Structurally homologous binding of plant calmodulin isoforms to the calmodulin-binding domain of vacuolar calcium-ATPase.

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Running title: Structurally homologous binding of soybean CaM isoforms
Summary

The discovery that plants contain multiple calmodulin (CaM) isoforms having variable sequence identity to mammalian CaM has sparked a flurry of new questions regarding the intracellular role of Ca\(^{2+}\) regulation in plants. To date, the majority of research in this field has focused on the differential enzymatic regulation of various mammalian CaM-dependent enzymes by the different plant CaM isoforms. However, there is comparatively little information on the structural recognition of target enzymes found exclusively in plant cells. Here we have used a variety of spectroscopic techniques including nuclear magnetic resonance, circular dichroism, and fluorescence spectroscopy to study the interactions of the most conserved and most divergent CaM isoforms from soybean, SCaM-1 and SCaM-4 respectively, with a synthetic peptide derived from the CaM binding domain of cauliflower vacuolar calcium-ATPase. Despite their sequence divergence both SCaM-1 and SCaM-4 interact with the calcium-ATPase peptide (CATPp) in a similar calcium-dependent, stoichiometric manner, adopting an antiparallel binding orientation with an \(\alpha\)-helical peptide. The single Trp residue is bound in a solvent inaccessible hydrophobic pocket on the C-terminal domain of either protein. Thermodynamic analysis of these interactions using isothermal titration calorimetry demonstrates that the formation of each calcium-SCaM-CATPp complex is driven by favorable binding enthalpy and is very similar to the binding of mammalian CaM to the CaM-binding domains of myosin light chain kinases and calmodulin dependent protein kinase I.

Introduction

The calcium ion (Ca\(^{2+}\)) is one of the most important signaling molecules in both plant and animal cells. Plant cells maintain a large asymmetry in subcellular Ca\(^{2+}\) distribution with submicromolar cytosolic levels, micromolar extracellular concentrations and millimolar levels in some organelles (1). Plants have utilized this steep Ca\(^{2+}\) concentration gradient by developing rapid signaling pathways using Ca\(^{2+}\) as the key secondary messenger molecule. Heat shock, cold, touch, hypoosmotic and oxidative stresses are a few of the many environmental stimuli that can trigger intracellular Ca\(^{2+}\) influx, with variations in spatial and temporal release, as well as the amplitude of the Ca\(^{2+}\) signal (2).

When Ca\(^{2+}\) is released into the cell it quickly binds to a variety of Ca\(^{2+}\)-binding proteins, some of which are simply involved in Ca\(^{2+}\)-storage and some of which regulate target enzymes
in a Ca\(^{2+}\)-dependent manner. One of the most important regulatory Ca\(^{2+}\)-binding protein is calmodulin (CaM). Mammalian CaM (mCaM) is a small, α-helical, dumbbell-shaped protein with four helix-loop-helix Ca\(^{2+}\)-binding loops commonly known as EF-hands. Calcium binding to mCaM causes a reorientation of these α-helices which results in the exposure of methionine rich hydrophobic patches on each of the protein’s two globular domains which it uses to bind to short peptide sequences in its target enzymes. The long flexible sidechains in these binding patches as well as the high polarizability of the Met sulfur atom, together with the considerable conformational flexibility of the solvent exposed linker region between the two globular domains allows Ca\(^{2+}\)-mCaM to bind to a wide range of target sequences having little sequence identity. In many proteins the CaM-binding domain is adjacent to an autoinhibitory domain, and CaM binding causes conformational changes which result in displacement of the autoinhibitory domain and subsequent activation of the target enzyme.

Higher plant species contain multiple CaM isoforms with varying degrees of sequence homology to the single CaM isoform encoded by three separate genes in mammalian cells. Of the plant CaM isoforms currently identified, those found in soybean provide one of the most intriguing systems to study since there are isoforms with more than 90% sequence identity to mCaM (SCaM-1, -2 and -3), as well as highly divergent isoforms with only 78% sequence identity (SCaM-4 and -5). Several studies have demonstrated that the different isoforms have dissimilar stimulatory affects on various mammalian target enzymes, including nitric oxide synthase (NOS), calcineurin, NAD kinase and myosin light chain kinases (MLCK’s) among others. Additionally these isoforms have different expression patterns in various plant tissue types suggesting that they likely play unique roles in the multiple Ca\(^{2+}\) signaling pathways of the plant.

One class of plant proteins that show CaM-dependent activation are the type IIb Ca\(^{2+}\)-ATPases, which are involved in restoring the intracellular Ca\(^{2+}\) concentration to resting levels by removing Ca\(^{2+}\) from the cytosol. This class is distinct from the type IIa class of Ca\(^{2+}\)-ATPases, which are not stimulated by CaM. Type IIb Ca\(^{2+}\)-ATPases show the highest homology to mammalian plasma membrane Ca\(^{2+}\)-ATPases (PMCA’s), but differ in that they have been found not only in the plasma membrane but also in the vacuole, endoplasmic reticulum, and plastid membranes. These plant homologs also differ from animal PMCA’s in that most have a truncated C-terminus and an extended N-terminus containing the CaM-binding domain, whereas
animal PMCA’s are lacking the N-terminal extension, but instead have an extended C-terminus containing the CaM-binding domain (17).

In cauliflower a Type IIb Ca\(^{2+}\)-ATPase (BCA1) was discovered in vacuolar membranes, with an N-terminal CaM-binding and autoinhibitory domain (19). The N-terminus was shown to interact with mCaM in a Ca\(^{2+}\)-dependent manner (20), and the activity of the protein was stimulated by mCaM in vitro (21). In the present work we have studied the interactions of a peptide corresponding to the CaM-binding domain of BCA1 (CATPp) with the most conserved and most divergent CaM isoforms from soybean, SCaM-1 and –4 respectively. The information obtained by various spectroscopic techniques all suggests that despite their sequence divergence both SCaM-1 and –4 bind to CATPp in a structurally homologous manner similar to Ca\(^{2+}\)-mCaM binding to many of its target peptides including those from CaM dependent protein kinase I (CaMKI), and the MLCK’s.

**Experimental Procedures**

**Protein purification and peptide synthesis:**

The 25 residue Ca\(^{2+}\)-ATPase peptide (CATPp) was synthesized at the Protein Chemistry Centre at the University of Victoria, Victoria, British Columbia, Canada. The sequence of the peptide Ac-ARQRWRSSVSIVKNRARRFRMISNL-amide corresponds to the amino acid sequence of residues 19 to 43 of BCA1 (19). The CATPp peptide used in the nuclear magnetic resonance (NMR) studies had an additional cysteine residue attached to its N-terminus to facilitate chemical labeling with fluorescent and paramagnetic probes. Peptide purity was confirmed by MALDI mass spectrometry, capillary electrophoresis, and amino acid analysis, while concentrations were determined by UV spectroscopy using the predicted molar extinction coefficient \(\varepsilon_{280} = 5690 \text{ M}^{-1}\text{cm}^{-1}\).

SCaM-1 was expressed and purified from *Escherichia coli* as previously described (22 and references therein). The pET-3d plasmid containing chimeric SCaM-4 (14) was cloned into E. coli expression strain ER2566, and then expressed and purified identically to SCaM-1. Protein identity and purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as well as MALDI and ESI mass spectrometry. For the purposes of this study Ca\(^{2+}\)-SCaM-1 and Ca\(^{2+}\)-SCaM-4 refer to the Ca\(^{2+}\)-saturated forms of each protein. For the preparation of both carbon-13 methyl-Met-labeled (\(^{13}\text{C-Met}\)) SCaM-1 and –4, 1 L cell
cultures were grown at 37°C to an OD\textsubscript{600} ~ 0.8 in Luria-Bertani (LB) broth with 100 mg/L ampicillin, and then harvested by centrifugation. Cell pellets were resuspended in 1 L of chemically defined MOPS medium (23) supplemented with all amino acids except Met at a concentration of 100 mg/L, and maintained in the shaker at 37°C for 30 minutes to deplete the media of any residual Met. Protein synthesis was then induced by the addition of 50 mg/L \textsuperscript{13}C-methyl-methionine and 100 mg/L IPTG. After a 4 hour induction time at 37°C, cells were harvested and the protein was purified identically to the unlabelled protein. Purified protein was dialyzed for a minimum of 60 hours against several changes of 8 mM ammonium bicarbonate and then lyophilized. The lyophilized protein was dissolved in appropriate buffer, and concentrations were determined using the molar extinction coefficients $\varepsilon_{276} \text{SCaM-1} = 1450 \text{ M}^{-1}\text{cm}^{-1}$, and $\varepsilon_{276} \text{SCaM-4} = 2900 \text{ M}^{-1}\text{cm}^{-1}$, or using the BioRad protein assay kit.

**Non-denaturing urea-polyacrylamide gel electrophoresis:**
Non-denaturing urea-PAGE mobility shift assays were performed in the presence of either 1.0 mM CaCl\textsubscript{2} or 2.0 mM EDTA using essentially the same procedure as described in reference (24).

**Isothermal titration calorimetry:**
All isothermal titration calorimetry (ITC) experiments were performed on a MicroCal VP-ITC microcalorimeter, similar to the procedure used by (25). Briefly, 3\textmu l volumes of 600 \textmu M CATPp in 20 mM HEPES, 100 mM NaCl, 2 mM CaCl\textsubscript{2}, pH 7.5 (ITC buffer) were injected from the syringe into the cell containing either 12 \textmu M SCaM-1 or 10 \textmu M SCaM-4 in the same buffer, at temperatures ranging from 10 to 30°C. Protein dimerization through disulfide bonding was prevented by incubating each protein for 24 hours in ITC buffer containing 10 mM dithiothreitol (DTT), and then desalting into ITC buffer without DTT using an Econo-Pac 10DG column immediately prior to ITC analysis. Both SCaM-1 and –4 were confirmed to be monomeric using non-denaturing polyacrylamide gel electrophoresis. The heat of dilution/mixing was measured in separate control experiments and subtracted in each case, and all of the data was analyzed using Microcal Origin software. For each titration the stoichiometry (N), association constant (K\textsubscript{a}) and enthalpy change (\Delta H) were directly obtained from the ITC data, and the Gibbs free energy (\Delta G), entropy (\Delta S), and heat capacity (\Delta C\textsubscript{p}) changes were calculated from equations [1], [2] and [3].

$$\Delta G = -RT \ln K_a$$  \[1\]
\[ \Delta G = \Delta H - T \Delta S \quad [2] \]
\[ \Delta C_p = \frac{d \Delta H}{dT} \quad [3] \]

**Circular Dichroism Spectroscopy:**

Circular dichroism (CD) spectra were acquired at room temperature on a Jasco J-715 spectropolarimeter in the Department of Chemistry, University of Calgary, Calgary, Alberta, Canada. Far-UV CD spectra were measured from 260-185 nm using a cylindrical quartz cuvette with a pathlength of 0.1 cm, and a volume of 300 µl, using the following parameters: 0.2 nm step resolution, 50 nm/min speed, 2 s response time, 1 nm bandwidth, 50 mdeg sensitivity. All spectra are the average of 10 scans with the background signal from buffer subtracted, and the data was then smoothed and converted to molar ellipticity using Jasco software. Samples consisted of 15 µM protein and/or peptide, in 10 mM Tris-HCl, 1 mM DTT, 2 mM CaCl_2, pH 7.5. Near-UV CD spectra were measured from 320-250 nm using a cylindrical quartz cuvette with a pathlength of 1.0 cm and a volume of 2 ml, using similar parameters to those above except with a sensitivity of 10 mdeg. All spectra are the average of 20 scans. Near-UV CD spectra were processed as above, except that they were not smoothed in order to preserve their fine structure. These samples contained 50 µM protein and/or peptide in 10 mM Tris-HCl, 1 mM DTT, 100 mM KCl, 2 mM CaCl_2, pH 7.5.

**Fluorescence Spectroscopy:**

Fluorescence spectra were acquired at room temperature on a Spex Fluorolog-22 spectrofluorimeter, using an excitation band width of 2 nm and emission band width of 10 nm. The lone tryptophan residue of CATPp was excited at 295 nm and fluorescence emission was measured from 300 to 450 nm. In this way the tryptophan can be selectively excited with minimal interference from the tyrosine residues of SCaM-1 and –4 (26). All samples contained 10 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM DTT, and 1 mM CaCl_2. All spectra presented are the average of three to six experiments, with the spectrum of the buffer subtracted. The concentration of CATPp was 8 µM, while that of SCaM-1 and –4 were each 10 µM to ensure complete saturation of the peptide. Quenching experiments were performed by titrating 5 M CsCl into 2 ml samples of CATPp or Ca\(^{2+}\)-SCaM-CATPp complexes, to a final concentration of 2 M CsCl. Fluorescence intensity was monitored at 355 nm for free CATPp and at 325 nm for Ca\(^{2+}\)-SCaM-CATPp complexes, and corrected for dilution effects. Quenching data is the average of three independent titrations.
Nuclear Magnetic Resonance Spectroscopy:
All NMR spectra were acquired at 298 K on a Bruker Avance 500 NMR spectrometer equipped with a triple resonance inverse Cryoprobe with a single axis z-gradient. Samples contained 400-650 µM \(^{13}\text{C}\)-Met-SCaM-1 or –4, or CATPp, in 100 mM KCl, 99.9% D\(_2\)O, pD 7.5 +/- 0.1 (not corrected for isotope effects) and 5 mM CaCl\(_2\). For the two dimensional (2D) \(^1\text{H}, \text{13C}\) HSQC spectra quadrature detection in the F\(_1\) dimension was obtained using echo/antiecho time-proportional phase incrementation. The sweep width in the \(^1\text{H}\) and \(^{13}\text{C}\) dimensions were 6009.6 Hz and 500 Hz respectively, while the carrier frequencies were 500.13235 MHz and 125.7596 MHz. The size of each spectrum was a 2048 x 64 real data matrix, with two scans for each experiment. For the assignment of CATPp in 30% aqueous trifluoroethanol (TFE) we also obtained two dimensional homonuclear \(^1\text{H}\) NMR spectra of a sample consisting of 2.5 mM CATPp in 30% deuterated TFE, 5% D\(_2\)O, 65% H\(_2\)O, pH 4.2 +/-0.1. Both Nuclear Overhauser effect (NOESY) spectra and total correlation (TOCSY) spectra were recorded at multiple mixing times (250 and 400 msec for NOESY, and 80 and 150 msec for TOCSY) to check for spin diffusion effects. All spectra were processed using NMRPipe [3.4] (27) and analyzed using NMRView [4.1.3] software (28) on workstations running Redhat [7.1] for Linux. Structure calculations were performed using Aria1.1/CNS1.1 (29;30). Proton chemical shifts were referenced to DSS (2,2-dimethyl-2-silapentane-5-sulfonate) as 0 ppm and \(^{13}\text{C}\) chemical shifts were referenced indirectly to DSS (31).

Results
Ca\(^{2+}\)-dependent, stoichiometric binding of SCaM-1 and –4 to CATPp.
To investigate the Ca\(^{2+}\)-dependence and stoichiometry of SCaM-1 and –4 binding to CATPp, we first performed urea-PAGE band shift assays under non-denaturing conditions. Urea is included in the samples to prevent any non-specific interactions from taking place (24). As seen in Figure 1A, a complete shift of both the SCaM-1 and –4 protein bands occurs in the presence of one molar equivalent of CATPp suggesting that both proteins bind the peptide with a 1:1 molar stoichiometry. In the presence of excess peptide no additional complexes are observed indicating that the 1:1 complex is the unique species in solution. In the presence of saturating amounts of the high affinity Ca\(^{2+}\)-chelator EDTA no band shifts are observed at any protein to peptide ratio, indicating that the interactions of SCaM-1 and –4 with CATPp are totally Ca\(^{2+}\)-
dependent, Figure 1B. The differences in the migration of the two proteins likely reflects dissimilarities in surface charge and/or small structural differences.

**Insert Figure 1**

*The interactions of Ca$^{2+}$-SCaM-1 and –4 with CATPp are thermodynamically similar and enthalpically driven.*

The binding affinity and thermodynamics of the interactions between the SCaM’s and CATPp were characterized using ITC. Representative titration data for both Ca$^{2+}$-SCaM-1 and –4 are shown in Figure 2. In agreement with our non-denaturing urea PAGE data, we obtained a stoichiometry (N) of binding for both proteins near 1.0 confirming that a specific 1:1 complex is formed in each case, Table 1. Of note, we found that the stoichiometry of binding to Ca$^{2+}$-SCaM-4 decreased slightly at lower temperatures, possibly due to increased aggregation. The association constants (K_a) for each protein binding to CATPp were near 10$^7$ M$^{-1}$ indicating the formation of tight complexes, however there was also slight temperature variation with the strongest interaction between 15-20°C and weaker binding at both higher and lower temperatures. We also found that the K_a for the interaction with Ca$^{2+}$-SCaM-1 was approximately three times larger than that with Ca$^{2+}$-SCaM-4 at each temperature used in the study indicating that Ca$^{2+}$-SCaM-1 consistently binds CATPp with higher affinity.

**Insert Figure 2**

**Insert Table 1**

In each titration with both proteins we obtained negative enthalpy ($\Delta H$) and entropy ($\Delta S$) values demonstrating that binding of each protein to CATPp is an exothermic event which is accompanied by increased order in the protein and/or peptide. The $\Delta S$ values indicate that the structure of the complex is more rigid than that of the free protein and peptide. Since a negative $\Delta S$ results in a positive $T\Delta S$ term in the calculation of Gibbs free energy change ($\Delta G$) through equation [2], our results show that the interaction of each protein with CATPp is driven by enthalpic and not entropic factors. Therefore the hydrophobic effect is not responsible for driving the formation of either Ca$^{2+}$-SCaM-CATPp complex. Importantly, although the $\Delta H$ and $\Delta S$ of Ca$^{2+}$-SCaM-1 binding were of greater magnitude than Ca$^{2+}$-SCaM-4 at each temperature, the values for both proteins had strong temperature dependence becoming more positive in a linear manner at lower temperatures, Figure 2C. As a result the $\Delta G$ of binding for each protein remained relatively constant over the temperature range tested, and had similar values to each
other, where the $\Delta G$ for $\text{Ca}^{2+}\text{-SCaM-1}$ was $-40.14 \pm 1.35 \text{kJ/mol}$ and $\text{Ca}^{2+}\text{-SCaM-4}$ was $-38.28 \pm 0.54 \text{kJ/mol}$, Table 1. This behavior which is referred to as enthalpy-entropy compensation, has been observed in numerous other protein-protein and protein-peptide interactions including $\text{Ca}^{2+}\text{-mCaM}$ binding to several of its target peptides (25;32-34).

In addition to these thermodynamic parameters, we also calculated the change in heat capacity ($\Delta C_p$) for binding of $\text{Ca}^{2+}\text{-SCaM-1}$ and $\text{SCaM-4}$ to CATPp from the slope of the $\Delta H$ versus temperature plot in Figure 2C through equation [3]. In all cases the enthalpy change as a result of linked protonation effects was assumed to be zero, based on the results from similar studies with mCaM (25;35), so $\Delta H$ is assumed to be entirely derived from the enthalpy of $\text{Ca}^{2+}\text{-SCaM-1}$ or -4 binding CATPp in each titration. Interestingly, despite the minor differences in all other thermodynamic parameters, the $\Delta C_p$ values for each protein binding to CATPp were found to be identical (-2.12 kJ mol$^{-1}$ K$^{-1}$). Therefore, since changes in heat capacity are predominantly due to changes in surface hydration of the protein and peptide, this result suggests that the interaction surfaces and thus the overall structures of the complexes are also highly alike. In similar studies with mCaM the $\Delta H$ values at 30$^\circ$C were not used in the calculation of $\Delta C_p$ since differential scanning calorimetry (DSC) experiments indicated that the central linker of mCaM began to unfold near this temperature (35). However, we did not observe this behavior in similar studies with the SCaM’s (results not shown), and our values at 30$^\circ$C fit the linear regression perfectly giving $R^2$ values over the entire temperature range of greater than 0.99. Therefore these values were included in our calculations.

**CATPp adopts an α-helical structure upon binding to $\text{Ca}^{2+}\text{-SCaM-1}$ and $\text{-4}$.**

CD spectroscopy is a technique commonly used to study the secondary structure of proteins and peptides in solution, and has been used extensively to study the interactions of mCaM with its target peptides (36-39). Like mCaM, the far-UV CD spectra of both $\text{Ca}^{2+}\text{-SCaM-1}$ and $\text{-4}$ are characteristic of highly α-helical proteins with intense minima near 208 and 222 nm and a maximum near 193 nm, Figures 3A and 3B. However the spectrum of $\text{Ca}^{2+}\text{-SCaM-4}$ is somewhat less intense suggesting that it may either have a lower proportion of α-helical structure, or that it may be more structurally dynamic than $\text{Ca}^{2+}\text{-SCaM-1}$. In the spectra of each protein in complex with CATPp there is a significant increase in peak intensity suggesting that

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\*The $\Delta G$ value at 10$^\circ$C for $\text{Ca}^{2+}\text{-SCaM-4}$ was considered to be inaccurate likely due to temperature dependent aggregation, and was therefore not included in the average or error calculations.
there is an even higher proportion of α-helical structure in both complexes than in the Ca\(^{2+}\)-bound proteins alone. Difference spectra clearly illustrate that the peptide-bound spectra are not a simple summation of the individual component spectra since the free CATPp produces a characteristic random coil spectrum with a single minimum near 197 nm, Figure 3C. Since it is well known that Ca\(^{2+}\)-mCaM undergoes little secondary structural changes upon binding to its target peptides (40-42), we suggest that both Ca\(^{2+}\)-SCaM-1 and –4 behave in a similar manner and thus the increase in α-helical structure can be attributed entirely to the bound peptide. This would suggest that binding to either protein forces the peptide from a random coil into an α-helix similar to the conformational changes induced by Ca\(^{2+}\)-mCaM binding many of its target peptides (36-38;43). Furthermore, the striking similarity in the difference spectra for Ca\(^{2+}\)-SCaM-1 and –4 suggests that the bound peptide adopts identical secondary structure when bound to each protein.

**Insert Figure 3**

TFE is well known to stabilize α-helical secondary structure in polypeptides with the propensity to form α-helices (44-46). Therefore, since CATPp was shown to bind both Ca\(^{2+}\)-SCaM-1 and –4 as an α-helix, we attempted to induce a similar structure in the peptide in the absence of protein using TFE. Although CATPp was a random coil in the absence of TFE, its α-helical content increased significantly during the titration with TFE up to a concentration of 30%, and then only moderate increases were observed at higher TFE concentrations (results not shown). This indicates that although the peptide is unstructured in aqueous solution, it has the propensity to form an α-helix similar to other CaM-binding domain peptides.

**CATPp adopts an antiparallel orientation with respect to the SCaM’s.**

Near-UV CD spectroscopy is very sensitive to the local environment of the aromatic side chains and is therefore a good technique to study protein tertiary structure and conformational changes (47). The technique has also been used extensively to study mCaM-peptide interactions where studies reveal that distinct spectral patterns are produced when the Trp residues from target peptides bind to the N- versus C-terminal domains of Ca\(^{2+}\)-mCaM (48-51). Binding of this Trp into the C-terminal hydrophobic pocket of Ca\(^{2+}\)-mCaM results in a broad positive intensity above 280 nm for the ^1L_a electronic transition of the indole ring, while binding to the N-terminal domain results in a strong negative intensity for this transition. Binding to either domain gives
rise to two weak negative bands at 282 and 290 nm for the $^1L_b$ transition, which gives the spectra fine structure consisting of two CD bands.

The near-UV CD spectra of Ca$^{2+}$-SCaM-1 and -4 show two intense negative peaks near 262 and 268 nm arising from Phe sidechains, and a more broad signal from 270 to 290 nm from their Tyr side chains, Figure 4. Neither protein contains a Trp residue in their sequence so no signals are observed at longer wavelengths. However, in the spectra of each Ca$^{2+}$-SCaM-CATPp complex two new positive signals are observed above 280 nm arising from the bound Trp5 sidechain of CATPp, suggesting that it is bound to the C-domain of both proteins. As expected due to its random coil structure observed in the far-UV CD spectra, no signal was observed for the free peptide (results not shown). The slightly more positive intensity near 277 nm in the spectrum of Ca$^{2+}$-SCaM-4-CATPp is likely due to the additional Tyr residue found in SCaM-4 which is not present in SCaM-1. Therefore, like the complexes of Ca$^{2+}$-mCaM bound to its target peptides from skeletal muscle MLCK (skMLCK), CaMKI and others (38;48-51), our results suggest that CATPp binds to both Ca$^{2+}$-SCaM-1 and -4 in an antiparallel orientation. Furthermore the high degree of similarity in the spectra suggests that the chemical environments of the Trp binding pockets of Ca$^{2+}$-SCaM-1 and -4 are also similar to each other, and to mCaM.

**Insert Figure 4**

**Trp5 of CATPp binds in a solvent shielded hydrophobic pocket.**

We next used steady state fluorescence spectroscopy of Trp5 of CATPp to study the interaction of the peptide with Ca$^{2+}$-SCaM-1 and -4. The broad unstructured fluorescence emission spectrum with emission wavelength maximum at 355 nm in the sample of free peptide is dominated by the electronic transition of the Trp indol ring to the $^1L_a$ state, typical of a fully solvent exposed Trp (52), Figure 5A. Upon addition of saturating amounts of Ca$^{2+}$-SCaM-1 or -4, a significant increase in fluorescence quantum yield is observed, as well as a structured blue shifted spectrum with two new emission wavelength maxima near 313 and 325 nm. The blue shift is characteristic of the Trp moving from solvent-exposed polar surroundings, into a more hydrophobic environment. In this hydrophobic environment, the electronic transition of the indole ring to the $^1L_b$ state becomes lower in energy than the $^1L_a$ transition, and this gives rise to the new structured emission spectrum (52;53). This structured emission can only be observed when narrow excitation slitwidths are used such as the 2 nm excitation slits used in these experiments, and indicates that the Trp is held very rigidly in the binding pocket. In fact, when
we performed the identical experiment using 10 nm excitation slits both spectra lost all fine structure and resembled the spectra reported in several other mCaM-peptide binding studies (26;38;39;54;55) (results not shown). The weaker intensity and decreased fine structure of the Trp fluorescence emission when bound to Ca\(^{2+}\)-SCaM-4 may be due to increased quenching either by the surrounding amino acid side chains, or from a greater exposure to solvent. However, it might also suggest that the Trp is less rigidly held in the binding pocket, although the Trp signal in our near-UV CD spectra were comparable when bound to each protein. To test whether this Trp had different solvent exposure we performed fluorescence quenching experiments using CsCl as the quenching molecule. The Stern-Volmer plot in Figure 5B shows that the Trp fluorescence of the free peptide is significantly quenched upon titration with CsCl, whereas the quenching molecule had almost no effect on the Trp fluorescence in either of the Ca\(^{2+}\)-SCaM-CATPp complexes. These results show that the Trp is highly solvent exposed in the free peptide yet that it is buried deep within the binding pocket of each protein and is almost entirely inaccessible to solvent in each complex. Therefore the increased quenching seen in the Ca\(^{2+}\)-SCaM-4-CATPp complex may be due in large part to more quenching from different side chains in the Trp binding pocket which includes an additional Met side chain in SCaM-4 (55).

**Insert Figure 5**

**The SCaM Met residues in the protein-peptide complexes are in similar chemical environments.**

It is well characterized that the hydrophobic binding patches of Ca\(^{2+}\)-mCaM are especially rich in Met residues and that these residues are very important in the promiscuous binding behavior of the protein (6-8;56). Therefore the Met residues in Ca\(^{2+}\)-mCaM and its complexes with target proteins have been the target of extensive investigations. In particular NMR spectroscopy studies have been used to monitor the chemical shift of the terminal methyl group of the Met side chain in complex with different target peptides (38;57-59). Since chemical shift is extremely sensitive to the precise chemical environment surrounding the nuclei, we studied the complexes of both \(^{13}\)C-Met-Ca\(^{2+}\)-SCaM-1 and –4 with CATPp by heteronuclear \(^1\)H, \(^{13}\)C-HSQC NMR spectroscopy as a more sensitive way to investigate structural similarities and differences between the two complexes. Although SCaM-1 has eight Met’s and SCaM-4 only seven, the majority of these residues are conserved in the two proteins, Figure 6, allowing for a direct comparison of their NMR spectra.
The two-dimensional $^1$H, $^{13}$C HSQC spectrum of $^{13}$C-Met-Ca$^{2+}$-SCaM-1 shows the presence of eight peaks corresponding to the eight Met residues of the protein, whereas the seven Met’s give rise to seven peaks in the spectrum of $^{13}$C-Met-Ca$^{2+}$-SCaM-4, Figure 7. Interestingly the peaks in both spectra have similar chemical shifts in both the $^1$H and $^{13}$C dimensions as well as comparable linewidths indicating that they may also have similar tertiary structures in the Ca$^{2+}$-bound state. In the presence of 0.5 molar equivalents of CATPp we found that the original peaks from Ca$^{2+}$-SCaM-1 or –4 were in slow chemical exchange with a completely new set of peaks corresponding to the Ca$^{2+}$-SCaM-peptide complexes. In the presence of 1.1 molar equivalents of peptide only the peaks arising from the Ca$^{2+}$-SCaM-CATPp complexes were detected confirming that both proteins bind the peptide with high affinity and with a 1:1 molar stoichiometry. The finding that all of the Met methyl resonances from both proteins are affected by CATPp suggests that both domains of each protein are involved in binding to the peptide. The chemical shifts of the Met methyl resonances in the two complexes are also notably similar to each other, Figures 7C and 7F. It is important to note that chemical shift is so sensitive to the precise chemical environment of a nucleus that even though Ca$^{2+}$-mCaM adopts highly homologous three-dimensional structures when bound to many of its target peptides, its Met residues have very different chemical shifts due to dissimilar peptide sequences (57-60). Therefore the considerable similarity in the spectra of the Ca$^{2+}$-SCaM-CATPp complexes suggests that the Met residue environment and thus the overall protein-peptide complexes are probably structurally homologous to each other.

**Insert Figure 6**

CATPp adopts a kinked, amphipathic $\alpha$-helical structure in 30% aqueous TFE.

Since our CD spectroscopy studies suggested that CATPp adopted a similar $\alpha$-helical structure when bound to both Ca$^{2+}$-SCaM-1 and –4, and that a comparable amount of helicity could be induced in the presence of TFE, we determined the structure of CATPp in 30% TFE. The resulting structure displays the expected $\alpha$-helical character for the peptide, however instead of forming a single long $\alpha$-helix in TFE like many mCaM target peptides, we found that CATPp can form two well-defined $\alpha$-helices from residues 5 to 16 and 18 to 23, with a kink between residues 17 and 18, Figure 8. While the N- and C-terminal helices can be overlaid separately with backbone RMSD’s of less than 1.0, the large degree of conformational flexibility of the two
helices around the kink did not allow for the lowest energy structures of the entire peptide to be superimposed. Interestingly the N-terminal helix was highly amphipathic with the sidechains from Trp5, Val9 and Val12 forming a large hydrophobic surface which seems like an excellent binding site for the C-domain binding pocket of either SCaM-1 or SCaM-4, while the Arg side chains extend towards the opposite face of the peptide for possible salt bridging with acidic residues on the surface of either protein. The C-terminal helix is also somewhat amphipathic with the sidechains from Phe19, Met21 and Ile22 forming a second hydrophobic surface that is involved in binding to the N-domain of the SCaM’s. The overall helicity and amphipathicity observed for CATPp is similar to many mCaM target peptides yet to our knowledge a similar kink in this region of a CaM-binding peptide has not been observed. Although this kink may have some functional significance (see discussion) it is probably due to charge repulsion in this region of the sequence which contains four basic Arg’s in a six residue sequence, and this repulsion may be partially neutralized by acidic residues on the surface of the SCaM’s in the actual protein-peptide complexes.

Insert Figure 8

Discussion

As with many CaM mutants and CaM isoforms from other species such as yeast, the divergent sequences of SCaM-1 and –4 have given each protein some unique functional characteristics. Since the majority of studies on the SCaM’s have focused on their capacity to activate mammalian target enzymes, we chose to perform structural studies on a CaM-binding domain (CaMBD) derived from a plant protein since it provides a more direct representation of the interactions with the plant CaM isoforms in vivo. Overall the results of our studies have demonstrated that the binding of both Ca\(^{2+}\)-SCaM-1 and –4 to CATPp is very similar to each other, and that each bind in a Ca\(^{2+}\)-dependent manner with a 1:1 molar stoichiometry. Furthermore we showed that both lobes of each protein are involved in binding to CATPp, and that the Trp5 residue near the N-terminus of the peptide binds into a solvent inaccessible hydrophobic pocket on the C-domain of either protein. This results in the protein and peptide having an antiparallel orientation with respect to each other. All of these characteristics of the Ca\(^{2+}\)-SCaM-CATPp complexes are common with many mCaM-target peptide complexes, especially peptides containing Trp residues near their N-termini such as skeletal and smooth
muscle MLCK’s and CaMKI. High resolution structures of their complexes with Ca\(^{2+}\)-mCaM have been reported (40;41;61), and in these structures the partially \(\alpha\)-helical central linker of Ca\(^{2+}\)-mCaM unravels to allow the N- and C-domains of the protein to wrap around a single long \(\alpha\)-helical peptide which spans the entire length of the channel formed by the protein. The similarity in CATPp binding compared to CaMKI and the MLCK’s is not surprising since their sequences share several conserved hydrophobic and basic residues especially near their N-termini, Figure 9. With all of these peptides and therefore likely with CATPp, binding is thought to be strongly driven by a primary interaction between the conserved Trp residue and the C-domain hydrophobic binding pocket, as well as many electrostatic interactions between the basic residues near the N-terminus of the CM-binding domain peptide and acidic residues on each protein (62;63). In the high resolution X-ray crystal structure of the intact CaMKI protein this Trp residue was shown to be in a highly solvent exposed loop region accessible to CaM, and enzymatic activation is thought to occur by CaM binding to this region which induces a coil-helix transition in the CaMBD resulting in autoinhibitory domain displacement (61;64;65).

Because of the sequence and structural similarities observed with CATPp, including a predicted autoinhibitory domain which is directly adjacent to the CaMBD, it appears that activation of CATPp occurs by a similar type of mechanism.

**Insert Figure 9**

In addition to the structural studies on Ca\(^{2+}\)-SCaM-1 and -4 binding to CATPp, we also performed thermodynamic characterization of these interactions by ITC. As with the spectroscopic results, our calorimetry data indicated that both proteins bound to the peptide in an almost identical manner. However the interactions with Ca\(^{2+}\)-SCaM-4 were found to be slightly weaker at all temperatures. The \(K_a\) for both proteins binding to CATPp was at least one order of magnitude weaker than that measured for Ca\(^{2+}\)-mCaM binding smooth muscle MLCK (smMLCK) under similar conditions, as well as being weaker than Ca\(^{2+}\)-mCaM’s interactions with several other peptides such as those derived from cerebellar NOS (cNOS) and cyclic nucleotide phosphodiesterase (PDE) (25;35). However they were close to the values obtained with the CaMKI peptide, and greater than those found for other peptides such as the CaMBD of caldesmon, suggesting that the affinity of the SCaM’s for CATPp is in a similar range as many of CaM-binding peptide interactions with Ca\(^{2+}\)-mCaM. We also found very similar \(\Delta H\), \(\Delta S\) and \(\Delta G\) values for binding of each protein, and like CaMKI and the MLCK’s, the Ca\(^{2+}\)-SCaM-
CATPp interactions were driven by large favorable $\Delta H$ values and were entropically unfavorable. Therefore, as described by Wintrode and Privalov (35), the formation of extensive Van der Waals contacts of CATPp with the protein is the major force driving the binding and not the hydrophobic effect. Nevertheless, Brokx et al (25) have shown that the binding of Ca$^{2+}$-mCaM to other CaMBD peptides such as the cNOS and PDE peptides are driven by favorable entropy, suggesting that various CaMBD peptides have different thermodynamic factors governing their binding to CaM’s. The increased order observed in the Ca$^{2+}$-SCaM-CATPp complexes is likely due to a combination of the coil to $\alpha$-helix transition of CATPp, as well as a decreased mobility of the two domains of the protein with respect to each other. The $\Delta C_p$ for Ca$^{2+}$-SCaM-1 and -4 were found to be identical to each other (-2.12 kJ mol$^{-1}$ K$^{-1}$), although they were slightly lower than the value measured for smMLCK (-2.7 kJ mol$^{-1}$ K$^{-1}$) and considerably less than CaMKI (-3.5 kJ mol$^{-1}$ K$^{-1}$). In fact, our values are somewhat intermediate to those reported earlier as being characteristic of peptide binding to both domains of Ca$^{2+}$-mCaM (~ -3.2 kJ mol$^{-1}$ K$^{-1}$) and binding predominantly to one domain (~ -1.6 kJ mol$^{-1}$ K$^{-1}$) (25). Since all of our other evidence suggests that the N-terminus of the peptide makes a high affinity interaction with the C-domain of each protein, these intermediate $\Delta C_p$ values suggest that binding of CATPp to the N-domain of the SCaM’s may be somewhat weaker than the interactions seen with Ca$^{2+}$-mCaM and either of the CaMKI or smMLCK peptides. However, we do not suggest that CATPp binds only to the C-domain of the SCaM’s since our NMR data clearly demonstrates that both domains of both Ca$^{2+}$-SCaM-1 and -4 are involved in the interaction. Recently Cho and coworkers presented evidence that Ca$^{2+}$-SCaM-1 is more specific for a 1-5-10 type of recognition motif, whereas Ca$^{2+}$-SCaM-4 is more specific for a 1-8-14 motif (66). Upon examination of the sequence of CATPp we find that it conforms to a 1-5-8-15 motif, where residue 1 corresponds to Trp5 of the peptides sequence, Figure 9. Therefore, residues 1, 5 and 8 at the N-terminus of CATPp seem ideal for interaction with either Ca$^{2+}$-SCaM-1 or -4, so it is not surprising to find that the N-terminus of CATPp binds strongly to each protein. However, Phe19 which likely acts as the C-terminal hydrophobic anchor residue at position 15 of the consensus motif, does not conform to the ideal recognition sequences for either protein, and this may partially explain the lower $K_a$ and intermediate $\Delta C_p$ values. It is also possible that the propensity of CATPp to form a kink between residues 17 and 18 may have a disruptive effect on the binding of the N-domain of each protein to this region of the peptide. However, this may
also provide flexibility in binding since the Phe sidechain is one residue farther downstream of the ideal position 14. This type of peptide flexibility has previously been observed in the CaMBD peptide from CaM-dependent protein kinase kinase (CaMKK) which forms a helix-loop structure when bound to Ca\(^{2+}\)-mCaM (62;63). Therefore the propensity to form a kink in this region may have structural or functional significance in the intact BCA1 protein, although this awaits structural elucidation.

Since our results demonstrate that Ca\(^{2+}\)-SCaM-1 and –4 have very similar secondary and tertiary structures in complex with CATPp, the different stimulatory capabilities of the two proteins must be due to very minor structural and chemical differences from individual amino acid substitutions in the sequences of each protein. This idea is supported by a study using mammalian cNOS where it was demonstrated that the single M144V point mutation is responsible for the inability of Ca\(^{2+}\)-SCaM-1 to activate the enzyme, whereas Ca\(^{2+}\)-SCaM-4 induced full activity since it did not have this mutation (67). In this complex M144 is directly involved in binding to cNOS and induces a conformational change that is critical for enzymatic activity, while the shorter Val144 is unable to perform a similar function. Another study using smMLCK showed that the residues in Ca\(^{2+}\)-SCaM-1 directly involved in the binding interface with the CaM-binding domain of the protein were not causing the differences in activation. Instead several residues on the surface of the protein-peptide complex including Lys30 and Gly40 form a local environment that is critical for Ca\(^{2+}\)-SCaM-1’s ability to activate smMLCK. In SCaM-4 the K30E and G40D mutations resulted in an inability to stimulate enzymatic activity, while binding to the CaMBD of smMLCK was still possible (68). The authors suggest that this site on the surface of Ca\(^{2+}\)-SCaM-1 makes critical interactions with an unknown region on the smMLCK enzyme that is absolutely necessary for its activation, while in Ca\(^{2+}\)-SCaM-4 the two negative charges on this surface disrupt the interaction. Together with our present study these studies demonstrate that the reciprocal regulation displayed by Ca\(^{2+}\)-SCaM-1 and –4 on target enzymes does not arise from a common structural difference, but instead from different local dissimilarities that are specific for each enzyme. Although the precise interactions that occur with the intact BCA1 protein are currently unknown, it is clear from our results that the structural recognition of the CaM-binding domain of this enzyme (CATPp) is very similar between Ca\(^{2+}\)-SCaM-1 and –4, and is comparable to the interactions between Ca\(^{2+}\)-mCaM and its binding domains from many CaM-dependent kinases.
Acknowledgements

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References


Abbreviations: calmodulin (CaM), calcium-ATPase peptide (CATPp), mammalian calmodulin (mCaM), soybean calmodulin (SCaM), nitric oxide synthase (NOS), smooth muscle myosin light chain kinase (smMLCK), skeletal muscle myosin light chain kinase (skMLCK), plasma membrane calcium-ATPase (PMCA), cauliflower vacuolar calcium-ATPase (BCA1), calmodulin dependent protein kinase I (CaMKI), isothermal titration calorimetry (ITC), dithiothreitol (DTT), circular dichroism (CD), trifluoroethanol (TFE), differential scanning calorimetry (DSC), calmodulin binding domain (CaMBD), cyclic nucleotide phosphodiesterase (PDE), calmodulin dependent protein kinase kinase (CaMKK).

Figure titles:

Figure 1: Non-denaturing (Urea) PAGE band shift analysis of the interactions of SCaM-1 and SCaM-4 with CATPp. Molar ratios of SCaM-1:CATPp were 1:0, 1:0.5, 1:1, 1:2 in lanes 1, 2, 3 and 4 respectively. Likewise the molar ratios for SCaM-4:CATPp were 1:0, 1:0.5, 1:1, 1:2 in lanes 5, 6, 7, and 8 respectively. Gel A was run in the presence of 1 mM CaCl₂, while gel B was run in the presence of 2 mM EDTA.

Figure 2: Calorimetric traces and derived binding isotherms for A) 12 µM Ca²⁺-SCaM-1 and B) 10 µM Ca²⁺-SCaM-4, each titrated with 600 µM CATPp, at 25°C. C) Plot of enthalpy change (ΔH) versus temperature for the interactions of CATPp with Ca²⁺-SCaM-1 (squares) and Ca²⁺-SCaM-4 (triangles). The slope of the linear regression of the data is equal to the heat capacity change (ΔCp) for the interaction.

Figure 3: Far-UV CD spectra of A) Ca²⁺-SCaM-1 (solid line), Ca²⁺-SCaM-1-CATPp (dashed line), B) Ca²⁺-SCaM-4 (solid line), Ca²⁺-SCaM-4-CATPp (dashed line), C) free CATPp (solid line), difference spectrum between Ca²⁺-SCaM-1-CATPp complex and Ca²⁺-SCaM-1 (dashed line), and difference spectrum between Ca²⁺-SCaM-4-CATPp complex and Ca²⁺-SCaM-4 (dotted line). All CD intensities are expressed as molar ellipticity (Θ).
Figure 4: Near-UV CD spectra of A) Ca$^{2+}$-SCaM-1 (solid line), Ca$^{2+}$-SCaM-1-CATPp (dashed line), and B) Ca$^{2+}$-SCaM-4 (solid line), Ca$^{2+}$-SCaM-4-CATPp (dashed line). All CD intensities are expressed as molar ellipticity ($\Theta$).

Figure 5: A) Steady state Trp fluorescence of free CATPp (solid line), Ca$^{2+}$-SCaM-1-CATPp (dotted line), and Ca$^{2+}$-SCaM-4-CATP (dashed line). B) Stern-Volmer plots of CsCl quenching titrations of CATPp (solid diamonds), Ca$^{2+}$-SCaM-1-CATPp (solid squares), and Ca$^{2+}$-SCaM-4-CATP (open triangles). $I_o$ is the initial fluorescence intensity before addition of CsCl and $I$ is the fluorescence intensity at each titration point after correction for dilution effects.

Figure 6: Sequence comparison of SCaM-1 and SCaM-4 with mCaM. The similarities in Met content are highlighted. ( * ) indicates identical residues; ( : ) indicates strongly conserved residues; and ( . ) indicates weakly conserved residues in all three proteins. Sequence alignment was generated using Clustal X 1.81.

Figure 7: Two dimensional $^1$H, $^{13}$C, HSQC NMR spectra of the Met methyl groups of $^{13}$C-Met-Ca$^{2+}$-SCaM-1 and $^{13}$C-Met-Ca$^{2+}$-SCaM-4 in the presence and absence of CATPp. Ca$^{2+}$-SCaM-1:CATPp ratios are as follows; A) 1:0, B) 1:0.5, C) 1:1.1. Likewise Ca$^{2+}$-SCaM-4:CATPp ratios are; D) 1:0, E) 1:0.5, F) 1:1.1. Note this similarity in chemical shifts for both Ca$^{2+}$-SCaM-1 and Ca$^{2+}$-SCaM-4, as well as their complexes with CATPp.

Figure 8: 20 lowest energy NMR structures of CATPp in 30% TFE, overlaid with respect to A) residues 5-16, and B) residues 18-23.

Figure 9: Sequence alignment of CATPp with the calmodulin binding domain from smooth muscle myosin light chain kinase (smMLCK), skeletal muscle myosin light chain kinase (skMLCK), calmodulin-dependent protein kinase I (CaMKI) and the ideal recognition sequences for SCaM-1 (1-5-10) and SCaM-4 (1-8-14) proposed by Choi et al, 2002 (66). The sequences are aligned with respect to their conserved Trp residue. Important hydrophobic residues are highlighted, and conserved basic residues are underlined.
Table 1: Thermodynamic parameters for the interactions of Ca$^{2+}$-SCaM-1 or Ca$^{2+}$-SCaM-4 with CATPp.

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<th>Temperature ($^\circ$C)</th>
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Figure 1:
Figure 2:
Figure 5

A

Fluorescence Intensity

320 360 400 440
Wavelength (nm)

B

$\frac{I_c}{I}$

0 0.5 1.0 1.5 2.0 2.5 3.0
[CsCl] (M)
Figure 7
Figure 9

CATPp  ARQRVSRSVSIVKNRARFRMISNL
smMLCK  ARRKKWQKTGHAVRAIGRLSS
skMLCK  KRRSKKNPIAVSAANRFKKISS
CaMKI   AKSKKQAFNATAVRHMRK

1-5-10 (SCaM-1)  ----H----H----H-----
1-8-14 (SCaM-4)  ----H-----H------H-
Structurally homologous binding of plant calmodulin isoforms to the calmodulin-binding domain of vacuolar calcium-ATPase
Aaron P. Yamniuk and Hans J. Vogel

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