The intracellular calcium ion is one of the most important secondary messengers in eukaryotic cells. Ca\(^{2+}\) signals are translated into physiological responses by EF-hand calcium-binding proteins such as calmodulin (CaM). Multiple CaM isoforms occur in plant cells, whereas only a single CaM protein is found in animals. Soybean CaM isoform 1 (sCaM1) shares 90% amino acid sequence identity with animal CaM (aCaM), whereas sCaM4 is only 78% identical. These two sCaM isoforms have distinct target-enzyme activation properties and physiological functions. sCaM4 is highly expressed during the self-defense reaction of the plant and activates the enzyme nitric-oxide synthase (NOS), whereas sCaM1 is incapable of activating NOS. The mechanism of selective target activation by plant CaM isoforms is poorly understood. We have determined high resolution NMR solution structures of Ca\(^{2+}\)-sCaM1 and -sCaM4. These were compared with previously determined Ca\(^{2+}\)-aCaM structures. For the N-lobe of the protein, the solution structures of Ca\(^{2+}\)-sCaM1, -sCaM4, and -aCaM all closely resemble each other. However, despite the high sequence identity with aCaM, the C-lobe of Ca\(^{2+}\)-sCaM1 has a more open conformation and consequently a larger hydrophobic target-protein binding pocket than Ca\(^{2+}\)-aCaM or -sCaM4, the presence of which was further confirmed through biophysical measurements. The single Val-144 → Met substitution in the C-lobe of Ca\(^{2+}\)-sCaM1, which restores its ability to activate NOS, alters the structure of the C-lobe to a more closed conformation resembling Ca\(^{2+}\)-aCaM and -sCaM4. The relationships between the structural differences in the two Ca\(^{2+}\)-sCaM isoforms and their selective target activation properties are discussed.

Calmodulin (CaM)\(^4\) is a multifunctional Ca\(^{2+}\)-sensor protein that mediates Ca\(^{2+}\) signals to trigger a variety of physiological responses. This small (≈17 kDa) acidic protein is found in all eukaryotic cells, and its amino acid sequence is strictly conserved. For example, the CaM sequence is invariant among the vertebrates (1). The three-dimensional structure of CaM consists of two similar globular domains, the N-lobe and C-lobe, which are connected by a highly mobile central linker region (2, 3). Each lobe possesses two helix-loop-helix EF-hand Ca\(^{2+}\)-binding motifs (4–6). In the absence of Ca\(^{2+}\), CaM adopts a “closed” conformation, where the four α-helices in each lobe are well packed in an almost parallel orientation. Upon binding Ca\(^{2+}\), CaM undergoes a large rearrangement of its helices altering the domain structure to the “open” conformation, whereby the hydrophobic methionine-rich interiors of each lobe become partially exposed to the solvent (7). The exposed hydrophobic pockets commonly serve as the primary binding interfaces for the CaM-binding domains (CaMBDs) of various CaM-target proteins mainly through hydrophobic interactions (reviewed in Refs. 8–11). A recently determined highly refined solution structure of vertebrate Ca\(^{2+}\)-CaM (12) is distinct from previously reported crystal structures (13, 14) in terms of the arrangement of the α-helices, which creates a difference in the size of the hydrophobic target-binding pocket.

In contrast to the extensive knowledge about the structural and biological properties of CaM in vertebrates and other animals (aCaM), relatively little is known about the CaM regulatory system in higher plant species. The Ca\(^{2+}\) level in plant cells changes through various extracellular stimuli such as light, cold/heat shock, touch, osmotic stress, wounding, hormones, and the presence of pathogens (15). These Ca\(^{2+}\) signals are absorbed by multiple CaM isoforms in contrast to the single CaM protein found in animals (16). For instance, the model plant Arabidopsis thaliana has nine CaM genes (CaM1–9) encoding seven different CaM isoforms (16, 17). Soybean has...
five distinct CaM genes (sCaM1–5) encoding four distinct isoforms of CaM (18). In the latter organism, sCaM1, -2, and -3 are highly homologous to aCaM with >90% sequence identity, whereas sCaM4 and -5 are divergent with only ~78% sequence identity. These sCaM isoforms show unique target activation profiles that have been categorized into three different groups (19, 20). The target enzymes belonging to group 1 include for example, myosin light chain kinases (MLCKs), and they are activated in a similar manner by both sCaM1 and -4. In contrast, group 2 and 3 target enzymes are exclusively activated by sCaM1 or sCaM4, respectively. It seems therefore that each CaM isoform is utilized to control enzymes involved in specific physiological responses in plants. For example, expression of the two most divergent isoforms, sCaM4 and -5, is markedly induced by treatment with a fungal elicitor or following a pathogen attack, and they both can activate the group 3 enzymes nitric-oxide synthase (NOS) (21). Generation of nitric oxide is thought to be one of the early events in plant defense reactions (22, 23). Interestingly, even though all sCaM isoforms retain the ability to bind strongly to enzymes belonging to the other group(s), they cannot properly activate them, which raises a question about how they distinguish their own target enzymes for activation rather than acting as a competitive inhibitor (19, 20). Site-directed mutagenesis studies of aCaM and sCaM isoforms have identified some of the residues that are responsible for the target selectivity. For example, the substitution of Lys-30 for Met in sCaM1 or sCaM4, respectively. The solution structures of these CaM isoforms have unexpectedly revealed that the C-lobe of Ca2⁺-sCaM1 has a more open conformation relative to those of Ca2⁺-sCaM4 and Ca2⁺-aCaM. Additional biophysical experiments further confirm the presence of an enlarged exposed hydrophobic pocket in Ca2⁺-sCaM1. Interestingly, the single Val-144 → Met substitution was found to reduce the size of this hydrophobic pocket of the C-lobe in Ca2⁺-sCaM1, which in turn restores its ability to activate NOS. Taken together our data provide unique insights into the relationship between the structural differences and the selective target activation properties of plant sCaM isoforms.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The Val-144 → Met mutant of sCaM1 (S1V144M) was generated using the QuikChange site-directed mutagenesis kit (Stratagene), and the presence of the mutation was confirmed by DNA sequencing and mass spectrometry. Unlabeled sCaM1, sCaM4, and S1V144M were overexpressed and purified from Escherichia coli, strain BL21(DE3), containing the plasmid pET-3d as the expression vector, grown in LB media. Uniformly 15N- and 15N,13C-labeled sCaM1, sCaM4, and S1V144M were prepared in M9 media containing 0.5 g/liter 15NH₄Cl and/or 3 g/liter 13C₆-glucose. All proteins were purified as described previously (18, 27). All NMR samples contained ~1 mM 15N- or 15N,13C-labeled sCaM1 or sCaM4, 6 mM CaCl₂, 100 mM KCl, 0.03% NaN₃, and 0.5 mM 2,2-dimethyl-2-silapentane 5-sulfonate in 90% H2O/10% D2O or 99.99% D2O (pH 6.8). In addition, 10 mM 2H-labeled dithiothreitol was also added to avoid dimerization caused by intermolecular disulfide bonding. Unlabeled sCaM1 and sCaM4 samples were also prepared in 99.99% D₂O. The samples used for residual dipolar coupling (RDC) measurements also contained 20 mM Bis-Tris (pH 6.8) and 16 mg/ml filamentous phage Pf1 (Asla Labs). The samples used for the chemical shift perturbation (CSP) studies using 1-anilino-8-naphthalenesulfonate (ANS) also contained an additional 20 mM Bis-Tris (pH 6.8).

**Fluorescence Spectroscopy**—Steady-state ANS fluorescence was recorded on a Varian Cary Eclipse fluorescence spectrophotometer (Varian Inc., Victoria, Australia), with an excitation wavelength of 370 nm, and the fluorescence emission was recorded from 400 to 600 nm. The excitation and emission slit widths were 2.5 nm and 5 nm, respectively, and a volume of 1 ml was used. The samples contained 60 μM ANS and 20 μM sCaM1, sCaM4, aCaM, or S1V144M in a buffer containing 20 mM HEPES (pH 7.5), 1 mM dithiothreitol, 100 mM KCl, and 1 mM EDTA for the apo-form or 1 mM CaCl₂ for the Ca²⁺-form. The concentrations of sCaM4 and aCaM were measured using a molar extinction coefficient of 2560 m⁻¹ cm⁻¹ at 280 nm. The concentration of sCaM1 and S1V144M was measured by using
a molar extinction coefficient of 1450 M$^{-1}$ cm$^{-1}$ at 280 and by using the Bio-Rad protein assay kit.

Isothermal Titration Calorimetry Experiments—The ANS-CaM interactions were monitored by ITC at 20, 25, and 30 °C in 20 mM HEPES (pH 7.5), 100 mM KCl, and either 5 mM CaCl$_2$ or 3 mM EDTA/EGTA. 5 mM ANS was titrated into a sample cell containing 50–70 μM protein. sCaM1, sCaM4, and S1V144M were incubated overnight at room temperature in the buffer containing an additional 5 mM dithiothreitol and then desalted into the buffer without dithiothreitol using Bio-Rad Econo-Pac 10DG column before the ITC experiments. All data were analyzed using the "one set of sites" model supplied in the MicroCal Origin software to determine association constant ($K_a$) as described in studies of methionine-modified CaMs (29).

NMR Measurements—All NMR experiments for structure determination were performed at 30 °C on Bruker Avance 500- or 700-MHz NMR spectrometers equipped with triple resonance inverse Cryoprobes with a single axis z-gradient. Sequential assignments of HN, N, CO, Ca, and Cβ resonances of sCaMs were achieved using two dimensional ($^1$H,$^15$N)-HSQC (HSQC) and three-dimensional experiments, including CBCANH, CBCA(CO)NH, HNCO, and HN(CO)CA. Side-chain assignments were obtained by three-dimensional C(CCO)NH-TOCSY, H(CCO)NH-TOCSY, HBHA(CBCACO)NH, (HB)CB(CGCD)HD (30), and (HB)CB(CGCDCE)HE (30) experiments. All NOESY experiments including the three-dimensional $^{15}$N-NOESY-HSQC, three-dimensional $^{13}$C-NOESY-HSQC and two-dimensional NOESY were measured with a mixing time of 100 ms. H-N RDC measurements were performed using the in-phase/anti-phase ($^1$H,$^15$N)-HSQC experiment (31). ($^1$H,$^15$N) NOE experiments were acquired on 700- and 500-MHz for sCaM1 and sCaM4, respectively, using a recycle delay of 5 s (32), and each experiment was repeated three times. Chemical shifts in all spectra were referenced using 2,2-dimethyl-2-silapentane 5-sulfonate to

![FIGURE 2. The assigned ($^1$H,$^15$N)-HSQC spectra of Ca$^{2+}$-sCaM1 (a) and Ca$^{2+}$-sCaM4 (b). Backbone amide signals are labeled with their amino acid residue number. Folded signals are indicated by an asterisk, and their actual $^{15}$N chemical shifts are indicated in parentheses in both spectra. The crowded region of each spectrum is expanded in the insets.]
obtain the ¹H, ¹⁵N, and ¹³C chemical shifts as described by Wishart et al. (33). All spectra were processed using the program NMRRpipe (34) and analyzed using the NMRView software (35).

**Structure Calculations**—Initial structures were calculated with CYANA (36) version 2.0 using distance restraints obtained from the automatic NOE assignment protocol, hydrogen bond restraints based on secondary structure from the chemical shift index, and dihedral angle restraints predicted by TALOS (37). Further structural refinements with the addition of RDC restraints were performed by XPLOR-NIH (38). Initial estimations for the axial component of the molecular alignment tensor (Da) and the rhombicity (R) were obtained for the structure calculated by CYANA using PALES (39). Finally, the 20 lowest energy structures from a total of 200 calculated were selected and analyzed. All molecular graphics are created using MOLMOL (40).

**RESULTS**

**Structure Determination**—Ca²⁺-sCaM1 and Ca²⁺-sCaM4 both generated well dispersed high quality ¹H, ¹⁵N-HSQC spectra (Fig. 2). Sequential assignments of the main-chain resonances were obtained from the HNCACB, HN(CO)CACB, HN(CA)CO, and HNCO experiments. Consequently, all amide resonances except for one Pro and a few of the N-terminal residues were successfully assigned in the ¹H, ¹⁵N-HSQC spectrum of both Ca²⁺-sCaM1 and Ca²⁺-sCaM4 (Fig. 2). The side-chain resonance assignments were mainly obtained from the C(CO)NH-TOCSY, H(CCO)NH-TOCSY, and HBHA(CO)NH spectra. The (HB)CB((GC)GD)HD and (HB)CB((GG)CDCE)HE experiments were also used for the assignment of aromatic ¹H resonances. Consequently, 96.4% and 98.5% of the total ¹H resonances are assigned and used for the structure calculations of Ca²⁺-sCaM1 and Ca²⁺-sCaM4, respectively. The NOE signals used to generate distance restraints were collected from the three-dimensional ¹⁵N- and ¹³C-NOESY-HSQC experiments as well as the two-dimensional NOESY experiment recorded in D₂O solution. Initially, a total of 3408 and 2808 NOE signals were manually identified, and 3008 and 2727 signals were successfully assigned by CYANA 2.0 with an automatic NOE assignment protocol, which generated 2019 and 1942 distance restraints for Ca²⁺-sCaM1 and Ca²⁺-sCaM4, respectively. The NOE signals determined for sCaM4 ranged from −24 and 21 Hz with a digital resolution of 2.5 Hz. The complete set of RDC values determined for sCaM4 ranged from −16 to 16 Hz with a digital resolution of 1.7 Hz. All of the restraints used for the final structure calculations of the four structures are summarized in Table 1. The backbone r.m.s.d. values of the final 20 structures of sCaM1 were 0.47 ± 0.06 Å and 0.32 ± 0.06 Å for the N- and C-lobe, respectively. Those for sCaM4 in the corresponding regions are 0.44 ± 0.07 Å and 0.36 ± 0.06 Å, respectively. The experimentally determined RDC values provide an excellent correlation with the best-fit RDC values calculated with the final structures of sCaM1 and sCaM4. The correlation (R)/quality (Q) factors of sCaM1 were 1.00/0.007 and 1.00/0.011 for the N- and C-lobe, respectively. These values for sCaM4 were 1.00/0.026 and 1.00/0.018 for the N- and C-lobe, respectively.

**Structural Comparisons of sCaM1, sCaM4, and aCaM**—Fig. 4 shows the superimposed ribbon diagrams of the lowest energy structures of sCaM1 and sCaM4 for the N- and C-lobe. These structures are as expected, based on sequence homology, very similar to one another. The backbone r.m.s.d. values in the helical regions are 0.88 Å and 1.50 Å for the N- and C-lobe, respectively. Consistent with this, the R/Q factors from the correlations between experimentally measured ¹H-¹⁵N RDC values of Ca²⁺-sCaM1 and the best-fit RDC values calculated with sCaM4 structures were 0.99/0.163 and 0.88/0.477 for the N- and C-lobe, respectively. The disagreement in the C-lobes seems to be mainly caused by a difference in the location of the H-helices (Fig. 4b). The structures were also compared with an available high resolution (1 Å) crystal structure of aCaM (PDB entry 3EXR (14)) and to NMR structures (PDB entries 1J7O and 1J7P (12)) of aCaM. The correlations of the RDC values that were measured for sCaM1 and sCaM4 to these aCaM structures and the resulting backbone r.m.s.d. values are summarized in Table 2. The N-lobes of both sCaM1 and sCaM4 show better agree-
TABLE 1

Experimental restraints and structural statistics of the 20 NMR structures

|                         | sCaM1 N-lobe (1–79) | sCaM1 C-lobe (80–149) | sCaM4 N-lobe (1–79) | sCaM4 C-lobe (80–149) |
|-------------------------|---------------------|-----------------------|--------------------|-----------------------|
| Number of experimental restraints | 959 | 982 | 976 | 978 |
| Distance restraints from NOEs | 259 | 268 | 285 | 295 |
| Intra-residue | 234 | 265 | 252 | 238 |
| Sequential | 272 | 231 | 255 | 258 |
| Medium range | 194 | 218 | 184 | 187 |
| Long range | 137 | 125 | 140 | 130 |
| Dihedral angle restraints (TAILOS) | 68 | 65 | 68 | 67 |
| Hydrogen bond restraints | 12 | 12 | 12 | 12 |
| Ca\(^{2+}\) ligand restraints | 61 | 62 | 66 | 67 |
| H-N RDC restraints | 0.049 ± 0.004 | 0.050 ± 0.002 | 0.049 ± 0.004 | 0.058 ± 0.005 |
| Dihedral angle restraint violation (°) | 0.29 ± 0.17 | 0.21 ± 0.19 | 0.24 ± 0.11 | 0.24 ± 0.11 |
| RDC restraint violation (Hz) | 0.098 ± 0.023 | 0.094 ± 0.015 | 0.153 ± 0.013 | 0.110 ± 0.013 |
| Average r.m.s.d. values from experimental data | 0.0043 ± 0.0005 | 0.0043 ± 0.0004 | 0.0040 ± 0.0002 | 0.0032 ± 0.0004 |
| Dihedral angle restraint violation (°) | 0.744 ± 0.024 | 0.675 ± 0.034 | 0.524 ± 0.009 | 0.460 ± 0.007 |
| RDC restraint violation (Hz) | 0.593 ± 0.035 | 0.566 ± 0.041 | 0.423 ± 0.020 | 0.380 ± 0.024 |
| Coordinate precision of folded regions | 0.47 ± 0.06 | 0.32 ± 0.06 | 0.44 ± 0.07 | 0.36 ± 0.06 |
| Backbone (Å) | 0.47 ± 0.06 | 0.32 ± 0.06 | 0.44 ± 0.07 | 0.36 ± 0.06 |
| All heavy atoms (Å) | 0.98 ± 0.05 | 0.95 ± 0.07 | 0.96 ± 0.07 | 0.90 ± 0.07 |

TABLE 2

Correlations between experimentally measured \(^1\)H–\(^{15}\)N RDC values of sCaM isoforms and best-fit RDC values calculated with the CaM structures

|                              | sCaM1 (N/C) | sCaM1 (x-ray) | sCaM4 (N/C) | sCaM4 (x-ray) |
|------------------------------|-------------|---------------|-------------|---------------|
| aCaM (NMR)                  | 0.994        | 0.932         | 0.965        | 0.971         |
| Q = 0.112                    | 0.932        | 0.932         | 0.947        | 0.947         |
| 0.65 Å                       | 0.932        | 0.932         | 0.947        | 0.947         |
| R = 0.994                    | 0.932        | 0.932         | 0.947        | 0.947         |
| Q = 0.248                    | 0.932        | 0.932         | 0.947        | 0.947         |
| 0.80 Å                       | 0.932        | 0.932         | 0.947        | 0.947         |

FIGURE 4. The backbone structures of Ca\(^{2+}\)-sCaM1 (yellow) and Ca\(^{2+}\)-sCaM4 (navy) are overlaid for the N-lobe (a) and C-lobe (b). Several residues that contribute to the different surface charges compared with aCaM are indicated. The positions of Val/Met-144 are also shown. The side chains of sCaM1 and sCaM4 are colored in green and purple, respectively. The helices are labeled as in the text.

Sized. As has been discussed, the crystal structure of Ca\(^{2+}\)-aCaM differs from the Ca\(^{2+}\)-CaM structures determined by NMR in terms of the arrangement of helices (12), where the solution NMR structure adopts a more “closed” conformation. Similar closed conformations are found in our N-lobe structures of both Ca\(^{2+}\)-sCaM1 and Ca\(^{2+}\)-sCaM4. The differences in the helix angles among all three NMR structures are small, <6° for both the B- and C-helix (Fig. 5a and Table 3). The C-helices however seem to have a larger variability in the arrangements of its helices among the various CaM structures (Fig. 5b). Similar to the N-lobe, the C-lobe of Ca\(^{2+}\)-aCaM adopts a more closed conformation in the NMR structure than what is seen in the crystal structure. In Fig. 5b, when the E- and H-helices are overlaid, the difference in the helix angles between Ca\(^{2+}\)-sCaM4 and Ca\(^{2+}\)-aCaM are relatively small, 4.6° and 11.4° for the F- and G-helix, respectively. However, those differences between Ca\(^{2+}\)-sCaM4 and Ca\(^{2+}\)-sCaM1 are more obvious, 19.5° and 16.0° for the F- and G-helix, respectively. Backbone r.m.s.d. values also show that the C-lobe of Ca\(^{2+}\)-sCaM4 is relatively similar to that of the NMR-determined Ca\(^{2+}\)-aCaM structure. On the other hand, the C-lobe of Ca\(^{2+}\)-sCaM1 is more closely related to the crystal structure of Ca\(^{2+}\)-aCaM (Table 2). The backbone r.m.s.d. values in the latter case are 1.14 and 0.90 Å to the NMR and the crystal structure, respectively. Based on these observations, it seems that the C-lobe of sCaM1 adopts a more open conformation in solution and has a larger hydrophobic targeting binding pocket than either Ca\(^{2+}\)-sCaM4 or Ca\(^{2+}\)-aCaM.
we studied the binding of the hydrophobic probe ANS to each protein using steady-state fluorescence spectroscopy and ITC measurements. The fluorescence intensity of ANS is known to be strongly dependent on the polarity of its environment, and in aqueous solution the fluorescence intensity of ANS is extremely small (41–43). Fig. 6 shows steady-state ANS fluorescence spectra with sCaM1, sCaM4, or aCaM, in the presence of either Ca$^{2+}$/H$_{11001}$ or EDTA. The apo-forms of sCaM1, sCaM4, and aCaM all give rise to very small enhancements in the fluorescence intensity of ANS, suggesting that the hydrophilic surfaces of the apo-CaMs bind poorly to ANS. From this observation, we can conclude that ANS is a suitable probe to provide a semi-quantitative estimate of the exposed hydrophobic surface area in the Ca$^{2+}$/CaMs. The fluorescence intensity enhancements seen for Ca$^{2+}$/sCaM4 and Ca$^{2+}$/aCaM are relatively similar at 33- and 26-fold, respectively, and are considerably smaller than the fluorescence intensity enhancement upon addition of Ca$^{2+}$/sCaM1, which is ~70-fold.

ITC experiments showed that ANS binding to Ca$^{2+}$/sCaM1, Ca$^{2+}$/sCaM4, and Ca$^{2+}$/aCaM was relatively weak ($K_a \approx 10^3–10^4$ M$^{-1}$) and exothermic in each case (Fig. 7). However, Ca$^{2+}$/sCaM1 gave rise to a much larger heat of binding to ANS than the other two Ca$^{2+}$/CaMs (Fig. 7). The overall affinity of Ca$^{2+}$/sCaM1 for ANS was also higher than for Ca$^{2+}$/sCaM4 or Ca$^{2+}$/aCaM at all temperatures tested (Table 4). No interaction between the apo-CaMs and ANS was detected by ITC, consistent with the very weak interactions observed in the fluorescence spectroscopy experiments.

Further characterization of ANS binding was achieved by NMR titration of each Ca$^{2+}$/CaM with 0.0, 0.4, 0.8, 1.2, 1.6, 2.0, and 2.8 molar equivalent of ANS. NMR CSP studies are well suited to provide detailed information on protein-ligand binding, particularly for relatively weak interactions (44). Fig. 8 shows the CSPs induced in the backbone amide resonances of Ca$^{2+}$/sCaM1 and Ca$^{2+}$/sCaM4 upon binding to ANS. In Ca$^{2+}$/sCaM1, many signals that belong to both the N- and C-lobe were perturbed when 0.4 molar equivalents of ANS were added (Fig. 8a). However, with Ca$^{2+}$/sCaM4, a characteristic sequential ANS binding was observed, where the first molar equivalent of ANS perturbs only residues in the N-lobe of sCaM4, and the second ANS equivalent perturbs mostly C-lobe residues. Similar sequential ANS binding was also observed with Ca$^{2+}$/aCaM (Fig. 8c). From these fluorescence, ITC, and NMR data, it is apparent that the C-lobe of Ca$^{2+}$/sCaM1 binds ANS more

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**TABLE 3**

The difference in the helix angles of sCaM1 from other isoforms

|          | sCaM1 | sCaM4 | aCaM (x-ray) | aCaM (NMR) |
|----------|-------|-------|--------------|------------|
| Helix B  | 5.5   | 4.0   | 13.2         | 5.5        |
| Helix C  | 1.9   | 5.6   | 18.4         | 1.9        |
| Helix F  | 14.5  | 19.5  | 12.2         | 14.5       |
| Helix G  | 7.4   | 16.0  | 10.0         | 7.4        |

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**Figure 5.** The backbone structures of Ca$^{2+}$/sCaM1 (yellow), Ca$^{2+}$/sCaM4 (navy), Ca$^{2+}$/sCaM (green) in solution, and Ca$^{2+}$/sCaM (red) determined by x-ray crystallography are overlaid using the A- and D-helices in the N-lobe (a), and using the E- and H-helices in the C-lobe (b). The differences in the angles between sCaM1 and sCaM4 for the B-, C-, F-, and G-helix are also indicated. All solution structures show similar arrangements of $\alpha$-helices in the N-lobe (a), whereas the C-lobe (b) shows more variability.

**Figure 6.** Ca$^{2+}$-induced exposure of the hydrophobic surfaces of sCaM1, sCaM4, and aCaM monitored by ANS fluorescence. The steady-state fluorescence emission spectra of ANS in the presence of Ca$^{2+}$/sCaM1 (a), Ca$^{2+}$/S1V144M (b), Ca$^{2+}$/sCaM4 (c), and Ca$^{2+}$/aCaM (d), e, the weak fluorescent emissions of ANS alone and in the presence of apo-forms of all CaM isoforms are overlapped. All experiments were carried out at 25°C.
Solution Structures and Activation Properties of Plant CaMs

The solution structures of Ca$^{2+}$-sCaM1 and Ca$^{2+}$-sCaM4 were found to be dumbbell-shaped structures with two lobes (N- and C-lobe) connected by a highly flexible linker similar to other CaM isoforms (11). In the N-lobe, no significant differences are found among all the solution structures (Fig. 5a), yet mutations in this region of sCaMs are known to cause changes in enzyme activation (24, 25). Because none of the amino acid substitutions change the tertiary structure of the N-lobe of the sCaM isoforms, we can conclude that the alterations of surface charges at the positions of residues 30 and 40 must be directly responsible for the different activation profiles reported for these two sCaM isoforms with NAD kinase and MLCKs (24, 25).

Even though the sequence identity between the C-lobes of Ca$^{2+}$-sCaM1 and Ca$^{2+}$-sCaM4 (80%) is higher than the sequence identity between the N-lobes of each protein (77%), the former shows a larger difference in the arrangement of the helices. From the overlay of the backbone structure of the C-lobe of Ca$^{2+}$-sCaM1 and Ca$^{2+}$-sCaM4, it is clear that the position of the H-helix has changed (Fig. 4b). Consequently, the C-lobe of Ca$^{2+}$-sCaM1 was found to be more opened, having a larger hydrophobic target-binding pocket than that of Ca$^{2+}$-sCaM4 (Fig. 5b). On the other hand, the helix arrangement and hydrophobic pocket of Ca$^{2+}$-sCaM4 are very similar to Ca$^{2+}$-aCaM. This outcome is particularly surprising, because the C-lobes of sCaM1 and aCaM have a higher amino acid sequence identity (87%) than the N-lobes of sCaM4 and aCaM (77%). One often assumes that a higher level of sequence similarity will lead to a closer structural agreement;

**TABLE 4**

The association constants for ANS binding to Ca$^{2+}$-CaM isoforms determined by ITC

| Temperature (°C) | 20       | 25     | 30     |
|------------------|----------|--------|--------|
| sCaM1            | $1.3 \pm 0.0 \times 10^4$ | $7.4 \pm 0.3 \times 10^3$ | $6.4 \pm 0.3 \times 10^3$ |
| sCaM4            | $7.8 \pm 0.7 \times 10^3$ | $6.3 \pm 0.4 \times 10^3$ | $4.7 \pm 0.3 \times 10^3$ |
| sCaM4M           | $4.8 \pm 0.3 \times 10^3$ | $4.1 \pm 0.1 \times 10^3$ | $3.7 \pm 0.2 \times 10^3$ |
| V144M            | $6.9 \pm 0.5 \times 10^3$ | $5.9 \pm 0.3 \times 10^3$ | $3.4 \pm 0.3 \times 10^3$ |

* Values were obtained from Yamniuk et al.*

* The experiment was repeated three times and averaged, whereas other errors were derived from curve fitting.

strongly than the C-lobes of Ca$^{2+}$-sCaM4 or Ca$^{2+}$-aCaM, which is consistent with the presence of a larger hydrophobic pocket in the solution structure of the C-lobe of Ca$^{2+}$-sCaM1.

**Effect of the Val-144 → Met Substitution on the Structure of Ca$^{2+}$-sCaM1**—To further investigate the relationship between the larger hydrophobic pocket that exists in the C-lobe of Ca$^{2+}$-sCaM1 and the inability of this protein to activate NOS, we generated the sCaM1 mutant, S1V144M (see Fig. 1). The complete NMR main-chain assignment of Ca$^{2+}$-S1V144M was achieved, and the amide CSP caused by the substitution was analyzed (Fig. 9). The impact of the single Val-144 → Met mutation was unexpectedly large, spanning all four helices in the C-lobe of Ca$^{2+}$-sCaM1. The steady-state fluorescence spectrum of ANS showed a remarkably reduced enhancement upon binding to Ca$^{2+}$-S1V144M compared with wild-type Ca$^{2+}$-sCaM1 (Fig. 6). A similar trend was also found in the ITC data in which Ca$^{2+}$-S1V144M creates a much smaller heat of binding to ANS with a slightly weaker affinity at all three temperatures that we have tested (Fig. 7 and Table 4). Consistent with these results, the NMR titration of Ca$^{2+}$-S1V144M with ANS showed a sequential ANS binding similar to that observed with Ca$^{2+}$-sCaM4 and Ca$^{2+}$-aCaM (Fig. 8d). We also detected the presence of a significant conformational alteration in Ca$^{2+}$-S1V144M using backbone H-N RDC NMR experiments. As expected from the CSP data, the RDC data for the Ca$^{2+}$-S1V144M N-lobe still show an excellent correlation with the N-lobe structures of both Ca$^{2+}$-aCaM and wild-type Ca$^{2+}$-sCaM1. However, the reduced $R$ factor and increased $Q$ factor of the C-lobe of the mutant protein compared with the wild-type Ca$^{2+}$-sCaM1, together with the increased $R$ factor and the reduced $Q$ factor compared with Ca$^{2+}$-aCaM (Table 5), provide evidence that the C-lobe of Ca$^{2+}$-S1V144M more closely resembles the solution structure of Ca$^{2+}$-aCaM.

Taken together, the NMR and biophysical data suggest that the single Val-144 → Met mutation altered the structure of the C-lobe of Ca$^{2+}$-sCaM1 to adopt a more closed conformation with a smaller hydrophobic target-binding pocket, similar to the C-lobe conformation of Ca$^{2+}$-aCaM and Ca$^{2+}$-sCaM4.

**DISCUSSION**

The solution structures of Ca$^{2+}$-sCaM1 and Ca$^{2+}$-sCaM4 were found to be dumbbell-shaped structures with two lobes (N- and C-lobe) connected by a highly flexible linker similar to other CaM isoforms (11). In the N-lobe, no significant differences are found among all the solution structures (Fig. 5a), yet mutations in this region of sCaMs are known to cause changes in enzyme activation (24, 25). Because none of the amino acid substitutions change the tertiary structure of the N-lobe of the sCaM isoforms, we can conclude that the alterations of surface charges at the positions of residues 30 and 40 must be directly responsible for the different activation profiles reported for these two sCaM isoforms with NAD kinase and MLCKs (24, 25).
however, our results show exactly the opposite for these three highly related proteins.

The presence of the larger hydrophobic pocket in the C-lobe of Ca\(^{2+}\)-sCaM1 was supported by our ANS binding experiments, where both the fluorescence intensity enhancement and the affinity for ANS were higher with Ca\(^{2+}\)-sCaM1 than with Ca\(^{2+}\)-sCaM4 or Ca\(^{2+}\)-aCaM (Figs. 6 and 7 and Table 4). Our NMR titration studies revealed that Ca\(^{2+}\)-sCaM4 and Ca\(^{2+}\)-aCaM bound ANS in a sequential manner, whereas both the N- and C-lobe of Ca\(^{2+}\)-sCaM1 bound ANS simultaneously (Fig. 8). Taking this into account, we propose that the more open conformation of the C-lobe of Ca\(^{2+}\)-sCaM1 may allow ANS to fit much deeper into its hydrophobic pocket, thereby leading to higher affinity binding.

Because mutation of Val-144 in sCaM1 to Met is known to restore the ability of this protein to activate NOS to ~60% of the sCaM4 activation level (26), we also studied this mutant form of sCaM1 (S1V144M). Fluorescence and ITC experiments indicate that Ca\(^{2+}\)-S1V144M binds ANS in a manner closely resembling Ca\(^{2+}\)-sCaM4 or Ca\(^{2+}\)-aCaM, which suggests that Ca\(^{2+}\)-S1V144M has a smaller hydrophobic pocket than wild-type Ca\(^{2+}\)-sCaM1. Consistent with this, the backbone H-N RDC analysis of Ca\(^{2+}\)-S1V144M clearly revealed that the single Val1144 → Met substitution altered the structure of the C-lobe.
of Ca\(^{2+}\)-sCaM1 to a conformation similar to that of Ca\(^{2+}\)-aCaM (Table 5). We note that the chemical shift differences (Fig. 9) as well as the changes in the H-N RDC values (data not shown) caused by the single substitution were found over all four helices of the C-lobe. Thus, our results suggest that the smaller hydrophobic pocket of the C-lobe of Ca\(^{2+}\)-s1V144M can form the same hydrophobic contacts to the CaMBD of NOS as Ca\(^{2+}\)-sCaM4 or Ca\(^{2+}\)-aCaM, thereby leading to enzyme activation. We have previously reported that the thermodynamic parameters for the binding of the two sCaM isoforms to a synthetic peptide corresponding to the CaMBD of cerebellar NOS were somewhat different (28). Therefore, from these results, we propose that the wide open conformation of Ca\(^{2+}\)-sCaM1 is likely responsible for its failure to activate NOS.

Even though the enthalpy and entropy of binding are different, the binding constants for the cerebellar NOS peptide are almost the same for Ca\(^{2+}\)-sCaM1 and Ca\(^{2+}\)-sCaM4 (28); therefore, a difference in affinity cannot explain the different activation properties. Here, we propose two possible explanations. First, similar to our observations with ANS, the large hydrophobic pocket of Ca\(^{2+}\)-sCaM1 could allow the anchoring residues of NOS to bind more deeply than with Ca\(^{2+}\)-sCaM4 and Ca\(^{2+}\)-aCaM, which may cause unfavorable conformational changes in the neighboring regions of the NOS enzyme, thereby prohibiting activity. Secondly, to make proper hydrophobic contacts between the wide open conformation of Ca\(^{2+}\)-sCaM1 and the CaMBD of NOS, Ca\(^{2+}\)-sCaM1 may have to adopt a different orientation of its N- and C-lobes in the complex compared with Ca\(^{2+}\)-sCaM4 and Ca\(^{2+}\)-aCaM. This would cause a difference in the charge distribution on the surface of the sCaM-CaMBD complex structure, which in turn would cause unfavorable repulsion/interactions for other parts of the NOS enzyme. In the case of Ca\(^{2+}\)-aCaM, it has also been demonstrated that such additional interactions with the target protein through the surface structure of Ca\(^{2+}\)-aCaM are required to remove the Ca\(^{2+}\)-aCaM bound inhibitory region from the catalytic cleft of the protein to give rise to activation of skeletal muscle MLCK (45).

In this work, we have discussed the structural differences of two Ca\(^{2+}\)-bound soybean CaM isoforms and Ca\(^{2+}\)-aCaM, and their relationship to their different target-enzyme activation properties. Consistent with the similarity in their amino acid sequences the two plant sCaM isoforms have very similar tertiary structures and dynamic properties. This agrees with previous studies that have shown that they can bind in a similar manner to most of the target enzymes. However, in solution, a variety of open conformations with a different size of the hydrophobic target-binding pocket in the C-lobe exists among the sCaM isoforms, and these lead to different hydrophobic contacts for the CaMBDs of some target enzymes, including NOS.

We note that many CaMBDs use a Trp side chain to anchor to the C-lobe of CaM, whereas NOS does not (10). Furthermore, the resulting charge distribution on the protein surface of the sCaM-CaMBD complexes, which would form additional contacts with the target protein, would also be essential. As we have discussed, this is apparently the case for the activation of NAD kinase and MLCKs. Therefore, future structural investigations of CaM complexes should aim to focus on these additional interactions, which would require studies with intact target-proteins/domains rather than short synthetic peptides. Such studies would allow for a better understanding of the role of plant CaM isoforms in fine-tuning the calcium-dependent response to various stimuli.

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