the peptide may have an a-helical structure. Fig. 4 summarizes the short and medium range NOE cross-peaks observed for the peptide. The NOE pattern indicates that the peptide is capable of adopting an a-helical structure. The inset of Fig. 5 shows the CD spectra of the peptide in aqueous solution at pH 5.0. The ellipticity minimum at 208 nm, which is indicative of an a-helix, is observed at pH 5.0. The maximum a-helical content of the peptide was estimated to be ~60% (Chang et al., 1985; Smith et al., 1985; Erickson-Viitanen and Degrado, 1987).

The CD Spectroscopy of the CaM-cNOS Peptide Complex—Many authors have studied peptide binding to CaM with CD spectroscopy, which almost invariably showed that the peptides bound in an a-helical conformation to CaM (Giedroc et al., 1985; Cox et al., 1985; Erickson-Viitanen and Degrado, 1987). Fig. 6 shows a small increase in the negative ellipticity at 222 and 208 nm for the CaM-cNOS peptide complex. This result is similar to that obtained with other CaM-binding peptides, showing that the cNOS peptide also binds in an a-helical manner to CaM.

DISCUSSION

It has been reported by several research groups that the activation of the constitutive nitric oxide synthase is mediated by Ca2+-CaM (Bredt and Snyder, 1990; Schmidt et al., 1992). By searching for a basic, hydrophobic region in the enzyme, a
strategy originally suggested by O'Neil and DeGrado (1990), a CaM-binding domain in constitutive NOS was proposed. However, to date no direct experimental evidence has been presented to show that this proposed CaM-binding region in cerebellar NOS is indeed involved in the binding of this enzyme to CaM. Indeed, this criteria does not always indicate the correct CaM-binding domain as shown in the case of caldesmon, for example (Leszyk et al., 1989; Zhan et al., 1991). In the present study, a synthetic peptide corresponding to the proposed CaM-binding domain of rat cerebellar NOS (residues 725-747) was found to bind in a calcium-dependent manner to CaM with 1:1 stoichiometry. The peptide can also compete with PDE for CaM, which indicates that NOS and PDE share at least in part the same binding regions on CaM. From the results of the competition experiment between PDE and the cNOS peptide, a relative $K_d$ of $\approx$2.2 nm for the binding of the peptide to CaM was determined. This value is close to the $K_d$ determined for the binding of the intact enzyme to CaM which was found to be $\approx$3.5 nm (Schmidt et al., 1991). While this paper was under review, Vorherr et al. (1993) showed that a longer 30-residue peptide also bound to CaM at 1:1 stoichiometry with identical affinity. These data suggest that the shorter cNOS peptide studied here probably contains the complete CaM-binding domain of the cNOS enzyme.

Several CaM-binding peptides can form a "nascent" $\alpha$-helical structure in aqueous solution. Alternatively, such a structure can be induced or further stabilized by addition of the $\alpha$-helix promoting solvent TFE (O'Neil and DeGrado, 1990; Munier et al., 1993; Zhang and Vogel, 1993b). Our 2D NMR studies of the cNOS peptide show that the peptide is capable of adopting a structure with $\alpha$-helical turns in aqueous solution. The consecutive NN(i,i+1) NOE connectivities and the medium range NOEs observed between residues Ala$^{728}$ and Met$^{744}$ suggest that an $\alpha$-helix can be formed in this part of the structure. The CD spectrum of the peptide obtained in aqueous solution shows that no regular $\alpha$-helix can be detected. This observation, in combination with the NMR data obtained in this environment, suggests that only a small portion of the peptide adopts an $\alpha$-helical conformation at a given time. Furthermore, such a nascent helical structure may deviate from ideal geometry and/or the ends of the $\alpha$-helix can fray (Dyson et al., 1992; Manning et al., 1988). However, upon addition of TFE the presence of an ordered $\alpha$-helix is indicated by the CD as well as the NMR data. In particular, the change in the ratio of the intensities of the NN(i,i-1) and the NN(i,i+1) NOEs suggests that the contribution from extended structures to the observed NOEs becomes less, and that an ordered $\alpha$-helix is now formed. CD experiments of the CaM-cNOS peptide complex (see Fig. 6) show the characteristic increase in negative ellipticity which has been reported for numerous CaM-binding peptides (Gierdroc et al., 1983; Cox et al., 1985; Erickson-Viitanen and DeGrado, 1987), and which indicates that these peptides bind in an $\alpha$-helical conformation.

The $\alpha$-helical structure of the cNOS peptide as revealed in a helical wheel projection shows the typical basic, apolar, and charged residues which are presumably present but they could not be observed because of resonance overlap. The dashed lines for the $\alpha$-helix NOEs represent the additional connectivities observed for the cNOS peptide in 30% TFE. The dashed line in the NN(i,i+1) region refers to the strong NOEs observed for the cNOS peptide in 30% TFE. The figure also shows the alignment of the amino acid sequence of the cNOS peptide with the CaM-binding domain of smooth muscle myosin light chain kinase (smMLCK) and skeletal muscle MLCK (skMLCK). The key amino acid residues which are believed to be critical for the binding of CaM to MLCK are highlighted in the boxes, and they are numbered starting from the first hydrophobic residue.

**Fig. 4.** Summary of the NOEs observed for the cNOS peptide. The thickness of the lines corresponds to the intensity of the crosspeaks, which were classified as weak (--), medium (+), and strong (++) by comparing the contour levels. The open boxes represent the cross-peaks which are presumably present but they could not be observed because of resonance overlap. The dashed lines for the $\alpha$-helix NOEs represent the additional connectivities observed for the cNOS peptide in 30% TFE. The dashed line in the NN(i,i+1) region refers to the strong NOEs observed for the cNOS peptide in 30% TFE. The figure also shows the alignment of the amino acid sequence of the cNOS peptide with the CaM-binding domain of smooth muscle myosin light chain kinase (smMLCK) and skeletal muscle MLCK (skMLCK). The key amino acid residues which are believed to be critical for the binding of CaM to MLCK are highlighted in the boxes, and they are numbered starting from the first hydrophobic residue.

**Fig. 5.** Titration of the cNOS peptide with TFE in 5 mM citric acid buffer (pH 5.0, 15 °C). The $\theta_{222}$ ellipticity is plotted versus the amount of TFE added. The inset shows the UV CD spectrum of the cNOS peptide in aqueous solution (+) and in 40% TFE (O).

**Fig. 6.** CD spectra of Ca$^{2+}$-CaM (O) and the CaM-cNOS peptide complex (O) at 1:1 ratio. The concentration of CaM was 11 μM in both experiments.
see Ikura et al., 1992), and a characteristic basic residue (Lys342) immediately before the second hydrophobic residue (Bagchi et al., 1992; Meador et al., 1992). Moreover, a hydrophobic residue (Val378) 7 residues downstream of the first hydrophobic residue is also found (Fig. 4). These 4 conserved amino acid residues are believed to form the key contacts with CaM in the CaM-MLCK peptide complexes (Ikura et al., 1992; Meador et al., 1992). In addition, it has recently been shown that the α-helical structures induced in the MLCK CaM-binding domain peptide by binding CaM or by adding TFE are identical (Zhang et al., 1993). Thus, our data suggest that the cNOS peptide can bind to CaM in a similar manner as the MLCK peptides.

REFERENCES


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