The solution structure of the Mg^{2+} form of soybean calmodulin isoform 4 reveals unique features of plant calmodulins in resting cells

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Abstract: Soybean calmodulin isoform 4 (sCaM4) is a plant calcium-binding protein, regulating cellular responses to the second messenger Ca^{2+} . We have found that the metal ion free (apo-) form of sCaM4 possesses a half unfolded structure, with the N-terminal domain unfolded and the C-terminal domain folded. This result was unexpected as the apo-forms of both soybean calmodulin isoform 1 (sCaM1) and mammalian CaM (mCaM) are fully folded. Because of the fact that free Mg^{2+} ions are always present at high concentrations in cells (0.5–2 mM), we suggest that Mg^{2+} should be bound to sCaM4 in nonactivated cells. CD studies revealed that in the presence of Mg^{2+} the initially unfolded N-terminal domain of sCaM4 folds into an α -helix-rich structure, similar to the Ca^{2+} form. We have used the NMR backbone residual dipolar coupling restraints $^1\text{D}_{\text{NH}}$, $^1\text{D}_{\text{C}\alpha\text{H}\alpha}$, and $^1\text{D}_{\text{C}'\text{C}\alpha}$ to determine the solution structure of the N-terminal domain of Mg^{2+} -sCaM4 (Mg^{2+} -sCaM4-NT). Compared with the known structure of Ca^{2+} -sCaM4, the structure of the Mg^{2+} -sCaM4-NT does not fully open the hydrophobic pocket, which was further confirmed by the use of the fluorescent probe ANS. Tryptophan fluorescence experiments were used to study the interactions between Mg^{2+} -sCaM4 and CaM-binding peptides derived from smooth muscle myosin light chain kinase and plant glutamate decarboxylase. These results suggest that Mg^{2+} -sCaM4 does not bind to Ca^{2+} -CaM target peptides and therefore is functionally similar to apo-mCaM. The Mg^{2+} - and apo-structures of the sCaM4-NT provide unique insights into the structure and function of some plant calmodulins in resting cells.

Keywords: soybean calmodulin; calmodulin; magnesium; protein structure; nuclear magnetic resonance spectroscopy; residual dipolar coupling; calcium regulatory protein; calcium-binding protein

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonate; apo-, metal-free; CaBP1, calcium-binding protein 1; CIB1, calcium- and integrin-binding protein 1; cTnC, cardiac troponin C; cTnI, cardiac troponin I; mCaM, mammalian calmodulin; Mg^{2+} -sCaM4-NT, magnesium-bound N-domain of soybean calmodulin isoform 4; NCS, neuronal calcium sensor; RDC, residual dipolar coupling; RMSD, root-mean-square deviation; sCaM1, soybean calmodulin isoform 1; sCaM4, soybean calmodulin isoform 4; sCaM4-CT, C-domain of soybean calmodulin isoform 4 (residues 78–148); sCaM4-NT, N-domain of soybean calmodulin isoform 4 (residues 1–77). Additional Supporting Information may be found in the online version of this article.

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Introduction

The ubiquitous mammalian protein calmodulin (mCaM) is one of the most important proteins in Ca^{2+} signaling pathways in mammalian cells.¹ mCaM has a highly conserved primary sequence formed by 148 amino acids and a dumbbell-shaped structure with two folded domains, which are connected by a flexible central linker.² The helix-loop-helix Ca^{2+} -binding motif is termed an EF-hand and is considered to be one of the most common protein folds in animal cells.^{3,4} mCaM has four EF-hands, two in each domain, therefore mCaM is capable of binding four Ca^{2+} ions in its fully active form. In eukaryotic cells, mCaM has well-folded structures in both the Ca^{2+} -bound^{5,6} and apo-state.^{7,8} In the apo-state, the four helices in each domain of mCaM are essentially aligned in parallel and they form a closed conformation. In contrast, in the Ca^{2+} -bound state, the helices in each domain form an opened conformation and a hydrophobic pocket is exposed. The exposed hydrophobic region allows mCaM to specifically interact with over 100 binding targets.¹ Hence, the extent of the exposure of the hydrophobic core is crucial for mCaM's function and, in turn, is of great interest for mCaM structural and functional studies. To date, more than 50 mCaM structures have been determined and deposited into the protein data bank (PDB), including apo-, Ca^{2+} -, and complexed structures with various binding targets. Among these reported mCaM structures, comparisons revealed that the differences mainly reside in the conformation of the flexible central linker between the two domains and the loops linking the EF-hands and the Ca^{2+} -binding site in each EF-hand, while the helical structures of CaM are relatively conserved.^{9,10}

In plant cells, calmodulin is also one of the primary Ca^{2+} -binding proteins. Soybean calmodulin has at least five isoforms (SCaM 1–5), when compared with the single isoform that is present in mammalian cells.¹¹ *Arabidopsis thaliana* appears to have a total of 7–9 calmodulins and ~50 calmodulin-like proteins.^{12,13} Genome-wide analysis of the rice genome revealed the presence of five calmodulins and 32 calmodulin-like proteins.¹⁴ Soybean calmodulin isoform 1 (sCaM1) has about 90% sequence identity to mCaM, whereas soybean calmodulin isoform 4 (sCaM4) is more divergent with about 78% sequence identity.¹¹ It has been reported that these soybean isoforms have different target activation profiles that have been categorized into three types.^{15,16} Recently, we also reported the Ca^{2+} -bound solution structures of two sCaM isoforms, sCaM1 and sCaM4.¹⁷ Both Ca^{2+} -sCaM1 and Ca^{2+} -sCaM4 are structurally similar to Ca^{2+} -mCaM and have four calcium-binding sites. Unexpectedly, the C-terminal domain of Ca^{2+} -sCaM1 was found to have a more opened conformation than both Ca^{2+} -sCaM4 and Ca^{2+} -mCaM even though sCaM1 shares higher

sequence identity (91%) with mCaM than sCaM4 (78%). It was considered plausible that the difference in the degree of exposure of their hydrophobic regions was responsible for their differing potentials in target activation.¹⁷

The role of Mg^{2+} in the stabilization of Ca^{2+} -binding protein structures in resting cells has been previously reported.^{18–20} In resting cells, the Ca^{2+} concentration in the cytosol is around 10^{-7}M , whereas the Mg^{2+} concentration in the cell is maintained at $0.5\text{--}2 \times 10^{-3}\text{M}$. During activation, the concentration of Ca^{2+} rapidly increases to $10^{-6}\text{--}10^{-5}\text{M}$, and the mCaM in turn binds Ca^{2+} and opens part of its hydrophobic core to interact with its binding targets. For most free EF-hand proteins, the Ca^{2+} dissociation constant falls in the range of $10^{-6}\text{--}10^{-5}\text{M}$, while the affinity for Mg^{2+} is in the mM range. It is not unexpected that the divalent Mg^{2+} binds to the Ca^{2+} -binding site of various Ca^{2+} -binding proteins,^{21–23} as Mg^{2+} and Ca^{2+} are in the same group of the periodic table and have similar chemical properties. On the other hand, because of the different sizes of Mg^{2+} and Ca^{2+} , they have different binding affinities for the same binding loops of mCaM.²² Moreover, Mg^{2+} also tends to retain several H_2O molecules, whereas Ca^{2+} readily releases them while bound to proteins.⁴ Therefore, Mg^{2+} is assumed to occupy the binding loops of some EF-hands in resting cells and it can be replaced by higher affinity Ca^{2+} , once the cells are activated.

Because of the importance of the Ca^{2+} /mCaM regulation, the effects of the binding of Ca^{2+} and Mg^{2+} as well as other metal ions to mCaM on the structure have been investigated extensively by experimental approaches^{22–25} and molecular simulations.²⁶ In the molecular simulation study, it has been reported that the short-range distances between Mg^{2+} ion and the coordinated side chains of mCaM range from 2.06 to 2.26 Å.²⁶ The Glu12 in the conserved metal ion-binding loop, which has been postulated to bind to Mg^{2+} in a monodentate manner, has been calculated to be at long range distances of 3.32 and 2.86 Å for the first and second EF-hands, respectively.²⁶ These studies have also shown that Mg^{2+} and Ca^{2+} bind to the same site but that Mg^{2+} binding only changes the local structure of the binding loop, while the global fold remained unchanged. Therefore, when bound to Mg^{2+} , the hydrophobic surface of the mCaM is not exposed.^{22,23} The key to the difference between Ca^{2+} binding and Mg^{2+} binding is that the Glu residue at the 12th position of the binding loop does not bind Mg^{2+} in a bidentate manner as Ca^{2+} does.^{22,23}

Despite extensive studies conducted on the Mg^{2+} -bound form of mCaM structure and functions, the Mg^{2+} -bound structure of native mCaM or any other CaM isoforms has still not been reported. In addition, even though the Ca^{2+} -bound sCaM1 and

Figure 1. HSQC spectra of apo-sCaM4 (A) N-terminal domain (NT) and (B) C-terminal domain (CT). Both samples contain 0.5 mM ^{15}N -labeled protein, 2 mM EDTA, 100 mM KCl, 5 mM DTT, and pH 7.0 ± 0.1 .

sCaM4 solution structures have been determined,¹⁷ there is still a shortage of knowledge on the corresponding structures of plant calmodulins in resting cells. In our studies using NMR and circular dichroism (CD) spectroscopy, we have found that the apo-form of sCaM4 is partially unfolded, which was unexpected as both the apo-sCaM1 and apo-mCaM have well-folded overall structures. Subsequently, the NMR backbone assignment of the N-domain of Mg^{2+} -sCaM4 was completed and the solution structure of the N-terminal domain of Mg^{2+} -sCaM4 was, in turn, determined with the residual dipolar coupling (RDC)-based structure determination approach.²⁷ The exposed hydrophobic surface area of the sCaM4 N-terminal and C-terminal domains was investigated by ANS fluorescence. Interaction studies of Mg^{2+} -sCaM4 with typical Ca^{2+} -CaM-binding peptides were also carried out using steady-state tryptophan (Trp) fluorescence spectroscopy.

Results

apo-sCaM4 is half folded, with its N-domain unfolded and the C-domain folded

The apo-sCaM4 HSQC spectrum shows that the apo-sCaM4 is only partially folded [Supporting Information Fig. 1(A)]. As calmodulin normally has a marked bilobal structure, the two domains were separately cloned, expressed, and purified. The HSQC spectra of apo-sCaM4-NT and apo-sCaM4-CT clearly indicate that the apo-sCaM4-NT is unfolded and the apo-sCaM4-CT has a well-defined fold [Fig. 1(A,B)]. The comparison between the HSQC spectra of apo-sCaM4 and the superposition of apo-sCaM4-NT and apo-sCaM4-CT suggests that the two domains of sCaM4 are essentially independent. A very small difference between these two spectra [Supporting Information Fig. 1(A,B)] indicates that the covalent connection between these two domains has only a very

small effect on each domain's structure. In the HSQC spectrum of apo-sCaM4-NT [Fig. 1(A)], the backbone NH peaks span only 0.4 ppm from 8.3 to 8.7 ppm in the ^1H dimension, clearly indicating an unfolded conformation of this protein lobe in solution. In contrast, apo-sCaM4-CT shows well-dispersed peaks in the HSQC spectrum, which is a characteristic of a well-folded protein [Fig. 1(B)]. The folding of apo-, Mg^{2+} -, and Ca^{2+} -bound sCaM4-NT and sCaM4-CT was also examined using CD spectroscopy (Fig. 2). In Figure 2(A), the CD trace of apo-sCaM4-NT has the biggest absorbance at 202 nm and no significant absorbance in the range of 225–230 nm, which suggests that the conformation of apo-sCaM4-NT is close to random coil. In contrast, the presence of Mg^{2+} ions drastically changed the absorbance of sCaM4-NT, and it shows a typical negative band for α -helical structure at 208 nm and a shoulder around 222 nm. The addition of Ca^{2+} ions slightly increases the helical contents of sCaM4-NT over Mg^{2+} ions. Similarly, the CD spectrum of sCaM4-CT indicates that Ca^{2+} promotes sCaM4-CT to take on a higher percentage of and/or a more stable α -helical structure. The CD profile of apo-sCaM4-CT shows a negative peak in the range of 204 nm and some negative absorbance in the range around 222 nm, indicating the existence of significant secondary structure [Fig. 2(B)]. In contrast, the apo-form of the sCaM4-NT displays a broad peak in the 198–202 nm range [Fig. 2(A)] when compared with the apo-sCaM4-CT [Fig. 2(B)]. In either sample, adding more Mg^{2+} or Ca^{2+} did not result in noticeable changes of the CD spectra, indicating that both samples were saturated and that the differences in the CD traces were caused by the intrinsic structural properties of Mg^{2+} -sCaM4-NT and Ca^{2+} -sCaM4-NT. Previous research on mCaM has shown that Ca^{2+} has at least a 100-fold higher binding affinity for mCaM than Mg^{2+} ,^{22,23} and the binding of

Figure 2. Circular dichroism (CD) spectra of sCaM4-NT (A) and sCaM4-CT (B) at apo- (—), Mg- (.....), and Ca- (---) solution conditions, recorded at room temperature with 10 μ M protein samples.

Ca^{2+} more effectively stabilizes the helical structure in mCaM when compared with Mg^{2+} . Taken together with the NMR data, these results support a model whereby apo-sCaM4 is a half-folded protein. The HSQC spectra of apo-sCaM4 were also acquired at various temperatures ranging from 5 to 40°C, and no substantial changes were observed (Supporting Information Fig. 2). Also, various KCl concentrations ranging from 100 to 500 mM were tested to show that the induced folding is specific for Mg^{2+} -addition and not a nonspecific ionic effect (data not shown).

Mg^{2+} binds to all four EF-hands of sCaM4 but binds to the first loop with higher affinity

The backbone assignment of Mg^{2+} -sCaM4 was achieved by analyzing ^{15}N , ^{13}C -labeled sCaM4 in the presence of 20 mM MgCl_2 . Only the N-terminal domain gave rise to a nearly complete assignment for residues 3–76, whereas the C-terminal domain has a significant number of peaks missing because of intermediate chemical exchange. In the assigned ^1H , ^{15}N -HSQC spectrum, the resonances corresponding to residue G25 in binding loop I were observed, whereas G61 in binding loop II is not observed (Fig. 3). For the C-terminal domain of apo-sCaM4, the backbone assignment in the apo-state was over 95% complete (results not shown), but the backbone assignment of the full-length sCaM4 in the Mg^{2+} -state cannot be completed. The key residues on binding loops have been respectively highlighted with circles for the first EF-hand and boxes for the second EF-hand, whereas all other peaks corresponding to residues on Mg^{2+} -sCaM4-CT loop III and loop IV are absent (Fig. 3), indicating the effect of line broadening caused by relatively weak Mg^{2+} interaction with the third and fourth EF-hands of sCaM4-CT. To summarize, all four EF-hands of sCaM4 seem to bind Mg^{2+} ,

and although the binding of Mg^{2+} to the first and second EF-hands in the N-domain converts this unfolded lobe to a well-folded conformation, the weak binding of Mg^{2+} to the third and fourth EF-hands in the C-domain causes the disappearance of several peaks from the HSQC spectrum. The observation of Mg^{2+} coordinating to G25 indicates that the first EF-hand has a somewhat higher binding affinity for Mg^{2+} .

Figure 3. Assigned HSQC spectrum of Mg^{2+} -sCaM4. The nearly complete assignment of the N-lobe could be obtained. However, the backbone assignment of the C-terminal domain could only be partially obtained because of intermediate exchange on the NMR chemical shift time scale. The regions in the C-lobe that could be identified are from the helices but the two Mg^{2+} -saturated EF-hand loops could not be detected. Similar problems were encountered when the structure of the Mg^{2+} form of mCaM was determined by NMR, necessitating the introduction of mutations in the calcium-binding loops (PDB code 2EQC). The peaks with circles are on the Mg^{2+} -binding loop 1 and the peaks with boxes are on the Mg^{2+} -binding loop 2. The peaks with asterisks are folded over in the HSQC spectrum.

Table I. Interhelical Angles of the EF-Hands in the sCaM4, mCaM, cTnC, and CaBP1

Helix pair	sCaM4-NT ^a		mCaM ^b			cTnC-CT ^c		CaBP1-CT ^d	
	Mg ²⁺	Ca ²⁺	apo	Mg ²⁺	Ca ²⁺	Mg ²⁺	Ca ²⁺	Mg ²⁺	Ca ²⁺
$\alpha 1/\alpha 2$ (EF1)	121 $\pm$ 1	104	131						
$\alpha 3/\alpha 4$ (EF2)	134 $\pm$ 2	101	132						
$\alpha 5/\alpha 6$ (EF3)			140	124	108	91	85	133	101
$\alpha 7/\alpha 8$ (EF4)			127	120	103	107	94	132	109

Residues in the helices are as follows:

^a Mg²⁺-sCaM4 and Ca²⁺-sCaM4 (PDB code [2ROA](#)): ($\alpha 1$) 6–18, ($\alpha 2$) 29–38, ($\alpha 3$) 45–55, and ($\alpha 4$) 65–73.

^b apo-mCaM (PDB code [1F70](#) for N-domain and [1F71](#) for C-domain): ($\alpha 1$) 6–18, ($\alpha 2$) 29–38, ($\alpha 3$) 45–55, ($\alpha 4$) 65–73, ($\alpha 5$) 82–91, ($\alpha 6$) 102–111, ($\alpha 7$) 118–127, ($\alpha 8$) 138–147. Mg²⁺-mCaM (E104D/E140D mutant, PDB code for C-domain [2EQC](#)): ($\alpha 5$) 83–92, ($\alpha 6$) 102–111, ($\alpha 7$) 118–128, ($\alpha 8$) 138–145. Ca²⁺-mCaM (PDB code for C-domain [1J7P](#)): ($\alpha 5$) 83–92, ($\alpha 6$) 102–111, ($\alpha 7$) 118–128, ($\alpha 8$) 138–145.

^c Mg²⁺-cTnC in complex with cTnI (PDB code for C-domain [1SBJ](#)): ($\alpha 5$) 94–104, ($\alpha 6$) 114–122, ($\alpha 7$) 130–140, ($\alpha 8$) 150–156. Ca²⁺-cTnC in complex with cTnI (PDB code for C-domain [1FI5](#)): ($\alpha 5$) 94–104, ($\alpha 6$) 114–122, ($\alpha 7$) 130–140, ($\alpha 8$) 150–157.

^d Mg²⁺-CaBP1 (PDB code for C-domain [2K7C](#)): ($\alpha 5$) 101–111, ($\alpha 6$) 121–130, ($\alpha 7$) 138–147, ($\alpha 8$) 158–166. Ca²⁺-CaBP1 (PDB code for C-domain [2K7D](#)): ($\alpha 5$) 102–111, ($\alpha 6$) 121–131, ($\alpha 7$) 139–147, ($\alpha 8$) 158–165.

The secondary structure of Mg²⁺-sCaM4-NT is conserved

Based on the backbone assignment and the subsequent chemical shift index analysis,²⁸ it was determined that Mg²⁺-sCaM4-NT adopts a typical helix-loop-helix structure conformation of two EF-hands (Supporting Information Fig. 3). The first EF-hand has two helices, including residues 6–18 and 29–38, with the binding loop (residues 19–28) in between. The second EF-hand includes residues 45–55/56–64/65–73 as the corresponding helix/loop/helix components. This secondary structure arrangement is not only similar to Ca²⁺-sCaM4-NT but also similar to many other calcium-binding proteins, such as mCaM and CaBP1.²⁹

Mg²⁺-sCaM4-NT has a closed structure, which is similar to the apo-form of mCaM

The sCaM4-NT structure calculation was primarily based on three sets of backbone RDC restraints, including ¹D_{NH}, ¹D_{C α H α} , and ¹D_{C' C α} . For the dihedral angles φ and ψ , the ratio of residues in favored regions is around 83%, and the ratios for residues in allowed regions is about 17% with no residues in disallowed regions (Supporting Information Table I). The experimental restraints and calculation statistics data are summarized in Supporting Information Table I. Two different starting models (Ca²⁺-sCaM4-NT structure (PDB code [2ROA](#)) and the apo-sCaM4-NT model) have been used in Xplor-NIH calculations to verify our approach, in which the apo-sCaM4-NT model was generated from the apo-mCaM-NT structure (PDB code [1F70](#)) by homology modeling. The backbone residues of the well-folded region between residues 6 and 73 were used to compare the two structures of Mg²⁺-sCaM4-NT obtained from the different starting models [Fig. 4(A)]. The RMSD of the apo- and Ca²⁺-starting models derived Mg²⁺-sCaM4-

NT structures is only 1.00 Å, indicating a good agreement using the same restraints but different starting models. It should be noted that this comparison not only includes the region of secondary structures but also the flexible Mg²⁺-binding loops. This indicates that the backbone RDCs and the Mg²⁺-binding loop restraints used²⁶ precisely defined the local structure of this relatively flexible area. The calculated Mg²⁺-sCaM4-NT structures both adopt a closed conformation, which is more similar to apo-mCaM than Ca²⁺-mCaM. Henceforth, the Mg²⁺-sCaM4-NT structure derived from the apo-starting model is used as the representative of Mg²⁺-sCaM4-NT (PDB code [2KSZ](#)). In Figure 4(B), the Mg²⁺-sCaM4-NT structure calculated from the apo-starting model was compared to the apo-mCaM-NT structure (PDB code [1F70](#)). This Mg²⁺-sCaM4-NT structure shows a high degree of similarity to the apo-mCaM-NT structure with an RMSD of 2.08 Å for the well-folded backbone region (residues 6–73). Interhelix angles were calculated using a homemade script to compare the Mg²⁺-sCaM4-NT and Ca²⁺-sCaM4-NT as well as the apo- and Ca-mCaM structures. The interhelix angle for each EF-hand provides a measure of the degree of opening or hydrophobic pocket exposure in EF-hand proteins.^{21,30} A smaller angle actually indicates a more opened conformation, whereas a larger angle indicates a more closed conformation and a less exposed hydrophobic region. In Table I, the angle between the two helices in the first EF-hand is 121° $\pm$ 1° for Mg²⁺-sCaM4-NT, which is quite different from the 104° for Ca²⁺-sCaM4-NT but just around 10° more opened than that of apo-mCaM (131°), suggesting that the binding of Mg²⁺ to the first EF-hand does not result in a significant opening of the hydrophobic core of sCaM4-NT. For the second EF-hand, the angle between the two helices in the Mg²⁺-sCaM4-NT structure derived from the apo-sCaM4-NT structure

is $134^\circ \pm 2^\circ$, when compared with 132° for apo-mCaM-NT, suggesting that the exposure of the hydrophobic core of the second EF-hand caused by Mg^{2+} binding is negligible. Compared with the Ca^{2+} -sCaM4-NT structure [Fig. 4(C)], Mg^{2+} -sCaM4-NT shows an RMSD of 3.58 Å in well-defined backbone region (residues 6–73), indicating a relatively larger difference. In terms of the opening of the helices of EF-hands, Ca^{2+} -sCaM4-NT has an angle of 104° corresponding to $121^\circ \pm 1^\circ$ for Mg^{2+} -sCaM4-NT for the first EF-hand and 101° for the second EF-hand corresponding to $134^\circ \pm 2^\circ$ for Mg^{2+} -sCaM4-NT (Table I). It is clear that Mg^{2+} -sCaM4-NT has a

relatively more closed conformation in both EF-hands when compared with the Ca^{2+} form.

The exposed hydrophobic core of Mg^{2+} -sCaM4-NT

To further test this calculated structural result, the fluorescence probe ANS has been used to detect the exposed hydrophobic area of apo-, Mg^{2+} -, and Ca^{2+} -sCaM4-NT. The aromatic fluorophore ANS shows a weak fluorescence signal in aqueous solution. When it binds to a nonpolar hydrophobic patch of proteins, its spectrum undergoes a noticeable blue shift and increased intensity. The detected fluorescence signal of Ca^{2+} -sCaM4-NT/ANS is significantly higher than either that of Mg^{2+} -sCaM4-NT/ANS or apo-sCaM4-NT/ANS. The Mg^{2+} - and apo-sCaM4-NT forms display a similar extent of ANS binding [Fig. 5(A)]. This result is consistent with our calculated structures. To estimate the degree of the opening of the C-lobe Mg^{2+} -sCaM4, we also performed ANS binding studies with the sCaM4-CT [Fig. 5(B)]. The results are almost identical to those obtained from the sCaM4-NT, suggesting that the two lobes of the protein have similar structural properties when saturated with Mg^{2+} .

Interaction with binding targets

Two peptides containing a Trp residue were selected to test the Mg^{2+} -sCaM4 target interactions, an approach that is useful as the protein itself does not possess any Trp. The peptides display an enhanced fluorescence signal and a blue shift upon interaction with their calcium-saturated binding target. In this study, one peptide was derived from plant glutamate decarboxylase (GAD) and the other one was derived from smooth muscle myosin light chain kinase (smMLCK). In Figure 6(A), apo-sCaM4, which does not bind to GAD peptide, shows the same trace as

Figure 4. The comparison of two Mg^{2+} -sCaM4-NT structures calculated with the same restraints but from two different starting models (A), and the comparisons of the Mg^{2+} -sCaM4-NT structure (PDB code 2KSZ) with the apo-mCaM-NT structure (PDB code 1F70) (B) and the Ca^{2+} -sCaM4-NT structure (PDB code 2ROA) (C). In (A), the blue structure is calculated from apo-sCaM4-NT as the starting model, which is generated from the apo-mCaM-NT structure (PDB code 1F70), and the red one is derived from Ca^{2+} -sCaM4-NT structure (PDB code 2ROA). The red and blue balls are the coordinated Mg^{2+} ions. The rmsd of the backbone structure for residues 6–73 between these two structures is 1.00 Å. In (B) and (C), the structures in magenta, blue, and green colors represent apo-mCaM-NT, Mg^{2+} -sCaM4-NT, and Ca^{2+} -sCaM4-NT structures, respectively. The superposition of residues 6–73 of Mg^{2+} -sCaM4-NT and apo-mCaM-NT results in an RMSD of 2.08 Å, and the corresponding one between Mg^{2+} -sCaM4-NT and Ca^{2+} -sCaM4-NT is 3.58 Å.

Figure 5. Fluorescence spectra of 1-anilino-8-naphthalene sulfonate (ANS) when it interacts with sCaM4-NT (A) and sCaM4-CT (B) recorded for apo-, Mg^{2+} -, and Ca^{2+} -solution conditions.

the peptide alone. In the presence of Mg^{2+} -sCaM4, the Trp residue in GAD shows a similar fluorescence pattern as apo-sCaM4, indicating no specific interaction between Mg^{2+} -sCaM4 and GAD. The positive control Ca^{2+} -sCaM4 shows an enhancement in the fluorescence intensity with a significant blue shift. Similarly, the smMLCK peptide does not interact with either apo- or Mg^{2+} -sCaM4 but interacts with Ca^{2+} -sCaM4 [Fig. 6(B)]. From the Trp fluorescence data, it can be concluded that the Mg^{2+} -sCaM4 is functionally similar to apo-sCaM4 despite its more ordered structure when compared with apo-sCaM4.

Discussion and Conclusion

The role of Mg^{2+} in Ca^{2+} signaling pathways has been investigated by focusing on mCaM and other related Ca^{2+} -binding proteins.^{18–21,29,30} The role of Mg^{2+} in stabilizing tertiary structures of EF-hand proteins has been reported in several cases,⁴ such as the neuronal calcium sensor-1 (NCS-1)¹⁸, CIB1,²⁰ and calexcitin B.³¹ For mCaM, many studies have

postulated that Mg^{2+} alters the local structure of the metal-binding loop, but that it has no substantial effect on its global structure.^{22,23} However, there are cases of other Ca^{2+} -binding proteins, where the situation is different. The Mg^{2+} form of cTnC, for example, complexed with the N-domain of cTnI, has been reported to adopt a very similar structure to its Ca^{2+} form with an RMSD of 0.94 Å for all backbone atoms.²¹ Therefore, the elucidation of the Mg^{2+} -saturated structure of calmodulin isoforms was needed to resolve these issues. However, to date, the weak binding affinity of Mg^{2+} to mCaM has hindered crystallization and, in turn, the determination of a high-resolution crystal structure. As an alternative approach, multidimensional NMR spectroscopy could be used, but conformational exchange makes this approach less straightforward. In this study, the

Figure 6. Fluorescence spectra of two CaM-binding peptides containing a Trp residue, studying the interaction between these peptides with sCaM4-NT at apo- (—), Mg - (---), and Ca - (...△...) solution conditions, recorded at room temperature with 10 μM peptide samples. (A) Interaction between plant glutamate decarboxylase (GAD) peptide and sCaM4; (B) interaction between smooth muscle form of myosin light chain kinase (smMLCK) and sCaM4.

RDC-based protein homology structure determination approach was implemented to determine the solution structure of Mg^{2+} -sCaM4-NT. Even when NOEs cannot be unambiguously identified, backbone RDCs can usually still be assigned with certainty. This RDC-based approach allows for a solution even for cases involving intermediate exchange NMR signal broadening. Another advantage of this approach is that it provides a quick and reliable way to determine the structure of protein homologs where the secondary structure is conserved and the overall tertiary structure of a homologous protein is available.²⁷

The protein sCaM4 was chosen for this structure determination as it unexpectedly has a half-folded structure in the apo-form. The CD and NMR results indicate that sCaM4 is half-folded in the apo-state, and interestingly the folded/unfolded fragments are clearly separated by the central linker between the N- and C-terminal domains, with the N-terminal domain being unfolded and the C-terminal domain folded. The calculated Mg^{2+} -sCaM4-NT structures from two different calculation starting models of apo-sCaM4-NT (a homology model generated from the apo-mCaM structure) and Ca^{2+} -sCaM4-NT structure show a good agreement with an RMSD of 1.00 Å for the backbone atoms of the well-defined regions. This good agreement provided a validation of the general approach. The examination of Mg^{2+} -sCaM4-NT revealed that the binding of Mg^{2+} changed the unfolded structure into a well-folded structure with two characteristic helix-loop-helix EF-hands and strong similarity to apo-mCaM-NT. The folded Mg^{2+} -sCaM4-NT is overall similar to the apo-mCaM-NT structure except for the local structure of the metal-binding site. The presence of Mg^{2+} just slightly opened the first EF-hand of sCaM4-NT when compared with apo-mCaM-NT. Mg^{2+} -sCaM4 displays a secondary structure similar to that of Ca^{2+} -sCaM4-NT; however, the orientation of the secondary structural components is different in the presence of Ca^{2+} and Mg^{2+} , especially with regards to the orientation of the helices in each EF-hand.

For sCaM4-NT, Mg^{2+} does not have significant effects on the exposure of the hydrophobic core. This result is similar to a recent study examining the Mg^{2+} - and Ca^{2+} -bound C-domain structures of calcium-binding protein 1 (CaBP1).²⁹ In the case of CaBP1, the Mg^{2+} -bound C-domain structure has an interhelical angle of 133° for the third EF-hand and 132° for the fourth EF-hand, whereas the Ca^{2+} -bound CaBP1 has a more opened conformation (101° for third EF-hand and 109° for the fourth EF-hand) [Table I, Fig. 7(A)]. Interestingly, the N-domain of CaBP1 does not bind Ca^{2+} , thus the Mg^{2+} -bound structure is the most likely form in both resting and activated cells, and it is similar to the apo-mCaM structure.²⁹

Figure 7. Comparisons of Mg^{2+} -structures to corresponding apo- or Ca^{2+} -structure for CaBP1, mCaM, and cTnC. (A) Superposition of Mg^{2+} -CaBP1 C-domain (blue, PDB code 2K7C) and Ca^{2+} -CaBP1 C-domain (red, PDB code 2K7D); (B) superposition of Mg^{2+} -cTnC C-domain in complex with cTnI (blue, PDB code 1SBJ) and Ca^{2+} -cTnC C-domain in complex with cTnI (red, PDB code 1FI5); (C) superposition of Mg^{2+} -mCaM E104D/E140D mutant C-domain (blue, PDB code 2EQC) and Ca^{2+} -mCaM C-domain (red, PDB code 1J7P); (D) superposition of apo-mCaM C-domain (purple, PDB code 1F70) and Mg^{2+} -mCaM E104D/E140D mutant C-domain (blue, PDB code 2EQC).

A different example is the C-domain of cTnC complexed with cTnI,²¹ where the Mg^{2+} -bound form is very similar to the Ca^{2+} -bound form, and the interhelical angle is 91° for the third EF-hand and 107° for the fourth EF-hand of Mg^{2+} -bound form, whereas the interhelical angles are 85° for the third EF-hand and 94° for the fourth EF-hand of Ca^{2+} -bound form. Therefore, even though the Mg^{2+} - and cTnI-bound cTnC structure is very similar to the Ca^{2+} -bound form, the EF-hands are still more closed than the Ca^{2+} -bound form [Fig. 7(B)]. Another interesting example is the C-domain of the mCaM E104D/E140D mutant, in which two mutations were introduced to improve the spectral quality for structure determination of the Mg^{2+} -bound mCaM E104D/E140D mutant (PDB code 2EQC). In this nonnative structure of mCaM, significant differences exist between the Mg^{2+} -bound structure and Ca^{2+} -bound wild-type mCaM: the interhelical angles are 124° and 120° for the third and fourth EF-hands of the Mg^{2+} -form, respectively, whereas the corresponding angles are 108° and 103° for the third and fourth EF-hands of the Ca^{2+} -form, respectively [Table I and Fig. 7(C)]. By comparison to apo-mCaM, this nonnative Mg^{2+} -loaded structure is more similar to the apo-form than the Ca^{2+} -loaded form [Fig. 7(C,D)].

Taken together, for many calcium-binding proteins, the binding of Mg^{2+} to EF-hands results in only a slight opening of the interhelical angle or shows no difference from the apo-structure.

Overall, the unique structural properties of sCaM4 may facilitate its biological role. The biosynthesis of this protein is markedly increased during the plant stress response,³² and this is accompanied by an increase in the cytoplasmic calcium concentration.³³ Be that as it may, the unexpected folding transition that accompanies Mg^{2+} binding to the N-lobe of sCaM4 is also expected to influence the calcium affinity of this protein.

Materials and Methods

Sample preparation

The clone of the sCaM4 fragment sCaM4NT (residues 1–77) was generated by mutating the codon encoding D78 to a stop codon using the Quick-Change site-directed mutagenesis kit (Stratagene). The C-terminal fragment of sCaM4 (sCaM4-CT: residues 78–148) was amplified from the sCaM4 gene by standard PCR techniques and then subcloned into the pET30b vector using NdeI/XhoI sites. ^{13}C , ^{15}N double-labeled sCaM4, ^{15}N -labeled sCaM4-NT, sCaM4-CT, and sCaM4 were expressed and purified as described previously.^{34,35} Protein concentrations were determined by using the Bio-Rad protein assay kit. The two calcium-binding peptides used were GADp (Ac-GSHKKT DSEVQLEMITAWKKFVEEKK KK-NH₂) and smMLCKp (Ac-ARRKWQKTGHAVRA IGRLLS-NH₂). Both synthetic peptides were purchased from AnaSpec (more than 95% pure as determined by mass spectroscopy and HPLC).

Circular dichroism spectroscopy

CD spectra were acquired at room temperature on a Jasco J-810 spectropolarimeter. Far-UV CD spectra were measured from 240–195 nm using a cylindrical quartz cuvette with a path length of 0.1 cm and a volume of 300 μL using the following parameters: step resolution 0.2 nm, speed 50 nm/min, response time 2 s, bandwidth 1 nm, and sensitivity 50 mdeg. All traces were the average of 10 scans, and the data were smoothed and converted to molar ellipticity using Jasco software. Samples consisted of 10 μM protein, 5 mM HEPES buffer (pH 7.5), and 1 mM dithiothreitol (DTT). The apo-sample contained 2 mM ethylenediaminetetraacetic acid (EDTA); the Mg^{2+} -sample contained 0.5 mM ethylene glycol tetraacetic acid (EGTA) and 3 mM MgCl_2 . The Ca^{2+} -sample contained 2 mM CaCl_2 .

Fluorescence spectroscopy

Fluorescence spectra were acquired at room temperature on a Varian Cary Eclipse spectrofluorimeter.

ANS fluorescence experiments were conducted with samples containing 60 μM sCaM4-NT, 1 mM DTT, 180 μM ANS, pH 7.6 with the same solution conditions for the apo-, Mg^{2+} -, and Ca^{2+} -samples as used for the CD experiments. The excitation wavelength was 370 nm and the recording emission was from 400 to 600 nm using excitation and emission slit widths of 5 and 10 nm, respectively. For the protein interaction fluorescence experiments, the peptides containing Trp were excited at 295 nm, and the fluorescence emission was measured from 300 to 450 nm, with excitation and emission slit widths of 5 and 10 nm, respectively. All samples contained 50 mM HEPES (pH 7.6), 1 mM DTT, and 10 μM sCaM4. Because of the previous observation that mCaM binds to the smMLCK peptide in a 1:1 ratio³⁶ and the GAD peptide in a 1:2 ratio,³⁷ the concentrations of the smMLCK peptide and the GAD peptide were 10 and 20 μM , respectively. The solution conditions for the apo-, Mg^{2+} -, and Ca^{2+} -samples were identical to those used in CD experiments.

Nuclear magnetic resonance spectroscopy

All NMR spectra were acquired at 303 K on a Bruker Avance 500 NMR spectrometer equipped with a triple resonance inverse Cryoprobe with a single axis z-gradient. Samples contained 500 μM ^{15}N -sCaM4-NT, ^{15}N -sCaM4-CT, ^{15}N -sCaM4, or ^{15}N , ^{13}C -sCaM4 in 100 mM KCl, 10% D₂O, pH = 7.0 $\pm$ 0.1, and the same solution conditions as the CD experiments for the apo-, Mg^{2+} -, and Ca^{2+} -samples were used, except that the MgCl_2 concentration was increased to 20 mM to counteract the added EGTA and to fully saturate the proteins. A ^{15}N -sCaM4 sample was used for RDC $^1\text{D}_{\text{NH}}$ acquisition and another ^{15}N , ^{13}C -sCaM4 sample was used to obtain the other two sets of RDCs ($^1\text{D}_{\text{C}\alpha\text{H}\alpha}$ and $^1\text{D}_{\text{C}'\text{C}\alpha}$). In both samples, 16 mg/mL pfl phage (Asla Biotech) was used to achieve partial alignment of protein molecules. Sequential assignments of HN, N, CO, C $_{\alpha}$, and C $_{\beta}$ resonances of Mg^{2+} -sCaM4 were achieved using three-dimensional experiments, including HNCACB, CBCA(CO)NH, HNCO, and HN(CO)CA. Three sets of backbone RDCs could be measured for sCaM4, including backbone $^1\text{D}_{\text{NH}}$, $^1\text{D}_{\text{C}\alpha\text{H}\alpha}$, and $^1\text{D}_{\text{C}'\text{C}\alpha}$. The $^1\text{D}_{\text{NH}}$ RDC was measured using 2D IPAP-HSQC³⁸ with complex points of 1024 $\times$ 1024. After linear prediction and zero filling, the digital resolution was 0.83 Hz/pt in the ^{15}N dimension. The RDC of $^1\text{D}_{\text{C}\alpha\text{H}\alpha}$ was measured using 3D IPAP-J-HNCO (CA),³⁹ with 1024 $\times$ 64 $\times$ 36 complex points. The digital resolution was 2.95 Hz/pt in F2 (^{13}C). A scale factor of 2 was used in the measurement of the $^1\text{D}_{\text{C}\alpha\text{H}\alpha}$ RDC. The RDC of $^1\text{D}_{\text{C}'\text{C}\alpha}$ was measured using 3D IPAP-J-HNCO,⁴⁰ with 1024 $\times$ 64 $\times$ 36 complex points, the same digital resolution as the $^1\text{D}_{\text{C}\alpha\text{H}\alpha}$ experiment was obtained.

Structural calculation

A two-step simulated annealing approach²⁷ using the program XPLOR-NIH 2.2⁴¹ was implemented for the structure determination of Mg^{2+} -sCaM4-NT. For step 1, two starting models, the Ca^{2+} -sCaM4-NT (PDB code 2ROA)¹⁷ and apo-sCaM4-NT, have been used. The apo-sCaM4-NT structure was generated from the apo-mCaM-NT structure (PDB code 1F70)²⁷ using the online server Geno3D⁴² based on primary sequence homology. After the homology model was created, Powell gradient energy minimization was implemented. The dihedral angle restraints were predicted from NMR chemical shifts using TALOS.⁴³ In the calculation using Ca^{2+} -sCaM4-NT as the starting model, the dihedral angle restraints, hydrogen bond restraints, and the Mg^{2+} coordination restraints were implemented in simulated annealing step 1. In simulated annealing step 1, cooling was achieved by lowering the temperature from 200 to 20 K with each step $\Delta T = 10$ K. The force constant of the dipolar coupling was ramped from 0.05 to 5 kcal mol⁻¹ Hz⁻². In simulated annealing step 2, cooling was achieved by lowering the temperature from 20 to 1 K with each step $\Delta T = 1$ K. The dipolar coupling force constant was kept static with 1 kcal mol⁻¹ Hz⁻². Instead of using the energy term of radius of gyration, a new term called the volume of gyration⁴⁴ was implemented to improve the packing of the protein, and it was kept static with a force constant of 1 kcal mol⁻¹ Å⁻³ throughout this two-step simulated annealing. In both steps, the dynamics time at each temperature step was optimized to be 4 ps. All other energy force constants in step 1 and step 2 are the same as reported elsewhere.²⁷ In the calculation, the RDC values of ¹D_{CαHα} and ¹D_{C' Cα} were normalized based on ¹D_{NH} RDC values. Separate calculations revealed that all three RDC restraints were required to achieve the lowest RMSD values (data not shown). Mg^{2+} -ligand coordinate restraints have also been used. These distance restraints are based on the simulated results with ±0.1 Å to allow for reasonable variations.²⁶ In fact, it has been reported that the distances between Mg^{2+} ion and the coordinated side chains range from 2.06 to 2.26 Å.²⁶ The Glu12 in the conserved metal ion-binding loop, which has been postulated to bind to Mg^{2+} in a monodentate manner, has been calculated to be at long range distances of 3.32 and 2.86 Å for the first and second EF-hands, respectively.²⁶ The Mg^{2+} -sCaM4-NT structure obtained from the apo-sCaM4-NT model has been deposited into PDB with the code 2KSZ.

Further characterization of calculated structures

The lowest dipolar energy Mg^{2+} -sCaM4-NT structures starting from apo-sCaM4-NT structure was

selected for further analysis. Interhelical angles were measured using a home-made script. Furthermore, the overall quality of the final structures was also assessed by using the program of Procheck.⁴⁵

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