Structural Analysis of a Calmodulin Variant from Rice

**THE C-TERMINAL EXTENSION OF OsCaM61 REGULATES ITS CALCIUM BINDING AND ENZYME ACTIVATION PROPERTIES**

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Received for publication, June 10, 2013, and in revised form, September 2, 2013 Published, JBC Papers in Press, September 19, 2013, DOI 10.1074/jbc.M113.491076

OsCaM61 is one of five calmodulins known to be present in Oryza sativa that relays the increase of cytosolic [Ca2+] to downstream targets. OsCaM61 bears a unique C-terminal extension with a prenylation site. Using nuclear magnetic resonance (NMR) spectroscopy we studied the behavior of the calmodulin (CaM) domain and the C-terminal extension of OsCaM61 in the absence and presence of Ca22+. NMR dynamics data for OsCaM61 indicate that the two lobes of the CaM domain act together unlike the independent behavior of the lobes seen in mammalian CaM and soybean CaM4. Also, data demonstrate that the positively charged nuclear localization signal region in the tail in apo-OsCaM61 is helical, whereas it becomes flexible in the Ca22+-saturated protein. The extra helix in apo-OsCaM61 provides additional interactions in the C-lobe and increases the structural stability of the closed apo conformation. This leads to a decrease in the Ca22+ binding affinity of EF-hands III and IV in OsCaM61. In Ca22+-OsCaM61, the basic nuclear localization signal cluster adopts an extended conformation, exposing the C-terminal extension for prenylation or enabling OsCaM61 to be transferred to the nucleus. Moreover, Ser172 and Ala173, residues in the tail, interact with different regions of the protein. These interactions affect the ability of OsCaM61 to activate different target proteins. Altogether, our data show that the tail is not simply a linker between the prenyl group and the protein but that it also provides a new regulatory mechanism that some plants have developed to fine-tune Ca22+-signaling events.

Ca22+ is an important second messenger whose cytosolic concentration in plant cells is increased in response toiotic and abiotic stimuli such as salinity (1), pathogen invasion (2), hormones (3), wounds (4), and developmental cues (5). Parts of these responses are mediated by the ubiquitous Ca22+-binding regulatory protein calmodulin (CaM)2 that is expressed in all eukaryotic cells. CaM is a well studied Ca22+-binding protein that is involved in many cellular events in mammals such as the regulation of protein kinases involved in cellular movement, e.g. myosin light chain kinase (MLCK) (6), and the regulation of cytoskeletal proteins, e.g. smooth muscle caldesmon (6). It is a highly conserved, acidic, heat-stable protein with an average molecular mass of 17 kDa (7). The protein has a flexible dumbbell structure and comprises an independently folded C-terminal lobe and a similar N-terminal lobe that are connected by a flexible linker (7). The two lobes of CaM are made up of two EF-hand motifs, allowing the protein to bind four Ca22+ ions (8).

Unlike animals that have only three CaM genes and only one isoform (9), plants have multiple CaM genes and isoforms (10, 11). For example, Arabidopsis has 11 CaM genes that generate at least seven distinct isoforms, soybean has at least five genes coding for four proteins (12), and rice has at least five CaMs (13). This shows the importance of these proteins in plant signaling, enabling the cells to respond to diverse stimuli. Different CaM isoforms in given plant species may be differentiated according to their expression patterns, cellular targets, Ca22+ and target protein binding affinities, and physiological functions. In addition to various types of CaMs, plants possess another set of proteins called the calmodulin-like (CML) proteins that have similarities to CaM but contain unique functions (14), and their compared sequence identity to CaM is lower (15). Over 50 genes identified in Arabidopsis (16) and 26 in rice encode putative CML proteins that have yet to be characterized completely. CMLs are involved in different cellular processes such as flowering of plants (where CML23 and CML24 act through the nitric oxide pathway) (17), establishment of cell polarity (CML12) (18), abiotic stress (CML9) (19, 20), and DNA lesion repair (CML19) (20). In addition, plants express numerous calcium-dependent protein kinases, protein

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**Background:** OsCaM61 is a plant CaM bearing a C-terminal extension and a prenylation motif.

**Results:** The C-terminal extension is partially helical and interacts with the CaM domain.

**Conclusion:** OsCaM61 uses the C-terminal extension to control its localization and enzyme activation ability.

**Significance:** This work provides structural details for a new plant regulatory mechanism.
kinases that contain a CaM-like domain (21, 22). Apart from the CMLs and the calcium-dependent protein kinases, a few proteins have been described in specific plants that have a CaM domain that is similar to other known CaMs, but they have an additional C-terminal extension (CTE). These extensions have been shown to provide unique characteristics to such variants of CaM. For example, *Oryza sativa* CaM61 (OsCaM61), which is expressed in rice, is made up of a conserved N-terminal 148 amino acids similar to all CaMs (92% similarity with *Homo sapiens* CaM; UniProtKB/Swiss-Prot accession number P62158) (Fig. 1) and possesses a CTE of 38 residues (Figs. 1 and 2). OsCaM61 is a heat-stable protein that can be purified by Ca$^{2+}$/H$^{1+}$-dependent phenyl-Sepharose chromatography and shows a Ca$^{2+}$/H$^{1+}$-dependent mobility shift on SDS-PAGE (23). The CTE of OsCaM61 is very rich in positively charged residues and carries a characteristic prenylation motif (CVIL) at the C terminus (24). A second related plant protein that is known to have a similar CTE with a prenylation site is the PhCaM53 protein found in *Petunia* (25). Although the extension of OsCaM61 has an amino acid sequence different from PhCaM53, they both contain multiple basic residues as well as a CXXL motif at the C terminus. The latter makes each protein a potential target for prenylation by geranylgeranyl transferase I, an enzyme known to be expressed in plants (25). Indeed both proteins have been shown to occur in a prenylated form in vivo (26, 27). Overexpression studies with a GFP-tagged version of OsCaM61 revealed that the prenylated protein localizes to the plasma membrane and other membranes (26). Also, the non-prenylated form of the protein localizes to the nucleus, suggesting the presence of specific CaM-binding targets of OsCaM61 inside the nucleus. Apart from OsCaM61 and PhCaM53, two other related proteins are *Arabidopsis thaliana* CaM-5 (AtCaM-5) and *Arabidopsis lyrata* CaM-2 (AlCaM-2). Both of these proteins contain a CaM domain and a CTE. Interestingly, their CaM domains are 100% identical to AtCaM-2, which does not contain a CTE. Although no studies have been conducted yet on AtCaM-5, it has been shown that AtCaM-2 has a higher ability to activate NAD kinase than AtCaM-4 or AtCaM-6 (28). In enzyme activation studies, the C-terminally truncated form (OsCaM61$_{148}$) (Fig. 2) has a higher binding affinity and shows higher activation of cyclic nucleotide phosphodiesterase when compared with the full-length protein (23). Also, mutational analysis of this protein has demonstrated that a few specific amino acids are responsible for activating some target enzymes less efficiently than either OsCaM1 or mammalian CaM (23). This may be due to differences in surface interactions of the protein with the enzymes (29). The fact that OsCaM61 can become anchored to the membrane because of prenylation combined with the inhibitory effect of the CTE on enzyme activation may represent a novel regulatory mechanism that could
potentially be used in specific plant cells to control Ca\(^{2+}\) signaling in response to various stimuli. All these data suggest that, although plant cells share numerous common features with mammalian cells in regard to Ca\(^{2+}\) signaling, plants have a more sophisticated Ca\(^{2+}\) regulatory system (15).

In this work, we studied the full-length OsCaM61 as well as its separate CaM domain using NMR spectroscopy. Our work demonstrates that the N-lobe and C-lobe have structures similar to mammalian and other plant CaMs. However, the linker region shows a different behavior between the two lobes when compared with animal CaM and soybean CaM1 and CaM4 (sCaM1 and sCaM4). Using \(^{1}H\),\(^{15}N\) heteronuclear NOE and dynamics data gathered from NMR relaxation measurements, we found that the two lobes of OsCaM61 interact with each other. Chemical shift information obtained from the NMR backbone assignment data show that the CTE is partially structured. We have demonstrated that the CTE interacts with the CaM domain in both the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms. Also, using chemical shift perturbations (CSPs) we have mapped the regions affected by the CTE in both the Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms. These data can explain why the full-length protein with the CTE has a weaker affinity and a lower activation ability for target enzymes. Finally, we used enzyme assays with constructs of OsCaM61 of different lengths (Fig. 2) to map the regions of the CTE that are most directly involved in the interaction with the remainder of the protein. Taken together, this work provides a structural basis for yet another theme in the control of plant Ca\(^{2+}\) signaling.

**EXPERIMENTAL PROCEDURES**

**Sample Preparations**—The OsCaM61 gene with optimized codons for expression in *Escherichia coli* was purchased from GeneArt. The gene was subcloned into the pET30 vector, and the protein was expressed in *E. coli* BL21(DE3) cells and purified using a previously published method used for CaMs (12). The C-terminally truncated forms of the OsCaM61 gene, Δ148 and Δ169, which lack the CTE, were also subcloned into a pET30 vector. Also, mutated forms of OsCaM61Δ148 were made using the Stratagene mutagenesis kit. These different forms of OsCaM61 were also purified using the standard protocol for animal CaM that relies on Ca\(^{2+}\)-dependent purification with phenyl-Sepharose (30). The protein purity was confirmed by SDS-PAGE. Finally, the CTE peptide encompassing residues 148–186 of OsCaM61 was subcloned into the pET15 vector as a small ubiquitin-like modifier fusion protein, and the protein was expressed in *E. coli* BL21 cells and purified using Ni\(^{2+}\)-nitrilotriacetic acid chromatography. Subsequently, the peptide was released from the small ubiquitin-like modifier portion by limited proteolysis. Its purity was confirmed by SDS-PAGE and mass spectrometry. Uniformly \(^{2}H\),\(^{13}C\),\(^{15}N\)- and \(^{2}H\),\(^{15}N\)-labeled OsCaM61 and uniformly \(^{15}N\)- and \(^{13}C\)-labeled truncated OsCaM61Δ148 were prepared using previously described protocols (31). All NMR samples for the Ca\(^{2+}\)-bound forms of the protein contained 1 mM \(^{15}N\)- or \(^{13}C\)-labeled truncated OsCaM61Δ148 or \(^{2}H\),\(^{15}N\)- and \(^{2}H\),\(^{13}C\),\(^{15}N\)-labeled full-length OsCaM61 with 3 mM CaCl\(_2\), 100 mM KCl, 0.03% NaN\(_3\), and 0.5 mM 2,2-dimethyl-2-silapentane 5-sulfonate in 90% H\(_2\)O, 10% D\(_2\)O (pH 6.8). Apo samples were prepared in a similar manner but they contained 5 mM EDTA instead of 3 mM CaCl\(_2\). In addition, 10 mM deuterated dithiothreitol (DTT) was added to prevent potential protein dimerization caused by intermolecular disulfide bonding.

**NMR Measurements**—All NMR experiments were performed at 25 °C on a Bruker Avance 500- or 700-MHz NMR spectrometer equipped with triple resonance inverse cryo-probes with a single axis z gradient. Sequential assignments of HN, N, CO, Ca\(_\alpha\), and CB resonances were achieved using several experiments including HNCA, CBCA(CO)NH (or HN(CO)-CACB in the case of the deuterated samples), HNCO, and HN(CA)CO. The full-length forms of apo- and holo-OsCaM61 were deuterated, and the backbone resonances were assigned using transverse relaxation optimized spectroscopy-based triple resonance experiments. The secondary structure prediction for the holo and apo forms of full-length OsCaM61 was done using the weighted average secondary shift method. The chemical shift differences for CO, Ca\(_\alpha\), and N in the protein structure and for random coil shifts were used according to different weighting functions to predict the secondary structure (32) as shown in Equation 1.

\[
W_{\text{ASS}} = \frac{1}{3} \left[ 3\delta_{\text{CO}} - \delta_{\text{Ca}_{\alpha}} + 1.7 \left( \delta_{\text{C}} - \delta_{\text{C}} \right) + 0.35 \left( \delta_{\text{N}} - \delta_{\text{N}} \right) \right]
\]

(Eq. 1)

Chemical shift differences between the holo and apo forms of full-length OsCaM61 as well as between the full-length and truncated forms of holo- and apo-OsCaM61 were analyzed using Equation 2 (32).

\[
\text{CSP} = \sqrt{\Delta H^2 + (\Delta N/5)^2}
\]

(Eq. 2)
Chemical shifts obtained for deuterated samples through transverse relaxation optimized spectroscopy-based experiments were corrected for isotope shifts before further analysis. \[\text{[H]}-^{15}\text{N} \text{n heteronuclear NOE experiments were conducted on deuterated} \ [^{15}\text{N}-\text{samples of full-length holo- and apo- OsCaM61 using 5-s trains of 120° pulses.} \ [^{15}\text{N} \text{T}_1 \text{data using inversion recovery were obtained with relaxation delay times of} 20, 490, 1190, 180, 90, 350, 890, 490, 90, \text{and} 700 \text{ms, respectively.} \ [^{15}\text{N} \text{T}_2 \text{relaxation data were acquired using a Carr-Purcell-Meiboom-Gill-type} \text{T}_2 \text{experiments with relaxation delays of} 8.8, 105.6, 44, 79.2, 140.8, 26.4, 61.6, 26.4, 123.2, \text{and} 79.2 \text{ms, respectively.}\]

The field strength of the 180° pulse in the T2 experiment was 5.6 kHz, and the 180° pulses were applied every 1 ms. All T1 and T2 experiments were repeated twice to measure uncertainty in peak intensities. \[^{15}\text{N} \text{T}_1 \text{and} \text{T}_2 \text{data were fitted using the program CurveFit (A. G. Palmer, Columbia University).}\]

Peaks representing residues with slow internal motions contribute to T1 relaxation were identified by having NOE <0.65 and were excluded from further analysis. Moreover, residues involved in chemical exchange that can affect T2 relaxation time were identified and removed as described (33). Finally, the rotational correlation time for the global tumbling \(\tau_m\) of each residue was calculated from the \(R_2/R_1\) ratio using the program \(R2R1_\text{quad}\) (A. G. Palmer, Columbia University).

14 mg/ml pf1 phage (Asla-Biotech) was added to partially align the protein molecules. Backbone H-N residual dipolar couplings (RDCs) were measured using a two-dimensional in-phase/antiphase heteronuclear single quantum correlation experiment with 2048 \(\times 512\) complex points (34). The data obtained were fitted to calculated RDCs determined from model structures using the PALES program (35). A linear correlation between the experimentally obtained RDCs and an existing protein structure with a high correlation factor \((R)\) is indicative of a good agreement. All spectra were processed using NMRPipe (36) and analyzed using NMRView (37) software. All molecular graphics used in this study were prepared using MOLMOL (38).

**Ca\textsuperscript{2+} Titrations**—A Ca\textsuperscript{2+}-free buffer consisting of 20 mM HEPES and 100 mM KCl was made using Chelex\textsuperscript{®} 100 resin. Protein was dialyzed against this buffer, and any residual Ca\textsuperscript{2+} was removed using a 1,2-bis-(\(\alpha\)-aminophenoxy)ethane-N\textsubscript{2}N\textsubscript{2}N\textsuperscript{3}N\textsuperscript{4},N\textsuperscript{4}-tetraacetic acid calcium sponge column (Invitrogen). Subsequently, 100 \(\mu\)M protein was prepared and loaded in the cell, and 5.1 mM Ca\textsuperscript{2+} \text{was titrated into the cell. All isothermal titration calorimetry (ITC) experiments were performed on a MicroCal VP-ITC microcalorimeter. The concentration of each protein was determined using the molar extinction coefficient (\(\varepsilon_{280} = 1490 \text{ cm}^{-1} \cdot \text{M}^{-1}\)). All titrations were performed at 25 °C, and the data were fit to a four-site sequential model using MicroCal Origin 7.0 software to obtain the dissociation constants \((K_d)\).

**Enzyme Assays**—MLCK and myosin light chain (LC20) were purified from chicken gizzards according to established procedures (39, 40). To measure the enzymatic activity, 0.1 \(\mu\)g/ml MLCK was preincubated at 30 °C with 50 \(\mu\)g/ml LC20, 25 mM Tris-HCl, pH 7.5, 60 mM KCl, 4 mM MgCl\textsubscript{2}, 0.1 mM excess CaCl\textsubscript{2}, 1 mM DTT, and different forms of OsCaM61 and mCaM for 2 min. The reaction was started with 0.2 mM \([\gamma\text{-32P}]\text{ATP}\).

After 7.5 min, the samples were withdrawn and spotted on P81 phosphocellulose paper (Whatman). Papers were washed with 3 \(\times\) 500 ml of 0.5% (v/v) \(\text{H}_2\text{PO}_4\) (5 min each) and once with 500 ml of acetone (5 min) and left to dry. Subsequently, \(32\text{P}\) was quantified by Cerenkov counting (41). Calmodulin kinase II (CaMKII) was also purified from chicken gizzards (42, 43). 0.2 \(\mu\)g/ml CaMKII was preincubated at 30 °C with 50 \(\mu\)M autocomtide 2 peptide substrate, 50 mM MOPS, pH 7.0, 60 mM KCl, 10 mM MgCl\textsubscript{2}, 0.2 mM excess CaCl\textsubscript{2}, and different forms of OsCaM61 and mCaM for 2 min. The remainder of the assay was as described for the MLCK activity assay.

**RESULTS**

**OsCaM61 CaM Domain and CTE Structural Characteristics**—Several structural studies of CaMs purified from mammalian and plant cells have indicated that these proteins have identical secondary structures (44). To obtain information about the structure of OsCaM61 in aqueous solution, the backbone chemical shifts were assigned for the apo and holo forms of the full-length protein using multidimensional NMR experiments. The assignment was complete for over 95% (supplemental Figs. 1–4). The weighted average secondary shift was calculated for each residue using the assigned chemical shifts where consecutive positive weighted average secondary shift values are indicative of the presence of a helix. OsCaM61 appears to be made up of four typical EF-hand helix-loop-helix motifs (Fig. 3). NMR residual dipolar couplings take advantage of the restriction on the molecular tumbling introduced by anisotropic media to address the relative orientation of secondary structural elements inside proteins (45). Hence, RDCs were measured for the apo and holo forms of the protein in the presence of phage pf1 (45, 46). Also, model structures of the N-lobe and C-lobe of OsCaM61 were created from available mCaM structures (apo N-lobe from Protein Data Bank code 1CFD, apo C-lobe from Protein Data Bank code 1CDF, holo N-lobe from Protein Data Bank code 1J7O, and holo C-lobe from Protein Data Bank code 1J7P) using the SWISS-MODEL server (Fig. 4) (47, 48). The RDC data obtained for OsCaM61 in the phage medium were fitted against the model coordinates (Fig. 4). This analysis demonstrates that the secondary and tertiary structures of the N-lobe and C-lobe of OsCaM61 are very similar to those of mCaM.

In mammalian CaM and soybean CaMs, a flexible linker is located between residues 75 and 81 that connects the two lobes of CaM (29, 49). This flexible linker enables CaM to interact with diverse targets inside the cell (8, 29). The flexibility of the linker region can be conveniently demonstrated using heteronuclear NOE experiments (29, 49). Interestingly, although its amino acid sequence is highly conserved (Fig. 1), the linker region of OsCaM61 shows significantly less flexibility than those of mCaM and soybean CaM isoforms 1 and 4 (29, 49). This is observed for both the apo and the Ca\textsuperscript{2+}-bound form of the protein (Fig. 3), suggesting that there could be an interaction between the C-lobe and N-lobe of OsCaM61. To further examine this possibility, we performed NMR relaxation experiments. The correlation time \(\tau_m\), calculated from the relaxation data is 14.99 ns for OsCaM61 in the holo form. The \(\tau_m\) values calculated for the N-lobe and C-lobe in the intact protein separately are 14.64 and 15.29 ns, respectively (Table 1). These
are much longer than the corresponding values for mCaM, which are 7.1 ns for the N-terminal lobe and 6.3 ns for the C-terminal lobe. This difference must be due to the fact that the two lobes of the CaM segment of OsCaM61 do not act in solution as two independent, non-interacting domains unlike mCaM or sCaMs (29, 49). Also, relaxation data obtained for OsCaM61\textsubscript{H9004}\textsubscript{148} show that the linker region behaves the same in the absence of the CTE, ruling out the possibility that the CTE is solely responsible for these interlobe interactions (data not shown). Moreover, after the removal of the tail, the N-lobe and C-lobe still interact with each other as measured for OsCaM61\textsubscript{H9004}\textsubscript{148} (see Table 1). This is also supported by dynamic light scattering data: the hydrodynamic radius of Ca\textsuperscript{2+}-mCaM is 3.0 nm, whereas that of Ca\textsuperscript{2+}-OsCaM61\textsubscript{H9004}\textsubscript{148} with the same number of amino acids as mCaM is 2.5 nm, suggesting a more compact structure than mCaM due to the interlobe interactions (see supplemental Fig. 5). To investigate the relationship between these interlobe interactions and the biological function, site-directed mutagenesis was conducted on OsCaM61\textsubscript{H9004}\textsubscript{148}. The substitutions A110T, G122E, and S126R have been tested here because these mutants had been shown previously to increase the enzyme activation properties of OsCaM61 (23). Interestingly, \(T_1\) and \(T_2\) analysis on these mutants demonstrated a drastic change in the dynamic behavior of the protein. Although \(T_1\), which is only dependent on molecular weight, temperature, and viscosity of the solution, is very similar between these mutants and the wild type protein, \(T_2\), which is also affected by conformational exchange, shows a faster relaxation rate for the mutants compared with the wild type (Fig. 5). This proposes a possibility that these mutations affect the interlobe interactions, allowing OsCaM61 to adopt new conformations favorable for enzyme activation.

As mentioned above, OsCaM61 has a 38-residue extension at the C terminus. Apart from bearing the C-terminal prenylation site, this region contains several basic residues. Secondary structure prediction (50) from the amino acid sequence of OsCaM61 and for the related PhCaM53 of Petunia hybrida suggests that the CTE is helical from Lys\textsuperscript{149} to Arg\textsuperscript{158} but is unstructured from Glu\textsuperscript{160} to Leu\textsuperscript{186} (Fig. 1). Interestingly, secondary structure analysis using NMR chemical shifts (51) demonstrates that the CTE has different structural characteristics in the apo and holo forms (Fig. 3). Our weighted average secondary shift data show that the apo form of OsCaM61 has an extended helix compared with the holo form, elongating the last helix of the CaM domain (which is shorter compared with other helices in CaM) to Arg\textsuperscript{158}. On the other hand, the Ca\textsuperscript{2+}-bound form of the protein seems to have a much shorter helix or is completely lacking a helical extension (Fig. 3). This can have a major effect on the behavior of the protein in the absence and presence of Ca\textsuperscript{2+}. Although the CTEs in
OsCaM61 and PhCaM53 seem to be partially helical, secondary structure predictions suggest that other similar proteins like AlCaM-2 and AtCaM-5 have no such helical structure (Fig. 1), indicating a behavior in these proteins different from that of OsCaM61.

**Effect of the CTE on Ca\(^{2+}\) Binding to OsCaM61**—Because of the presence of an extended helix in OsCaM61 compared with mCaM, the effect of this change was examined on the Ca\(^{2+}\) binding ability of OsCaM61. Ca\(^{2+}\) titration experiments with full-length and truncated versions of OsCaM61 demonstrate

### TABLE 1
**Calculated rotational correlation times of OsCaM and mCaM in nanoseconds**

Data for holo- and apo-mCaM are taken from Tjandra et al. (56) and Barbato et al. (49), respectively. N-term, N-terminal lobe; C-term, C-terminal lobe; —, not determined.

|       | Apo N-term | Apo C-term | Apo Total | Holo N-term | Holo C-term | Holo Total |
|-------|------------|------------|-----------|-------------|-------------|------------|
| OsCaM61 | 12.67      | 13.37      | 13.07     | 14.64       | 15.29       | 14.99      |
| OsCaM61Δ148 | —         | —          | —         | 12.72       | 12.21       | 12.45      |
| mCaM   | 7.1        | 6.3        | —         | 7.9         | 7.4         | —          |

OsCaM61 and PhCaM53 seem to be partially helical, secondary structure predictions suggest that other similar proteins like AlCaM-2 and AtCaM-5 have no such helical structure (Fig. 1), indicating a behavior in these proteins different from that of OsCaM61.
that the CTE influences the Ca\(^{2+}\) binding properties of the protein. Ca\(^{2+}\) titrations were followed by NMR and showed that upon addition of Ca\(^{2+}\) to OsCaM61 the signals for Gly25 and Gly98 of Ca\(^{2+}\)-OsCaM61 appear first in the low field region of the spectrum (~10 ppm) followed by the appearance of Gly134 and Gly61 signals (Fig. 6). This indicates a specific order of Ca\(^{2+}\) binding to the four Ca\(^{2+}\)-binding loops of OsCaM61. The order of binding of Ca\(^{2+}\) changes in the truncated OsCaM61\(^{148}\) protein, which no longer has the CTE. Now the Gly98 and Gly134 signals appear first followed by Gly25 and Gly61. This is similar to other known CaMs for which the two Ca\(^{2+}\)-binding loops in the C-lobe bind Ca\(^{2+}\) first. Ca\(^{2+}\) titrations with OsCaM61\(^{169}\) show that the pattern of binding is similar to that of full-length OsCaM61 (data not shown). These data are consistent with Ca\(^{2+}\) titrations using ITC showing that OsCaM61 and OsCaM61\(^{169}\) have a very similar Ca\(^{2+}\) binding pattern that is, however, different from that of OsCaM61\(^{148}\) (Fig. 7). Although OsCaM61 and OsCaM61\(^{169}\) have an endothermic reaction with Ca\(^{2+}\), OsCaM61\(^{148}\) shows a mixed endothermic and exothermic reaction with Ca\(^{2+}\). Also, the first and second binding sites in OsCaM61 and OsCaM61\(^{169}\) show similar affinities for Ca\(^{2+}\) and are 20-fold weaker than those of OsCaM61\(^{148}\). Binding constants obtained from ITC and the order of site filling taken from the NMR data reveal that in OsCaM61 and OsCaM61\(^{169}\) Gly\(^{134}\) shows a weaker binding than Gly\(^{25}\) (~20-fold), but after the removal of the helical extension of the protein, Gly\(^{134}\) shows stronger binding to Ca\(^{2+}\) compared with Gly\(^{25}\). This proposes a role for the helical region in the CTE in Ca\(^{2+}\) binding (Table 2 and Figs. 6 and 7).

**The CTE and the CaM Part of OsCaM61 Interact with Each Other**—Previous work on OsCaM61 had demonstrated that the protein was a better activator of the phosphodiesterase enzyme...
when the CTE region was removed from the protein (26). This suggests that the CTE might be interacting with the rest of the protein, thereby inhibiting the interaction with some target enzymes inside the cell. {1H}-15N NOE experiments in both apo form and Ca2+-bound form demonstrated that the {1H}-15N NOE values decrease in the CTE region, demonstrating its increased flexibility (Fig. 3). Values lower than 0.5 are indicative of a flexible region in a globular protein. Intriguingly, the NOE and $R_1$ values start to increase from Gly170 to Arg179 (Fig. 3). This is seen in both the apo and Ca2+-bound forms of

**TABLE 2**

|             | $K_1$           | $K_2$           | $K_3$           | $K_4$           |
|-------------|-----------------|-----------------|-----------------|-----------------|
| OsCaM61     | $3.1 \times 10^4 \pm 2.8 \times 10^2$ | $3.7 \times 10^4 \pm 3.4 \times 10^3$ | $1.1 \times 10^3 \pm 1.1 \times 10^2$ | $139 \pm 18$    |
| OsCaM61Δ148 | $7.2 \times 10^5 \pm 5.1 \times 10^3$  | $1.4 \times 10^5 \pm 2.8 \times 10^3$  | $2.4 \times 10^3 \pm 72$  | $435 \pm 15$   |
| OsCaM61Δ169 | $3.2 \times 10^6 \pm 4.7 \times 10^2$  | $7.1 \times 10^6 \pm 2.1 \times 10^3$  | $3 \times 10^4 \pm 2.1 \times 10^2$ | $168 \pm 22$   |

FIGURE 6. Ca2+ titration of different isoforms of OsCaM61 using NMR illustrates the order of Ca2+ binding to the Ca2+-binding loops of OsCaM61 (A) and OsCaM61Δ148 (B) by monitoring the appearance of the glycine peaks in the expanded NMR spectrum. Only the region for the four glycine residues that are located in position 6 of the Ca2+-binding loops is shown.

FIGURE 7. Ca2+ titration of different isoforms of OsCaM61 using ITC. OsCaM61 and OsCaM61Δ169 show a very similar binding pattern, which is distinct from that of OsCaM61Δ148.
OsCaM61, suggesting that this region might have an interaction with the rest of the protein, leading to a decrease in the flexibility of this region. To further analyze the possibility of such an interaction, the OsCaM61Δ148 form of the protein was expressed in an isotope-labeled form and purified. Subsequently, the backbone chemical shifts for the truncated form in the absence and presence of Ca²⁺ were assigned (supplemental Fig. 1–4), and the chemical shift difference between atoms corresponding to a specific residue were calculated (Fig. 8 A). Interestingly, in the Ca²⁺-bound state, there is a large chemical shift difference for the residues that are located in the linker region (residues 77–81). Also, significant changes are observed forThr⁴⁴, Ala⁵⁷, and Thr¹¹⁷. In the apo form, a change is observed for residues Val⁵⁵, Asp⁵⁸, Asn⁶⁰, Met⁷², Arg⁷⁴, Asp⁸⁰, Ala⁸⁸, Gly⁹⁸, and Arg¹¹⁵ (Fig. 8 B). Titration of a peptide corresponding to residues 149–186 of OsCaM61 into a sample of ¹H,¹⁵N-labeled OsCaM61Δ148 produced similar NMR results, confirming these data (data not shown). Overlaying the CSP data for both A and B in Fig. 8 demonstrated that a common pattern is observed for the regions that are mostly affected as depicted in Fig. 8 C. Interactions of the CTE with these regions of the CaM-like part of OsCaM61 might be responsible for the lower activity of the full-length protein compared with OsCaM61Δ148. Although the full-length OsCaM61 is stable even in the apo form, the truncated apo form of this protein seems very unstable in the absence of Ca²⁺. This caused severe broadening of the NMR signals and led to an incomplete assignment of the backbone (supplemental Fig. 2). These observations also corroborate our earlier finding that the extension of the last α-helix stabilizes the apo form.

A Specific Part of the CTE Might Be Involved in the Interaction with the CaM Domain—Having determined that the CTE interacts with the CaM domain of OsCaM61, [¹H]-¹⁵N NOE and CSP data were used to map the regions of the CTE that are involved in the interaction. As depicted in Fig. 3, there is an increase in the heteronuclear NOE value for residues Gly¹⁷⁰–Arg¹⁷⁹ that could be explained by a possible interaction with the CaM domain. Interestingly, CSPs measured for the Ca²⁺-bound and apo forms of OsCaM61 revealed that residues Lys¹⁴⁹–Arg¹⁵⁸ (the polybasic cluster) and Ser¹⁷² and Ala¹⁷³ (in

**FIGURE 8.** Shown is CSP calculated between OsCaM61 and OsCaM61Δ148 in the presence of Ca²⁺ (A) and the absence of Ca²⁺ (B). C demonstrates an overlay of A (black) and B (gray). Although the residues with large changes are not identical, these data show that the same pattern is obtained with changes occurring around residues 55–60, 77–81, and 115–117.
the unstructured region) of the CTE show a large change in chemical shifts after Ca\(^{2+}\)/H1100 binding to the CaM domain (Fig. 9A). This chemical shift change is comparable with other parts of the protein that undergo major conformational changes after the binding of Ca\(^{2+}\). This suggests that at least this part of the CTE is directly involved in the interactions with the CaM domain and is affected by their conformational changes upon Ca\(^{2+}\) binding. Moreover, Ca\(^{2+}\) titration data also demonstrated a possible role for the helical part of the CTE (Lys149–Arg158) in the apo form by stabilizing the EF-hands. To confirm these observations, enzyme assays were conducted to measure the ability of the different forms of OsCaM61 to activate MLCK and CaMKII. As illustrated in Fig. 9, B and C demonstrate the ability of different forms of OsCaM61 to activate MLCK (B) and CaMKII (C). Error bars represent S.D.

**DISCUSSION**

Because of the presence of multiple CaM variants and CMLs, Ca\(^{2+}\) signaling in plants has been shown to be very diverse (52). Interestingly, CaMs containing a CTE with a prenylation site have been studied *in vivo* in two different plants (53). Undoubtedly, more related proteins will be found as more plant genomes are sequenced. PhCaM53 and OsCaM61 comprise a CaM domain, which is highly similar to other CaM proteins, and a C-terminal extension with a CXXL prenylation motif (X is any amino acid) (25, 26, 53, 54). It has been shown that prenylation of PhCaM53 is prevented under certain metabolic conditions such as the inhibition of mevalonic acid synthesis or moving the plants to the dark, and the protein localizes to the nucleus (25). Surprisingly, although the prenylation process of the isolated CXXL motif linked to GFP still occurs *in vitro*, it is not sufficient to recruit PhCaM53 to the plasma membrane *in vivo* conditions (25), and the presence of the basic cluster located in the...
...the CaM domain. This leads to a higher transition energy compared with the apo state for several peaks even for Gly¬96, which is located in one of the Ca²⁺-binding loops. This could be due to the fact that the apo state is not stabilized by the Ca²⁺ ions, making the apo state more reliant on the stabilizing effects of the CTE compared with the holo state. Moreover, it seems that apo-OsCaM61 unwinds or loses its extended helix positioned in the CTE after binding Ca²⁺ (Figs. 3 and 9). Previously, it was suggested that the prenylation and localization of PhCaM53 are not affected by the absence or presence of Ca²⁺, unlike the myristoylated proteins whose membrane recruitment depends on the change in CaM conformation upon binding Ca²⁺ (25). The experiment used to support this idea showed that GFP could still be recruited to the nucleus or the membrane using just the CTE of PhCaM53. Of course, the CTE in that construct would have been in an extended or unstructured conformation, which is required to interact with the nuclear localization signal receptors or to be prenylated. This would be similar to the conformation of the CTE after the binding of Ca²⁺ to OsCaM61 or the structure of the intact CTE in solution (data not shown). We think that in the open conformation of holo-OsCaM61 the absence of the interhelical interactions might lead to the exposed conformation of the basic cluster in the CTE of holo-OsCaM61 (Fig. 10). This would expose the polybasic residues, enabling them to act as a nuclear localization signal or as an enhancer of prenylation.

Although somewhat less studied than PhCaM53, here we have chosen to study the rice OsCaM61 protein because of the importance of rice in agriculture. In this study, we examined the structural and dynamic characteristics of the CaM domain and the CTE of OsCaM61 in an attempt to describe their roles in the context of the function of OsCaM61. Secondary structure predictions suggest that apo-OsCaM61 may have a longer final helix than mammalian CaM that extends into the CTE (Fig. 1 and 4). This seems to be a pattern in many but not the entire group of plant CaMs bearing such an extension. Our work has shown that the extended helix provides extra interactions and increases the structural stability of the closed conformation of the CaM domain. This leads to a higher transition energy compared with the open conformation and in turn decreases the Ca²⁺ binding affinity (7). In the presence of the CTE, because of these extended interhelical interactions, the fourth Ca²⁺-binding loop has a lower binding affinity for Ca²⁺ compared with the first loop of the protein. Upon removal of the CTE, the affinities of the third and fourth Ca²⁺-binding loops increase 10 times, most likely due to the decrease in the stability of the apo form. This phenomenon has not yet been reported for other known CaMs, emphasizing the unique function of the extended α-helix in CaM variants such as OsCaM61 and PhCaM53. Because EF-hands usually act as a pair (55), extra interhelical interactions in the fourth EF-hand also increase the stability of the coupled third EF-hand and in turn decrease the affinity of this Ca²⁺-binding loop for Ca²⁺. The stabilizing effect of the CTE seems to be particularly important for the apo state of OsCaM61. This can be observed by the severe NMR peak broadening that occurs upon removal of the CTE in OsCaM61Δ148 for the apoprotein but not for the holoprotein (supplemental Figs. 2 and 4). Also, there are drastic changes in the chemical shifts of the apo state for several peaks even for Gly¬96, which is located in one of the Ca²⁺-binding loops. This could be due to the fact that the apo state is not stabilized by the Ca²⁺ ions, making the apo state more reliant on the stabilizing effects of the CTE compared with the holo state. Moreover, it seems that apo-OsCaM61 unwinds or loses its extended helix positioned in the CTE after binding Ca²⁺ (Figs. 3 and 9). Previously, it was suggested that the prenylation and localization of PhCaM53 are not affected by the absence or presence of Ca²⁺, unlike the myristoylated proteins whose membrane recruitment depends on the change in CaM conformation upon binding Ca²⁺ (25). The experiment used to support this idea showed that GFP could still be recruited to the nucleus or the membrane using just the CTE of PhCaM53. Of course, the CTE in that construct would have been in an extended or unstructured conformation, which is required to interact with the nuclear localization signal receptors or to be prenylated. This would be similar to the conformation of the CTE after the binding of Ca²⁺ to OsCaM61 or the structure of the intact CTE in solution (data not shown). We think that in the open conformation of holo-OsCaM61 the absence of the interhelical interactions might lead to the exposed conformation of the basic cluster in the CTE of holo-OsCaM61 (Fig. 10). This would expose the polybasic residues, enabling them to act as a nuclear localization signal or as an enhancer of prenylation. This is different from the previous thought that the induced conformational change in CaM has no effect on its localization or prenylation. It is important to note from our ITC results that the affinity of the fourth Ca²⁺-binding loop is too weak to bind Ca²⁺ under physiological conditions. Therefore, it is currently not clear whether Ca²⁺ binding to only the third Ca²⁺-binding loop is enough to carry out the above mentioned functions. Also, the Ca²⁺ binding affinity is normally increased in the presence of target enzymes (7). Further investigations are ongoing to address this question.

NMR dynamics studies revealed a highly flexible character for the unstructured region of the CTE. Interestingly, residues 170–179 show an increase in their NOE value, meaning that there is some restriction to their movement; most likely this region folds back and interacts with the CaM domain of the protein. Our NMR CSP data support this hypothesis as they show that certain parts of the CaM domain of OsCaM61 interact with the CTE in the absence and presence of Ca²⁺. In the presence of Ca²⁺, there seems to be an interaction between the CTE and the linker region. We think that these interactions inhibit OsCaM61 from adopting a proper target-binding surface, leading to an increase in the $K_m$ for some target enzymes. Once OsCaM61 binds to the enzymes, it can activate them to the maximum level, and for that reason the $V_m$ does not change (Table 3). Recent studies have shown that the presence or absence of the CTE does not have any effect on $K_m$ and $V_m$ in the activation of O. sativa Ca²⁺/CaM-binding protein kinase (OsCBK), a reaction that is thought to only be activated by OsCaM61 but not by OsCaM1 (23). This might suggest that the CTE increases the selectivity to activate specific targets and enables OsCaM61 to have a rather specific control during Ca²⁺

### Table 3: Kinetic data for the activation of MLCK and CaMKII by different OsCaM61 constructs

| Protein        | $K_m$ (μM) | $V_m$ (μmol Pi/min/mg) | $K_m$ (μM) | $V_m$ (μmol Pi/min/mg) |
|----------------|------------|------------------------|------------|------------------------|
| OsCaM61        | 1982 ± 230 | 2.1 ± 0.1              | 89.6 ± 12.7 | 127.9 ± 4.2            |
| OsCaM61Δ148    | 92.5 ± 11.5 | 1.8 ± 0.05             | 14.5 ± 2.3  | 132.5 ± 4.1            |
| OsCaM61Δ169    | 642.3 ± 63.2 | 2.4 ± 0.05             | 93.5 ± 14.6 | 126.1 ± 4.1            |
| mCaM           | 0.4 ± 0.03 | 2.5 ± 0.03             | 4.3 ± 0.8  | 143 ± 7                |

...
Our NOE and $R_1$ data demonstrate that the region of the CTE that interacts with the CaM domain is located between residues 170 and 179. This is consistent with the CSP data between holo- and apo-OsCaM61 showing a large change in particular for residues Ser172 and Ala173. To validate this, OsCaM61/H9004169 was generated, and its ability to activate different enzymes was compared with OsCaM61 and OsCaM61Δ148. In the case of MLCK, omitting the last 16 residues of OsCaM61 enhances its activation capability. Interestingly, two key residues involved in the interaction of the CaM domain and CTE are alanine and serine. Previously, it was thought that basic residues present in the CTE are solely...
responsible for the characteristics of the CTE; however, our data suggest that residues other than basic residues could be involved in the function of the CTE. Also, our enzyme assays showed that OsCaM61A148 has a lower $K_m$ compared with OsCaM61, but the $V_{max}$ was constant, indicating that the CTE inhibits the binding of the protein to the target enzyme rather than interfering with the activation. For CaMKII, on the other hand, OsCaM61A169 can interact with CaMKII with almost the same $K_m$ as the full-length protein. However, OsCaM61A148 has a 6-fold increase in its affinity for CaMKII, suggesting that the residues involved in the inhibition are in the region 149–159 and that they are located in the positively charged region of the CTE. This region may give rise to unfavorable interactions between the protein and CaMKII.

Apart from the effect of the CTE on the behavior and function of OsCaM61, it seems that unique residues in the CaM domain of OsCaM61 contribute to its function. It appears that the interlobe interactions in OsCaM61 force the two CaM lobes to adopt unique conformations that lead to a specific activation capability. Our mutational analysis suggests that by mutating A110T, G122E, and S126R the protein undergoes conformational exchange. More drastic changes were observed for the A110I mutant (position 110 is occupied by an Ile residue in sCaM4, a soybean CaM with two independent domains): most of the peaks originating from the N-lobe in the heteronuclear single quantum correlation spectrum disappeared by introducing this single mutation in the C-lobe (Fig. 11). Under these conditions that give rise to the breakage of the interactions between the two lobes, the two domains can adopt new conformations and better activate the target enzymes. This suggests that the interlobe interactions are a key aspect of the structure of the protein and are directly related to its function.

Overall, it seems that Ca$^{2+}$-binding plant proteins use various themes to relay Ca$^{2+}$ signals. This control includes expressing several CaM variants as well as CMLs in the cell. Moreover, we have shown here that the variant of CaM that has a CTE that exists in some plant cells gives rise to unique properties and functions. The CTE includes a prenylation motif that helps CaM to localize in a specific region of the cell, enabling the cell to have better spatial control over Ca$^{2+}$ signaling. Also, the CTE can interact directly with the CaM domain, thereby modulating the affinity for Ca$^{2+}$ and controlling its ability to bind to different enzymes inside the cell (Fig. 10).

Acknowledgment—We thank Dr. Rustem Shaykhutdinov for the maintenance of the NMR instrumentation in the Bio-NMR Centre.

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