The Crystal Structure of the Novel Calcium-binding Protein
AtCBL2 from Arabidopsis thaliana*

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Arabidopsis thaliana calcineurin B-like protein (AtCBL2) is a member of a recently identified family of calcineurin B-like calcium-binding proteins in A. thaliana. The crystal structure of AtCBL2 has been determined at 2.1 Å resolution. The protein forms a compact α-helical structure with two pairs of EF-hand motifs. The structure is similar in overall folding topology to the structures of calcineurin B and neuronal calcium sensor 1, but differs significantly in local conformation. The two calcium ions are coordinated in the first and fourth EF-hand motifs, whereas the second and third EF-hand motifs are maintained in the open form by internal hydrogen bonding without coordination of calcium ions. Both a possible site and a feasible mechanism for the target binding to AtCBL2 are discussed based on the three-dimensional structure.

Calcium signaling mechanisms are widely employed by all eukaryotic organisms to regulate gene expression and a variety of cellular processes. In plants, many extracellular signals, such as light, drought, cold, salinity, and stress factors, elicit changes in cellular calcium concentration (1, 2). The calcium sensor protein often changes its conformation in a calcium-dependent manner and interacts with other calcium-binding proteins to relay the signal. Several families of calcium sensor proteins have been identified in higher plants. One of the most fully characterized sensor proteins is calmodulin, which has four EF-hand motifs for calcium binding (3).

Recently, novel calcium sensor proteins with EF-hand motifs from Arabidopsis thaliana have been identified (4, 5), referred to as AtCBL1 (A. thaliana calcineurin B-like protein). AtCBL is also referred to as SCaBP (SOS3-like calcium-binding proteins), where SOS3 (salt overlay sensitive 3) is the first sensor protein identified in this family. These proteins show substantial sequence similarity with the regulatory B subunit of calcineurin (CNB) and neuronal calcium sensor 1 (NCS-1) from animals (Fig. 1). AtCBLs are encoded by a multigene family of at least 10 members in Arabidopsis and are predicted to have three EF-hand motifs for calcium binding with lower affinity than calmodulin (6). Furthermore, other members of the AtCBL family, such as AtCBL 1, 4, and 8, contain a putative N-terminal myristoylation motif. In fact, AtCBL4 (SOS3) requires N-myristoylation for plant salt tolerance (6).

AtCBL2 is a CBL that has no myristoylation motif in the N-terminus, and its expression profile suggests a role in light-signal transduction (17). AtCBL2 interacts prominently with AtSR1, which is identical to AtCIPK14 in the CIPK family. AtSR1 accumulates in response to light (18), evidence that...
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* Table I

| Phasing                  | Native-1 | Native-2 | TMLA* | LuCl3 | K$_2$Pt(NO$_2$)$_4$ |
|--------------------------|----------|----------|-------|-------|---------------------|
| Resolution (Å)           | 2.1 (2.17-2.10)$^b$ | 2.5 (2.59-2.50) | 2.5 (2.59-2.50) | 3.2 (3.31-3.20) | 3.5 (3.62-3.49) |
| Reflections              | 80668    | 62590    | 103928 | 30395 | 20183              |
| Unique reflections       | 14398    | 8671     | 8939   | 4717  | 3259               |
| Completeness (%)         | 98.0 (96.3) | 98.5 (99.0) | 98.4 (95.5) | 97.5 (100.0) | 96.7 (96.0) |
| R$_{sym}$ (%/f)          | 5.5 (26.6) | 4.9 (23.5) | 6.1 (32.8) | 10.7 (30.6) | 10.9 (28.0) |
| Mean I/σ                 | 8.5 (1.9) | 11.6 (2.9) | 8.6 (2.0) | 6.5 (2.3) | 5.5 (2.4) |
| R$_{free}$/R$_{sym}$ (%) | 14.3     | 12.1     | 12.1   | 25.1  |                    |
| Phasing power (a/c) (%)  | 0.970.77 | 0.580.43 | 0.730.63 |       |                    |
| Mean figure of merit     | 0.37     |          |        |       |                    |

| Refinement               | Native    |
|--------------------------|-----------|
| Resolution range (Å)     | 35.58–2.1 |
| Atoms included           | 1544 for protein, 138 for water, 2 for Ca$^{2+}$ ion |
| R$_{cryst}$/R$_{free}$ (%)| 20.4/24.8 |
| Mean B factor (Å$^2$)    | 36.8      |
| r.m.s.d. bonds (Å)       | 0.013     |
| r.m.s.d. angles (°)      | 1.415     |

$^a$ TMLA, trimethyllead acetate.
$^b$ Values in parentheses are for the outermost resolution shell.

**Materials and Methods**

Expression, Purification, CD Spectroscopy, and Electrospary Ionization Mass Spectra—Details on the expression, purification, and crystallization of AtCBL2 and AtSR1 have been reported previously (30). In brief, the AtCBL2 encoding *A. thaliana* 1-226 was expressed as a fusion protein with glutathione-S-transferase. It was then purified by three column chromatography steps using glutathione Sepharose, HiTrap Q, and Superdex 75 (Amersham Biosciences).

The CD spectra of calcium-bound AtCBL2 were measured on a JASCO J-720W spectrometer using a 0.1-mm quartz cuvette in a stock solution (5 mM Tris-HCl buffer, pH 7.5, 1.0 mM NaCl, 1.0 mM diethylenetriol, and 1.0 mM CaCl$_2$). Successively, the CD spectra were also recorded after the addition of 1.25 mM EGTA.

Electrospary ionization mass spectra were acquired on a Micromass Q-ToF2 mass spectrometer. A solution of 50 μM calcium-bound AtCBL2 in the stock solution was applied on a hand-filled short size-exclusion chromatography column packed with Amersham Sephadex G-25, and rapid in-line desalting was achieved prior to electrospary ionization-mass spectroscopy analyses (49). After the equilibration of the size-exclusion chromatography short column with 10 mM ammonium acetate, 5 μL of the sample solution was injected. The mobile phase was introduced at 5 μL/min, and electrospary mass spectra were obtained.

Crystalization and Data Collection—Crystals of AtCBL2 were obtained using polyethylene glycol 8000 as a precipitant. The crystals belong to space group C222$_1$, with unit-cell parameters of a = 83.9 Å, b = 118.1 Å, and c = 49.1 Å. A summary of data collection statistics is given in Table I. All the sets of diffraction intensity data for structural analysis (data-sets of Native-2, trimethyllead acetate, LuCl$_3$, and K$_2$Pt(NO$_2$)$_4$ in Table I) were collected at 100 K using Cu-K$_α$ radiation with a Rigaku R-AXIS IV++ Imaging Plate diffractometer equipped with Osmic confocal mirror optics, the whole being mounted on a Rigaku FR-D ultra-high brilliant rotating-anode x-ray generator operated at 50 kV and 60 mA. High-resolution native data (data set of Native-1 in Table I) were collected at 100 K using a Jupiter 210 (Rigaku MSC) on the BL14XU beam line at SPring-8, Harima, Japan. The wavelength was set to 1.02 Å with a crystal-to-detector distance of 190 mm and an exposure time of 30 s per degree of oscillation. All the data sets were processed using CrystalClear (31).

Structural Determination and Refinement—No successful results were obtained by the molecular replacement method, in which AMoRe (32) was applied to the structure of the human calcineurin-B subunit (Protein Data Bank code 1AUI) (22) or on the structure of NCS-1 (Protein Data Bank code 1GB1) (19) as a search model. This was attributed mainly to a rather low sequence homology with either of these models, as well as to structural differences. The structure was therefore solved by a multiple isomorphous replacement method. Experimental phases were calculated up to 2.5 Å resolution with SOLVE (33) and improved by solvent flattening with RESOLVE (34). An initial model built with O (35) was refined with crystallography-NMR software (36) to an R value of 33.5%. After several cycles of rebuilding and refinement with REFMAC (37), the model finally converged, resulting in a crystallographic R value of 20.4% and a free R value of 24.8% for all diffraction data up to 2.1 Å resolution. The Ramachandran plot of the final model, containing 189 amino acid residues from Asp-32 to His-220 plus two calcium ions and 138 water molecules, shows that all of the amino acid residues are in the most favored and allowed region defined by the program PROCHECK (38). The structural determination and refinement statistics are summarized in Table I. Accessible surface areas were calculated using the Protein-Protein Interaction server (39). The figures are displayed by GRASP (40), MolScript (41), and Raster3d (42).

**Results**

Overall Structure—The polypeptide chain of AtCBL2 is folded into two globular domains (N-terminal and C-terminal domains) composed of an α-helical structure with nine α-helices (αA-αI), two β$_{3}_{β_2}$-helices (αJ and αK) and four short β-strands. These two domains are connected by a short linker (Figs. 1 and 2a) and superimposed with a root mean square deviation (r.m.s.d.) of 2.0 Å for Ca atoms corresponding to four α-helices (αB-αE, αF-αI) and the structure of AtCBL2 is similar in overall folding topology to the structures of the related proteins, CNB and NCS-1 (respective 23 and 22% sequence identities with AtCBL2), neurocalcin and recoverin (Fig. 1). However, AtCBL2 contains an additional helix (αN) in the N terminus and a long C-terminal region including αJ and αK, compared with the structure of CNB. In addition, significant differences are observed in local conformation at the domain-domain interface between AtCBL2 and these related proteins. In fact, the superposition of AtCBL2 on CNB for Ca atoms corresponding to eight α-helices yields the large r.m.s.d. of 2.3
Å, whereas the N-terminal and C-terminal domains are both well superimposed, with r.m.s.d of 1.1 and 1.6 Å, respectively. This indicates that the different domain-domain hinge motions occur between AtCBL2 and the related proteins. In fact, when the N-terminal domains of AtCBL2 and CNB are superimposed, 30° swiveling of the C-terminal domain is observed (Fig. 2b).

 Likewise, 15° rotations are observed between AtCBL2 and NCS-1 (19) and between AtCBL2 and neurocalcin (20). Loop structures connecting α-helices at the opposite side of the EF-hands are significantly different between AtCBL2 and CNB. In the loop connecting C and D, the residues located at the end of C and the loop (Leu-74 and Phe-77) make intimate interactions with the residues in the C-terminal region (Tyr-206, Pro-215, and Phe-217). As a result, C and the loop orient inward (Fig. 2c). In contrast, CNB contains a short α-helix in the corresponding loop region and the loop protrudes toward the solvent. These structural differences induce a large conformational change in the loop region and a significantly different interhelical angle of C and D (AtCBL2, 50°; CNB, 132°).

Although the sequence and length of the loop connecting G and H is similar in AtCBL2 and CNB, the loop conformation and interhelical angle of G and H are different (AtCBL2, 97°; CNB, 126°). This is mainly because of the interactions among the residues (Met-147 and Glu-154) in the G and the loop, and the C-terminal region (Leu-198 and Asn-201) and the loop C-D (Lys-78). EF-1 (residues 58–71; Fig. 4a) displays a large conformational change due to a characteristic sequence in AtCBL2 (see “EF-hand”).

Compared with the amino acid sequences of CNB and NCS-1, AtCBL2 has a 30-residue segment extending to the N terminus. This segment is disordered in the crystal and considered to be functionally unimportant, because the deletion mutant of the first 30 amino acids from the N terminus retains the interaction activity (21).

Hydrophobic Crevice Shielded by C Terminal Region—The C-terminal regions, including 310-helices J and K of the related proteins, adopt various conformations. The conformation affects the degree to which the hydrophobic crevice is exposed. The crevice is located at the opposite sides of EF-hands (Figs. 2a and 3a) and is involved in target recognition by the CNB in complex with calcineurin A (CNA) (22, 23). The helix J of neurocalcin and recoverin extend toward this crevice, and the helix K of recoverin is exposed to the solvent region (24, 25). The helix J of NCS-1 differs from the helices of neurocalcin and recoverin and is oriented along the edge of the crevice.

In contrast to the related proteins, the C-terminal region that includes two 310-helices of AtCBL2 is plunged into the crevice so as to shield the crevice from the solvent (Fig. 3a), whereas the crevices of NCS-1, recoverin, and neurocalcin are fully or partially exposed into the solvent region. The surface area of the interface between the crevice and the C-terminal region of AtCBL2 is estimated to be ~1000 Å², which is within the ranges usually found for the interfaces of protein complexes (26) but is much smaller than the interface of CNB-CNA complex (~1800 Å²). The electrostatic surface potential map shows that the crevice is hydrophobic, and the interactions between the crevice and the C terminus are formed by several
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Fig. 2. Overall structure of AtCBL2. a, ribbon representation with numbering scheme of secondary structure elements. α-Helices and 3_{10}-helices, colored blue and green, respectively, are sequentially labeled αA–αK. β-strands, colored orange, are also labeled β1–β4, and calcium ions are depicted by yellow balls. b, ribbon representation superimposed with CNB. AtCBL2 and CNB are represented with blue and red ribbons, respectively. Two ribbon structures were superimposed, with their corresponding α-helices of N-terminal domains shown in a paler shade. N-terminal regions are omitted for clarity. Rotation angle of the C-terminal domains of AtCBL2 and CNB is ~30°. c, superimposition of AtCBL2 (blue) and CNB (red) viewed from the opposite side of α. Segments displaying large displacements are highlighted, and the residues discussed are displayed.

hydrophobic residues. Because the residues in the C-terminal region (Met-202, Leu-204, Leu-207, Phe-214, and Phe-217) are secondary structure elements, they are highly conserved in the AtCBL family except for AtCBL8, the shielding mechanism may be commonly shared.

EF-hand—The canonical EF-hand motif is helix-loop-helix and is characterized by a sequence with the pattern X, Y, Z, −Y, −X, −Z, where X, Y, Z, −Y, −X, and −Z are the ligands that participate in calcium coordination. AtCBL2 is predicted to have three or four EF-hand motifs (EF-1 ~ EF-4) (Figs. 1 and 4a) from the amino acid sequence, and two calcium peaks can be clearly identified in EF-1 and EF-4 from the omit map contoured at the 7σ level (Fig. 4b). We also confirmed the number of calcium ions coordinated in AtCBL2 by electrospray ionization mass spectra. The spectra indicated that a series of ions corresponding to uptake of 0, 1, or 2 calcium ions per mole equivalent of protein, associated with Na⁺ adducts. After the deconvolution process of the acquired spectrum, three peaks at M̃_c 25953.4 (apo), M̃_c 25991.5 (+1 Ca²⁺), and M̃_c 26029.9 (+2 Ca²⁺) were recognized associated with Na⁺ adducts. No ions with three or four Ca²⁺ adduct were observed. This suggests that the protein binds two calcium molecule equivalents per mole protein, and that some calcium ions bound to the protein are removed during the ionization or desalting process.

The Lys at the Y position of EF-4 is exceptional for a classical EF-hand structure, but the calcium ion is coordinated in a typical EF-hand fashion of a pentagonal-bipyramidal geometry with the side chain carboxylate of Asp-176 (X), Asp-180 (Z), Glu-187 (−Z), the main chain carbonyl of Lys-178 (Y), and that of Lys-182 (−Y), and a water molecule (−X) (Fig. 4b). The sequence of EF-1 differs markedly from that of the classical EF-hand. EF-1 lacks highly conserved Asp residue at the X position, and three residues are inserted between the X and Y positions. The sequence also differs from that of the S100 protein family, which is well known to have a non-classical EF-hand structure. A calcium ion is, however, identified in EF-1 and coordinated in the fashion of a pentagonal-bipyramidal geometry with the side chain carboxylate of Asp-64 (Z), Glu-71 (−Z), the main chain carbonyl of Lys-178 (Y), and that of Lys-182 (−Y), and a water molecule (−X) (Fig. 4b).

The sequences of EF-2 and EF-3, which are similar to the sequence of EF-4, are almost canonical except for Lys at the Y position, but no calcium ion is identified in EF-2 or EF-3. The structures of EF-2 and EF-3 are stabilized with many electrostatic non-covalent internal interactions (Fig. 4c). In EF-2, Asp-95 at the X position is hydrogen-bonded to the main chain atoms of Gly-100 and the side chain atom of Asn-99, and Gly-106 (−Z) is hydrogen-bonded to Asp-95 and Asn-99 through a water molecule. In EF-3, Asp-132 (X) interacts with the main chain atoms of Gln-136 and Gly-137, and water-mediated hydrogen bonds are also observed among Lys-134, Gln-136, and Gly-140. In EF-hand structures that lack calcium ions, similar structural features appear for some calcium-binding proteins. p11 (S100A10) makes hydrogen bonds between the side chain of Asp (X) and main chain atoms (27), whereas recoverin has a salt-bridge between Lys and Glu (24).

All EF-hands in CNB have calcium ions, whereas neurocalcin, NCS-1, recoverin, and guanylyl cyclase activating protein-2, which belongs to the recoverin family (28), has a disabled EF-1 hand (in addition, recoverin has a disabled EF-4 hand), because their Lys and Cys residues are unsuitable for the formation of calcium ligands.

Structural analyses of several calcium-binding proteins have shown that when both EF-hands from a domain pair are occu-
The present study is the first crystal structure analysis of proteins belonging to the AtCBL family. It revealed that AtCBL2 has two calcium-loaded EF-hands despite atypical sequence for EF-hand, indicating that, among members of this family, the number of calcium ions depends on the sequence alignment (Fig. 4a). For example, the Y position of EF-2 in AtCBL7 is occupied by Asn residue, which is suitable for the coordination to calcium ion. Likewise, each Y position of EF-3

**Fig. 4.** Representation of the four EF-hand motifs (EF-1, EF-2, EF-3, and EF-4). a, sequence alignment in the EF-hand regions of the proteins in the CBL family (AtCBL1–9). The residues involved in the calcium coordination are marked at the top of the sequence alignment of each EF-hand motif by (X, Y, Z, X, Y, Z) according to the classical EF-hand. The consensus residues of canonical EF-hands (43) are shown at the bottom. Amino acid residues identical to the consensus are colored yellow. b, calcium-bound EF-hand motifs (EF-1 and EF-4). Calcium ions are represented as yellow balls. An omit map, contoured at the 7σ level around each calcium ion, is superimposed. Amino acid residues involved in the calcium coordination are shown. c, calcium-unbound EF-hand motifs (EF-2 and EF-3). Non-covalent interactions within EF-hands are represented as dotted lines.

**Fig. 5.** CD spectra of calcium-loaded AtCBL2 (solid line) and that treated with 1.25 mM EGTA (dotted line).

... pied by calcium ions, the helices of each EF-hand exhibit open conformation related by −90°, and in calcium-free EF-hands the helices exhibit closed conformation with nearly anti-parallel arrangement. All the α-helices in the four EF-hand structures of AtCBL2 are well superimposed, with r.m.s.d. values of 1.3–1.5 Å for corresponding α-helices, whereas the angle between the two α-helices within each EF-hand structure ranges from 77° to 123°. This indicates that the four EF-hands in AtCBL2 adopt open conformations.

As shown in Fig. 5, the CD spectrum of AtCBL2 was markedly different between a holo-form and an apo-form; the latter is derived from treating the holo-form with ethylene glycol bis(2-aminoethyl ether) tetraacetic acid (EGTA), a chelating reagent. This result suggests that the AtCBL2 changes its conformation in a calcium-dependent manner and thereby functions as a molecular switch through the EF-hands.

**DISCUSSION**

The present study is the first crystal structure analysis of proteins belonging to the AtCBL family. It revealed that AtCBL2 has two calcium-loaded EF-hands despite atypical sequence for EF-hand, indicating that, among members of this family, the number of calcium ions depends on the sequence alignment (Fig. 4a). For example, the Y position of EF-2 in AtCBL7 is occupied by Asn residue, which is suitable for the coordination to calcium ion. Likewise, each Y position of EF-3...
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in AtCBL1, 6, or 9 is either an Asp or an Asn residue. These structural characteristics favor the coordination of calcium ions in the respective EF-hand motifs. In contrast, the fact that four amino acid residues are deleted around the Y position of EF-1 in AtCBL6 (Fig. 4a) indicates that AtCBL6 is unlikely to contain calcium ions in EF-1. Such differences in calcium coordination within the CBL family might be related to the specificity of target recognition in calcium signaling. Ishitani et al. (6) showed that the mutant of a three-amino acid deletion in the EF-2 in SOS3 (AtCBL4) revealed little or no calcium binding while showing disruption of the interaction between SOS3 and SOS2. The EF-2 in AtCBL2 has no calcium ion and SOS3 probably does not contain it, because the amino acid sequences are homologous in this region. Therefore, the deletion may not cause a loss of calcium but may instead disrupt the conformation of the EF-hand kept in the open form. This would decrease the affinity for calcium ion while also increasing disruption of the target protein.

It is known that the β-sheet of the C-terminal domain of calmodulin, to which calcium binds with high cooperativity, is highly twisted in the apo form, and calcium binding removes this twist (44–46). Therefore, this conformational change in the β-sheet may play an important role for the cooperative calcium binding, by which calcium binding in one EF-hand can affect the other EF-hand to form the calcium binding conformation. Cooperativity was observed for the pair of sites in each domain, but not between the N- and C-terminal domains. For example, unmyristoylated recoverin has one functional EF-hand in the domain and exhibits uncooperative binding of two calcium ions (47). Because AtCBL2 has one functional EF-hand in each domain with no myristoylation, this protein would exhibit uncooperativity, and thus β-sheet would not be related with the cooperative binding. Moreover, AtCBL2 functions as a sensor with two EF-hands (EF-1 and -4), and this structural study shows the disabled EF-2 and -3 because of the internal interactions.

The target recognition mechanism by calcium-binding proteins with EF-hand motifs has been extensively studied for calmodulin, which is recognized by its interaction with the short helices in the calmodulin binding domain of each target protein (29). In the crystal structure of CNB in complex with CNA, the hydrophobic crevice of CNB recognizes a five-turned α-helix protruding from CNA (Fig. 3b). The helix J of CNB is pushed aside along the edge of the crevice. The affinity for the target protein might be stronger than that for the C-terminal region, because the interface surface area between CNB and CNA in CNB-CNA complex is significantly (~1.8 times) larger than that between the hydrophobic crevice and the C-terminal region of AtCBL2. Assuming that the recognition mechanism between AtCBL2 and CIPK is similar to that between CNB and CNA in CNB-CNA complex, the C terminus region of AtCBL2 is released from the hydrophobic crevice so that it can interact with the CBL binding domain of CIPK when AtCBL2 interacts with CIPK. It is therefore indicated that the C terminus region of AtCBL2 blocks the adventitious binding of various proteins to AtCBL2 in the absence of CIPK. As stated above, the hydrophobic crevice of AtCBL2 corresponds to that of CNB and is ~36 Å, long enough to recognize 21 or 24 amino acid residues. It should also be noted that the NAF domain or FISL motif necessary for CBL binding consists predominantly of hydrophobic residues. These structural features suggest that the CBL binding domain of CIPK interacts hydrophobically with CBL.

Structural studies of recoverin family have shown that a hinge motion between N- and C-terminal domains is dependent on the calcium binding state (48). The binding of calcium ion to the EF-hand in recoverin leads to local structural changes within the EF-hand that alter the domain interface and cause a swiveling of the N- and C-terminal domains. Likewise, it is supposed that the calcium binding to the EF-hands of AtCBL2 induces local structural changes, and a backbone hinge rotation at the domain linker allows a re-arrangement of the domain interface. The C-terminal region in recoverin partially shields the crevice, and this region has similar conformation in apo- and holo-state. Therefore, the C terminus region of AtCBL2 would have the same conformation in apo- and holo-state to shield the hydrophobic crevice.

The calcium-bound structures, guanylyl cyclase activating protein-2 (28), NCS-1 (19), and neurocalcin (20), show similar swiveling angle of the N- and C-terminal domain. Their structures were determined by different methods, NMR (guanylyl cyclase activating protein-2) and x-ray (NCS-1 and neurocalcin), and x-ray structures were solved in the different crystal packing. These facts suggest that the swiveling angle is intrinsic and not an indication of an artifact due to crystal packing interactions.

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