Characterization of the Basic Amphiphilic α-Helix Calmodulin-Binding Domain of a 61.5 kDa Tobacco Calmodulin-Binding Protein†

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Received August 23, 1996; Revised Manuscript Received November 25, 1996⊗

ABSTRACT: A 19-amino acid residue peptide, Gly-Trp-Leu-Lys-Ile-Lys-Ala-Ala-Met-Arg-Trp-Gly-Phe-Phe-Val-Arg-Lys-Lys-Ala, corresponding to the basic amphiphilic α-helix (BAA) motif at the C-terminus of a recombinant tobacco calmodulin-binding protein, TCB60, was synthesized. The interaction of the synthetic binding domain with calmodulin (CaM) was analyzed by gel mobility shift assays, phosphodiesterase competition assays, and fluorescence, circular dichroism, and nuclear magnetic resonance spectroscopy. Mobility shift assays showed an apparent 2 kDa increase in CaM M_r in presence of synthetic peptide and $CaCl_2$ in 4 M urea polyacrylamide gel electrophoresis. HPLC measurements of hydrolysis of cyclic AMP by CaM-dependent phosphodiesterase indicated the synthetic peptide competitively inhibits ($K_i = 15-20$ nM) stimulation of phosphodiesterase activity by CaM. Upon binding CaM, the fluorescence emission maximum of the synthetic peptide, which contained two tryptophanyl residues, shifted toward blue and increased in intensity. The circular dichroism spectra indicated the ellipticity of CaM increased at 208 and 222 nm upon complex formation with the synthetic peptide. ^{1}H NMR studies showed that the peptide interacts with the aromatic residues in domains I and III of CaM. Taken together, these data provide direct evidence that the structurally conserved basic amphiphilic α-helix CaM-binding domain of the recombinant tobacco CaM-binding protein interacts with CaM at physiologically significant nanomolar concentrations and the microenvironments of both CaM and the synthetic binding domain are modified upon complex formation.

Calmodulin (CaM)[1] is one of the best characterized components of the Ca^{2+}-signaling pathway. Analysis of crystal structures indicate CaM is a small dumbbell-shaped molecule with two globular Ca^{2+}-binding domains connected by a long extended α-helix (James et al., 1995; Babu et al., 1985). Calmodulin itself does not have any enzymatic activity, but, upon binding Ca^{2+}, it acts as molecular switch and modulates a number of enzymic and non-enzymic proteins. In the absence of Ca^{2+} the hydrophobic sites of CaM are concealed, but upon binding Ca^{2+} the two hydrophobic patches become exposed and interact with the target sites of many structurally and functionally diverse proteins (Meador et al., 1993; Ikura et al., 1992; James et al., 1995). Although the sequence of CaM is highly conserved among plants, animals, and yeast, the sequences of CaM-binding sites of the target proteins vary considerably (Harrington et al., 1994).

How calmodulin binds to and regulates the activities of structurally and functionally diverse target proteins is intriguing (O'Neil & DeGrado, 1990). Since the CaMBPs play important roles in many Ca^{2+}-mediated cellular activities and signal transduction pathways, it is essential to understand the molecular mechanisms underlying the interactions between CaM and the CaM-binding target proteins (Arazi et al., 1995; Reddy et al., 1996). The large size and unknown three-dimensional structure of the target proteins are a major obstacles in elucidating the structural interactions by which CaM modulates the activities of so many proteins. In several animal CaMBPs basic amphiphilic α-helix (BAA) motifs have been identified as the CaM-binding domain. The BAA is structurally conserved and is characterized by a sequence of 15−20 amino acids that form a helical wheel projection with the predominance of basic residues on one face and hydrophobic residues on the other. Another common feature of BAA CaM-binding domains is the presence of a tryptophan residue. Recently, it has been possible to identify the BAA motifs by secondary structural analysis of CaMBPs. Several studies using synthetic BAA-binding domains and hydrolytic fragments of CaMBPs have shown that CaM interacts with BAA motifs with high affinity and broad specificity (O'Neil & DeGrado, 1990).

The helical wheel projection of the deduced amino acid residues encoded by the cDNA clone pTCB60 using computer-assisted protein secondary structure prediction program of Chou and Fasman (1978) and gel overlay assays of cDNA deletion constructs (Lu & Harrington, 1994) indicated the presence of a putative BAA at the C-terminus of the protein. To date, little is known about the molecular mechanisms of the interaction of a BAA plant CaM-binding domain with calmodulin. Thus, a 19-amino acid residue peptide, Gly-Trp-Leu-Lys-Ile-Lys-Ala-Ala-Met-Arg-Trp-Gly-Phe-Phe-Val-Arg-Lys-Lys-Ala, corresponding to the

† This research was supported by the U.S. Department of Agriculture under CSRS/NRI Grant #92-37100-7737 to H.M.H., Hawaii Institute of Tropical griculture and Human Resources, Journal Series No. 4220.

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⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1997.

[1] Abbreviations: BAA, basic amphiphilic α-helix; CaM, calmodulin; CaMBP, calmodulin-binding protein; cAMP, adenosine cyclic 3′,5-phosphate; CD, circular dichroism; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PDE, phosphodiesterase; TCB60, tobacco calmodulin-binding protein.

S0006-2960(96)02167-8 CCC: $14.00

basic amphiphilic α-helix motif of a recombinant tobacco calmodulin-binding protein (CaMBP) sequence, was synthesized. In this study, we have characterized the interaction of the synthetic BAA CaM-binding domain of TCB60 protein with CaM by gel mobility shift assays, phosphodiesterase competition assays, fluorescence, circular dichroism, and nuclear magnetic resonance spectroscopy.

MATERIALS AND METHODS

Peptide Synthesis and Purification. The synthetic peptide, 19 amino acid residues corresponding to the basic amphiphilic α-helix motif of the fusion protein, was made by standard solid-phase synthetic methods using an Applied Biosystems 430 A automated synthesizer (University of Hawaii Biotechnology Facility). The synthetic peptide was purified by reverse-phase high pressure liquid chromatography (HPLC) on a C_{18} column, and the most prominent peak eluted with gradients of 0.1% (v/v) aqueous trifluoroacetic acid and acetonitrile was lyophilized and dissolved in nanopure deionized water. A small fraction of the peptide was hydrolyzed in 5.7 N HCl (containing phenol and β-mercaptoethanol), heated under vacuum at 110 °C, and dried. The dried sample was dissolved in sodium citrate buffer, pH 2.0, for amino acid analysis using a Beckman Model 6300 amino acid analyzer. The peptide concentration was determined using molar extinction coefficient of 11 000 cm^{-1} M^{-1} at 280 nm due to the presence of two tryptophan residues in the peptide.

Gel Mobility Shift Assays. The ability of the peptide to bind CaM was confirmed by relative mobility shifts of CaM 4 M urea polyacrylamide gel electrophoresis (Erickson-Viitanen & De Grado, 1987) in the presence of the peptide and $CaCl_2$. Urea gels contained 12.5% acrylamide, 4 M urea, 0.375 M Tris-HCl, pH 8.8, and 0.1 mM $CaCl_2$ or 2 mM EGTA and run at constant voltage of 100 V in electrode buffer consisting of 25 mM Tris-HCl, 192 mM glycine, pH 8.3, and 0.1 mM $CaCl_2$ or 2 mM EGTA. Calmodulin (100 nM) and increasing concentrations of synthetic peptide (100, 150, 200, 250, and 300 nM) were incubated at room temperature for 1 h in 100 mM Tris-HCl, pH 7.2, 4 M urea, and 0.1 mM $CaCl_2$ or 2 mM EGTA. Glycerol (50%) with tracer bromophenol blue was added before the samples were loaded on the gel.

Phosphodiesterase Competition Assay. In phosphodiesterase competition assays the hydrolysis of cyclic AMP by phosphodiesterase was measured by HPLC (Watterson et al., 1980). The reaction mixture contained 40 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 0.4 mM $MnCl_2$, various concentrations of cyclic AMP, and enzyme preparations with or without CaM. The basal level of enzyme activity was determined in the absence of CaM, and the stimulated activity was determined in the presence of CaM and $CaCl_2$. Increasing concentrations of synthetic peptide were used to inhibit stimulation of phosphodiesterase activity in the presence of varying concentrations of CaM. The column was washed with 50 mM KH_2PO_4, pH 4.0, before starting each experiment.

Fluorescence Spectroscopy. The presence of a tryptophan residue is a unique feature of most basic amphiphilic α-helix CaM-binding domains identified and characterized so far. Thus, the changes in the microenvironments of the tryptophan residues of the peptide upon binding calmodulin were monitored by fluorescence spectroscopy (Malenick & Anderson, 1984) using a SLM 8000C spectrofluorimeter (SLM Aminco, Champaign, IL) modified with data acquisition electronics and software from ISS (Champaign, IL). The excitation and emission slit widths were 2 nm, and the emission spectra scanning was done at 10 nm/min with a 1-cm path length cuvette. The synthetic peptide (5 μM) incubated in 5 mM Tris-HCl, pH 7.3, and 0.5 mM $CaCl_2$ at room temperature for 30 min was excited at the excitation wavelength for tryptophan residues at 295 nm, and the fluorescence emission spectra in the range 290−440 nm were recorded. Calmodulin (to a final concentration 5 mM) was added to the same cuvette from a highly concentrated stock solution to minimize dilution effects. Emission maximum shifts between 290−440 nm were recorded in the presence of 0.5 mM $CaCl_2$. EGTA, 2 mM (from a 1 M stock solution) was added to the peptide+$CaCl_2$ sample and the peptide+$CaCl_2$+CaM sample, and the emission spectra were again recorded.

Circular Dichroism. Circular dichroism spectra were recorded on a Jasco 600 spectropolarimeter equipped with an HP 7475A plotter (Hewlett Packard). Each spectrum was the average of four scans and smoothing of base-line-corrected spectra done by digital filtering. The spectra of 20 μM synthetic peptide and 20 μM CaM in 5 mM Tris-HCl, 0.5 mM $CaCl_2$ (pH 7.3) were recorded independently between 190 and 250 nm using a 1.2-mm cell path length, sensitivity of 10 mdeg/cm, time constant of 16 s, and scan speed of 10 nm/min. The peptide (20 μM) and CaM (20 μM) were mixed in 5 mM Tris-HCl, 0.5 mM $CaCl_2$, pH 7.3, CaM and incubated for 30 min at room temperature, and the spectrum was recorded. To the same sample was added EGTA (1 M stock solution) to a final concentration of 2 mM, and the change in spectrum was recorded.

Nuclear Magnetic Resonance Experiments. The ^1H NMR studies were done at the NMR Facility of the Chemistry Department, University of Hawaii. Both CaM and peptide samples were deionized by passing through neutral Chelex-100. Neutral Chelex was prepared by washing 10 g of dry resin with 50 mL of 2 N HCl. The slurry was applied to a 5 cm × 0.5 cm column and washed with deionized distilled H_2O until the eluant was neutral. The pH of ^2H_2O from (99.98%, low in paramagnetic impurities, Aldrich Chemical Company was adjusted to 7.0. The eluant from the Chelex-100 column was freeze-dried and dissolved in ^2H_2O. The sample was placed in a 5 mm OD NMR tube (William Glass Co.) and freeze-dried. The final sample was prepared by adding 300 μL of ^2H_2O (99.98%) to 0.088 μmol of CaM in an NMR tube. When present together the ratio of CaM to synthetic peptide was 1.2:1. All spectra were recorded on a GE GN-Omega 500 Fourier transform NMR spectrometer operating at 500.11 MHz for ^1H NMR. All spectra were recorded at 25 °C and referenced to external dioxane in D_2O (δ = 3.53 ppm). One-dimensional spectra were recorded using a presaturation pulse sequence (t = 1.0 s irradiation time) and a compensated pulse with a 90° flip angle. The spectra were digitized into 8 K complex data points using quadrature detection and Bessel filtration. Time domain data were analyzed with an exponential weighing function of 0.5 Hz, Fourier transform, and phase correction.

RESULTS

Previously, we described the isolation of cDNAs encoding CaMBPs using a ligand screen (Lu & Harrington, 1994).

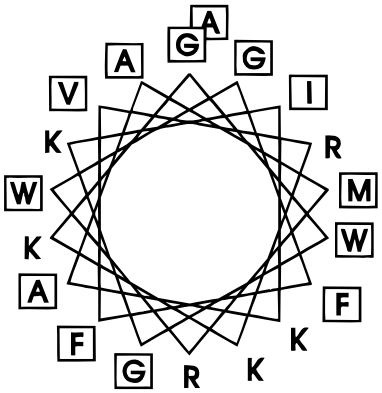

FIGURE 1: Chou-Fasman helical wheel projection of the basic amphiphilic α-helix CaM-binding domain at the C-terminus of the recombinant tobacco CaM-binding protein TCB60. Residues with squares are hydrophobic.

Several of these clones have been characterized in detail including pTCB60. These studies indicate that CaM binds to a C-terminal area of the TCB60 fusion protein (Lu & Harrington, 1994). Recent efforts have determined the full-length sequence of TCB60 mRNA and protein. To better determine the identity of the binding domain under controlled conditions, a 19 amino-acid peptide corresponding to residues 521−539 in native TCB60 was synthesized for direct measurements of interactions with CaM. The predicted structure of the synthetic peptide (Figure 1) fits the general model for a basic amphiphilic helix calmodulin-binding domain (O'Neil and DeGrado, 1990). While structurally conserved, this binding domain lacks specific sequence conservation. The other proteins that bind to CaM through this class of domain have pronounced hydrophobic and positively charged faces. In comparsion, the TCB60 binding domain has a strong positively charged region represented by KKR residues adjacent to each other as well as a hydrophobic face. The domain contains two W residues which are essential for high-affinity, reversible CaM-binding activity (O'Neil & Degrado, 1990). Also present are two F residues which may be involved in binding since CaM binds to phenathiazine drugs and phenyl Sepharose in a Ca^{2+}-dependent manner.

Gel Mobility Shift Assays. Complex formation between CaM and the peptide was confirmed by gel electrophoresis in the presence of 4 M urea, as low-affinity and nonspecific complexes are dissociated by urea and only tight and specific complexes were observed in the gel. In the presence of equimolar amounts of peptide, the mobility of CaM decreased (Figure 2A, lane 1 vs 2) indicating the binding of synthetic peptide to CaM (Erickson-Viitanen & DeGrado, 1988) stop increasing samples before concentrations of the synthetic peptide (150, 200, 250, and 300 nM) resulted in additional decreases in mobility relative to the CaM band (Figure 2A, lane 1). No shift was observed on addition of 5 mM EGTA to the samples, indicating that binding is Ca^{2+}-dependent (Figure 2B).

Competition Assays of the Peptide with CaM-Dependent Phosphodiesterase. Competition assays using a CaM-activated enzyme, cyclic nucleotide phosphodiesterase, were designed to determine if the synthetic binding domain interacts with calmodulin at physiological nanomolar concentrations. Under conditions employed here, phosphodiesterase activity was stimulated approximately 4-fold by 0.1 nM CaM (data not shown). Measurements of the conversion of cyclic AMP to AMP by phosphodiesterase in the

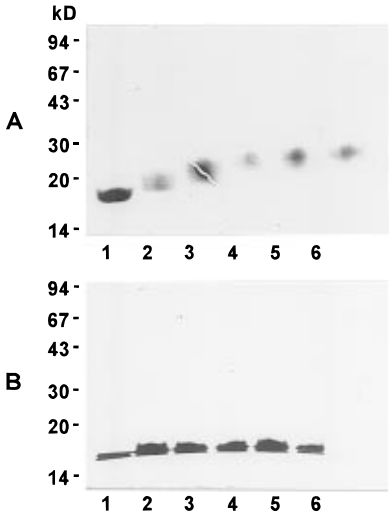

FIGURE 2: Electrophoretic mobility shift of CaM upon binding the synthetic CaM-binding domain. Mobility shift was observed in 4 M urea gels in presence of 0.1 mM $CaCl_2$ (panel A) or 2 mM EGTA (panel B). Lane 1, CaM (100 nM); lanes 2, 3, 4, 5, and 6, CaM (100 nM) plus 100, 150, 200, 250, and 300 nM peptide, respectively.

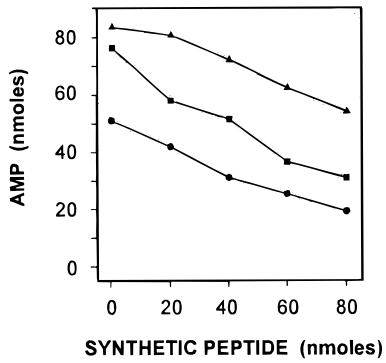

FIGURE 3: Inhibition of CaM-stimulated PDE activity by TCB60 synthetic binding domain. PDE activity was assayed in presence of varying concentrations of CaM 10^{-6}, M (▲), 10^{-7} M (■), or 10^{-8} M (●) and synthetic peptide.

presence of increasing concentrations of synthetic peptide and $CaCl_2$ indicate that the synthetic peptide competes with phosphodiesterase to bind CaM (Figure 3). Based on a Dixon−Webb plot of the quantity of AMP produced by CaM-stimulated (10^{-8}, 10^{-7}, and 10^{-6} M CaM) phosphodiesterase in the presence of 20, 40, 60, and 80 nM synthetic peptide, the K_i (inhibition constant) of the synthetic peptide is estimated to be 15−20 nM (Figure 4).

Fluorescence Emission Spectra. Since calmodulin has no tryptophan residues and the synthetic binding domain contains two tryptophan residues, the conformational changes of the binding domain during complex formation with CaM were analyzed from the fluorescence emission spectra of the CaM-bound peptide excited at 295 nm. The emission maximum of the peptide excited at 295 nm depends on the rigidity and polarity of the molecular environment surrounding the indole group of tryptophan (O'Neil & DeGrado, 1990). The emission maximum of the peptide in the buffer only was 356 nm (Figure 5). Upon addition of calmodulin and $CaCl_2$, the fluorescence emission maximum of the synthetic peptide shifted to shorter wavelengths (from 356 to 335 nm) and increased in intensity (Figure 5), indicating that the tryptophan residues were in a hydrophobic environment such as the interior of CaM. Addition of 2 mM EGTA to the same reaction mixture completely reversed the shift in the emission spectrum. These results agree with previous

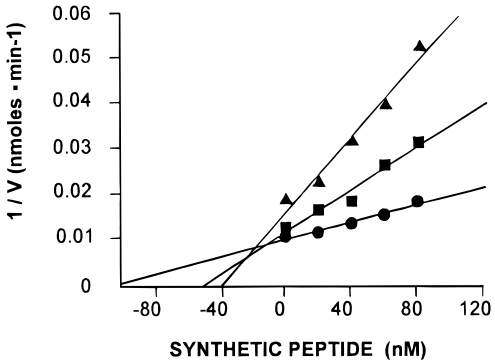

FIGURE 4: Dixon−Webb plot of synthetic TCB60 CaM-binding domain inhibition of CaM-dependent phosphodiesterase (PDE). Nanomolar concentrations of peptide were assayed for competition with PDE for calmodulin. The K_i (inhibition constant) for the peptide was determined from the intercept of the Dixon−Webb plot. CaM 10^{-6} M (●), 10^{-7} M (■), or 10^{-8} M (▲). The concentration of PDE (4 μg) was held constant.

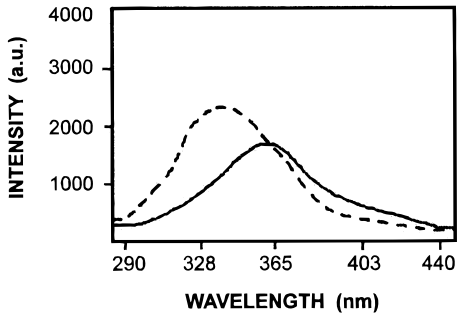

FIGURE 5: Comparison of the fluorescence emission spectra of the free peptide and CaM/peptide complex in presence of $CaCl_2$. Soild line, peptide + 0.5 mM $CaCl_2$; dashed line: peptide+CaM and 0.5 mM $CaCl_2$.

studies on myosin light chain kinase CaM-binding domain which indicate that the microenvironment of the tryptophan residues changes when calmodulin is bound to the target protein (Malenick et al., 1982; Lukas et al., 1986).

^{1}H NMR of the Interaction of TCB60 Binding Domain with Calmodulin. The ^{1}H NMR study of the interaction between the binding domains and CaM leads to the identification of the domains in CaM that interact with the peptide (Klevit, 1987; Vorherr et al., 1990). The changes in the aromatic portion of the ^{1}H NMR spectra of CaM and CaM/peptide complex were analyzed. The assignments given to the resonances are in general agreement with previous studies (Seamon, 1980; Krebs & Carafoli, 1982; Ikura et al., 1985; Dalgarno et al., 1984; Vorherr et al., 1990). The aromatic portion of the ^{1}H NMR spectra of the free peptide is shown in Figure 6A. A comparison of the NMR spectra of CaM and CaM/synthetic peptide complex suggests that the interaction occurs with aromatic residues in both C-terminal and N-terminal half of calmodulin (Figure 6 B and 6C). The resonances of the phenylalanine F89/F16 residues were more perturbed than the remaining aromatic resonances. The F16 residue is present in domain I, and the F89 residue is present in domain III at the opposite end of the calmodulin molecule. Thus, the peptide interacted with the leading helices of domains I and III.

Circular Dichroism. Circular dichroism (CD) is a powerful technique to investigate the changes in secondary structures of proteins (Klevit et al., 1985). The CD spectrum of CaM displayed the minima at 208 and 222 nm and a maximum at 195 nm, typical of most helical proteins.

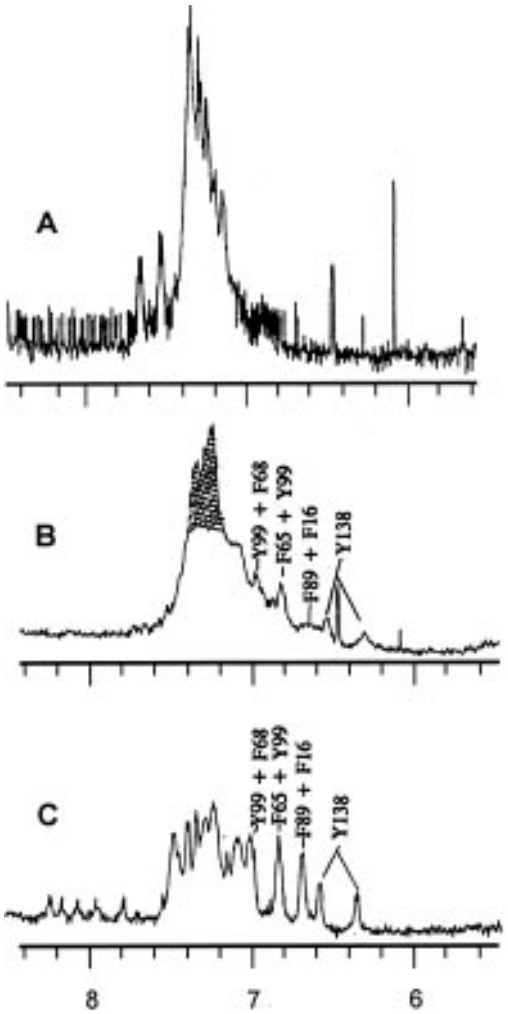

FIGURE 6: Aromatic region of the 500 MHz NMR spectra of the (A) synthetic binding domain, (B) calmodulin, and (C) calmodulin−peptide complex. The assignments given were in general agreement with those found in the literature as mentioned in the results and discussion. The sharp peaks in the cross-hatched region between 7.2 and 7.5 ppm (B) were due to the free tryptophan resonances of the unbound peptide.

Analysis of the CD spectra of the free peptide, free CaM, and the CaM/peptide complex in presence $CaCl_2$ showed the calmodulin peaks at 208 and 222 nm shifted and increased in intensity upon binding CaM (Figure 7). The ellipticity of the CaM/peptide complex at 222 nm was more negative than free CaM and peptide, which suggested a significant increase in the α-helix content during complex formation. The binding was partially reversed by addition of 2 mM EGTA (Figure 7), indicating that the interaction was Ca^{2+}-dependent.

DISCUSSION

Calmodulin modulates a large number of enzymes and proteins with diverse structures and functions; however, little is known about the molecular basis of such interactions. Many CaM-dependent enzymes are activated by conformational changes resulting from the binding of the Ca^{2+}/CaM complex. Thus, it is essential to understand the structural requirements and properties of the CaM-binding domains in the target proteins as well as interactions with the Ca^{2+}/CaM complex with the microenvironment of the target protein itself. This study provides the first detailed analysis of a plant CaM-binding domain using both biochemical and biophysical methods. Taken together, these data indicate that

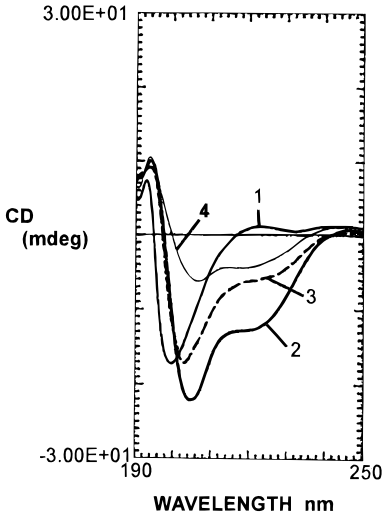

FIGURE 7: Circular dichroism spectra of calmodulin, peptide, calmodulin−peptide complex. Line 1, peptide+0.5 mM $CaCl_2$; 2, peptide+CaM+$CaCl_2$; 3, CaM + $CaCl_2$; 4, peptideCaM+$CaCl_2$+2 mM EGTA.

a domain with a basic amphilic α-helix is responsible for the ability of TCB60 to bind to CaM.

The shift in relative mobility of CaM upon binding equimolar amounts of synthetic peptide in presence of $CaCl_2$ with an apparent 2 kDa increase in CaM M_r was observed in 4 M urea gels. This increase is in agreement with the calculated molecular weight of the peptide. Additional peptide caused further decreases in CaM mobility, suggesting that up to 2 mol of peptide/mol of CaM may bind under these conditions. The peptide was assayed for competition with CaM-dependent phosphodiesterase activation. A Dixon and Webb plot of the competition assay indicated the inhibition was competitive with a K_i of 15−20 nM, characteristic of a high-affinity interaction between the synthetic binding domain and CaM in the physiological range. Previous studies characterizing the CaM-binding domains of various proteins indicate that CaM has very high affinity for the target sites (O'Neil and DeGrado, 1990). Synthetic peptides corresponding to the binding domains skeletal muscle phosphorylase kinase (Dasgupta et al., 1989; DeGrado et al., 1987) and myosin light chain kinase (Blumenthal et al., 1985), also have inhibition constants in the nanomolar range. The increased intensity and shift of the fluorescence maximum of the peptide toward shorter wavelength upon binding Ca^{2+}/CaM indicate some change in the microenvironments of the two tryptophanyl residues of the peptide due to complex formation with CaM, as shown in the previous studies.

^{1}H NMR studies demonstrate that the peptide interacts with the aromatic residues of the leading helices of domains I and III of CaM. This may lead to the folding of calmodulin so as to facilitate its interaction with a small specific site of the target protein. However, as reported previously (Vorherr et al., 1990) it is difficult to make conclusive interpretations of the spectral changes due to interaction between CaM and the peptide on the basis of 1D NMR.

The circular dichroism spectra of CaM/peptide complex relative to the CD spectra of free peptide and CaM suggested that the helicity of the peptide was altered when it binds CaM. This result is in agreement with previous studies on CaM-binding domains of animal proteins, which show that the peptides form helices when they bind CaM, thus increasing the intensity of the peaks (Cox et al., 1985; DeGrado et al., 1985; McDowell et al., 1983; Giedroc et al., 1983). Similar changes in ellipticity were observed in cases of both myosin light chain kinase intact protein as well as the synthetic binding domain (Blumenthal & Krebs, 1987). Taken together, these data confirm that a structurally conserved basic amphiphilic α-helix CaM-binding domain, similar to most CaM-binding proteins in animal systems is present in the 61.5 kDa tobacco calmodulin-binding protein. Furthermore, its interaction with CaM at nanomolar concentrations suggests that this as yet unidentified protein plays some physiolgical role in plants.

ACKNOWLEDGMENT

We thank Prof. David Jameson for his help in fluorescence studies and Dr. Robert C. Bugos for his help in HPLC analysis.

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BI9621674