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Expression, purification, crystallization and preliminary X-ray analysis of calmodulin in complex with the regulatory domain of the plasma-membrane Ca^{2+}-ATPase ACA8

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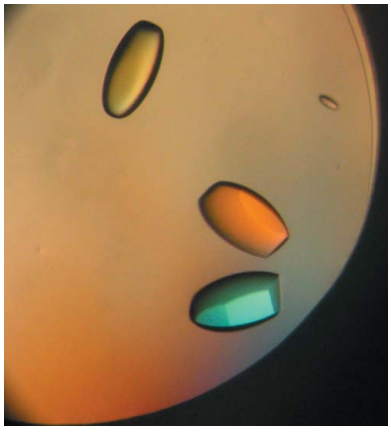
Plasma-membrane Ca^{2+}-ATPases (PMCAs) are calcium pumps that expel Ca^{2+} from eukaryotic cells to maintain overall Ca^{2+} homoeostasis and to provide local control of intracellular Ca^{2+} signalling. They are of major physiological importance, with different isoforms being essential, for example, for presynaptic and postsynaptic Ca^{2+} regulation in neurons, feedback signalling in the heart and sperm motility. In the resting state, PMCAs are autoinhibited by binding of their C-terminal (in mammals) or N-terminal (in plants) tail to two major intracellular loops. Activation requires the binding of calcium-bound calmodulin (Ca^{2+}-CaM) to this tail and a conformational change that displaces the autoinhibitory tail from the catalytic domain. The complex between calmodulin and the regulatory domain of the plasma-membrane Ca^{2+}-ATPase ACA8 from *Arabidopsis thaliana* has been crystallized. The crystals belonged to space group $C2$, with unit-cell parameters a = 176.8, b = 70.0, c = 69.8 Å, β = 113.2°. A complete data set was collected to 3.0 Å resolution and structure determination is in progress in order to elucidate the mechanism of PMCA activation by calmodulin.

1. Introduction

Plasma-membrane Ca^{2+}-ATPases (PMCAs) are high-affinity calcium pumps that export Ca^{2+} from the cytosol to the extracellular environment of eukaryotic cells and thus maintain overall Ca^{2+} homoeostasis and provide local control of intracellular Ca^{2+} signalling (reviewed in Di Leva *et al.*, 2008; Brini, 2009). They belong to the P_{2B} subfamily of P-type ATPases (Axelsen & Palmgren, 1998) and are of major physiological importance, with different isoforms being essential, for example, for presynaptic and postsynaptic Ca^{2+} regulation in neurons, feedback signalling in the heart and sperm motility (Strehler *et al.*, 2007). Compared with other P-type ATPases, plasma-membrane calcium ATPases contain an additional autoinhibitory or regulatory domain. Based on the location of this domain, plasma-membrane Ca^{2+}-ATPases can be subclassified into PMCAs (plasma-membrane Ca^{2+}-ATPases) from animals and ACAs (autoinhibited Ca^{2+}-ATPases) from plants (Baekgaard *et al.*, 2006). In the resting state, these calcium pumps are autoinhibited by binding of their C-terminal (in mammals) or N-terminal (in plants) tail to two major intracellular loops. Activation requires the binding of calcium-bound calmodulin (Ca^{2+}-CaM) to a calmodulin-binding domain (CaMBD) within this tail and a conformational change that displaces the autoinhibitory tail from the catalytic domain (Carafoli, 1994; Penniston & Enyedi, 1998). The autoinhibitory and calmodulin-binding domains at least partly overlap in both plants and mammals (Enyedi *et al.*, 1989; Harper *et al.*, 1998). For ACA8 it has been shown that the CaMBD comprises significantly more residues than the autoinhibitory domain (Baekgaard *et al.*, 2006).

Calmodulin is a small Ca^{2+}-binding protein which is one of the most ubiquitous, abundant and conserved proteins in eukaryotes. It interacts with a large number of proteins and modulates physiological processes as diverse as muscle contraction, apoptosis, inflammation, metabolism, fertilization, immune response and cell proliferation (Hoeflich & Ikura, 2002; Chin & Means, 2000). Calmodulin contains four EF-hand motifs, each of which binds a Ca^{2+} ion. Its approxi-



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                  40         50         60         70         80         90
      Sequence    SIERLQQWRK AALVLNASRR FRYTLDLKKE QETREMRQKI RSHAHALLAA NRFMDM

Secondary structure   ?hhhhhhhhh hhhhh????? heeeeccchh hhhhhhhhhh hhhhhhhhhh hhhhhh
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Figure 1
Sequence of the ACA8 regulatory domain used in this study. The consensus secondary-structure prediction is indicated (h, helix; e, sheet). The putative CaM-binding region of ACA8 is underlined (Baekgaard *et al.*, 2006).

mately symmetrical N- and C-terminal domains are separated by a flexible hinge region. Calcium binding causes a conformational change in calmodulin, leading to the exposure of hydrophobic methyl groups. This hydrophobic surface can in turn bind to hydrophobic regions of basic amphiphilic helices on the target protein. A comparison of various calmodulin target structures (Hoeflich & Ikura, 2002) revealed different protein-activation mechanisms by calmodulin: relieving autoinhibition (Crivici & Ikura, 1995; Meador *et al.*, 1992, 1993), active-site remodelling (Drum *et al.*, 2002) or dimerization (Schumacher *et al.*, 2001). Thus, calmodulin seems to adopt its conformation according to its binding partners (Hoeflich & Ikura, 2002).

In order to shed light on the structural basis of the activation of plasma-membrane Ca^{2+}-ATPases by calmodulin binding, we have initiated structure determination of the N-terminal regulatory domain of ACA8 (residues 40–95) in complex with calmodulin. Here, we describe the expression, purification, crystallization and preliminary X-ray analysis of this complex from *Arabidopsis thaliana*.

2. Materials and methods

2.1. Cloning, protein expression and purification

The DNA encoding *A. thaliana* calmodulin (CaM7) was cloned into a pET42a-derived vector with an N-terminal fusion consisting of a His_6 tag and a TEV protease cleavage site. The DNA encoding residues 40–95 (the region including the calmodulin-binding site; Baekgaard *et al.*, 2006) of the plasma-membrane Ca^{2+}-ATPase ACA8 from *A. thaliana* (Fig. 1) was cloned into a pRSET-derived vector with an N-terminal fusion consisting of a His_6 tag, a lipoyl domain and a TEV protease cleavage site. The protein complex was co-expressed in *Escherichia coli* C41 cells (Miroux & Walker, 1996) for 12 h at 293 K using simultaneous selection by ampicillin and kanamycin and was purified using standard His-tag purification protocols followed by TEV protease digestion, a second Ni-affinity chromatography step to separate the HisLipoTEV tag and a final gel-filtration step. The protein was kept in storage buffer (25 m*M* Tris pH 7.0, 50 m*M* NaCl, 10 m*M* β-mercaptoethanol, 5 m*M* $CaCl_2$) and flash-frozen in liquid nitrogen. Protein homogeneity and purity were assessed using mass spectrometry and SDS–PAGE.

2.2. Crystallization

Initial crystallization conditions were screened for using commercially available matrices from Hampton Research, Jena Biosciences, Qiagen, Molecular Dimensions and Emerald BioSystems. More than 1800 conditions were tested using the vapour-diffusion technique in sitting drops prepared using an Innovadyne Screenmaker robot (Innovadyne Inc., Santa Rosa, USA). The protein complex at a concentration of 16 mg ml^{-1} was mixed with an equal volume of reservoir solution at 290 K. Small protein crystals were only obtained in one condition consisting of 2.0 *M* ammonium sulfate, 0.1 *M* CAPS pH 10.5, 0.2 *M* lithium sulfate (final pH 8.2). Larger crystals were reproduced and optimized by mixing 1.5 µl CaM7–ACA8(40–95) solution with 1 µl reservoir solution and equilibrating against 600 µl reservoir solution at 293 K. Crystal growth took more than two months.

3. Results and discussion

3.1. Purification and crystallization of the complex between the regulatory domain of the plasma-membrane Ca^{2+}-ATPase and calmodulin

The N-terminal region of ACA8, the plasma-membrane Ca^{2+}-ATPase from *A. thaliana*, contains its regulatory domain, which is the target of calmodulin binding leading to subsequent activation of the pump. Secondary-structure prediction indicated the presence of a folded domain comprising residues 40–95 (Fig. 1), while the N-terminus is predicted to be unstructured. This folded region contains the calmodulin-binding site (Baekgaard *et al.*, 2006). Attempts to express this region of ACA8 alone in *E. coli* did not yield soluble protein. However, co-expression of ACA8(40–95) with CaM7 (calmodulin from *A. thaliana*) allowed purification of the complex in high yields (>10 mg pure protein per litre of *E. coli* culture). After cleavage by TEV protease and subsequent removal of the fusion partners, the CaM7–ACA8(40–95) complex could be isolated from excess unbound calmodulin by gel filtration. The retention time, as well as small-angle X-ray scattering analysis (SAXS), indicated the presence of a 1:1 complex (data not shown). The protein complex was stable; it could be concentrated to up to 80 mg ml^{-1} and was flash-frozen until further use.

Large single crystals with dimensions of 0.6 × 0.3 × 0.15 mm (Fig. 2) were obtained after several months using 2.0 *M* ammonium sulfate, 0.1 *M* CAPS pH 10.5, 0.2 *M* lithium sulfate (final pH 8.2) as reservoir solution. SDS–PAGE analysis of the mother liquor after six months and of a dissolved crystal confirmed the presence of the

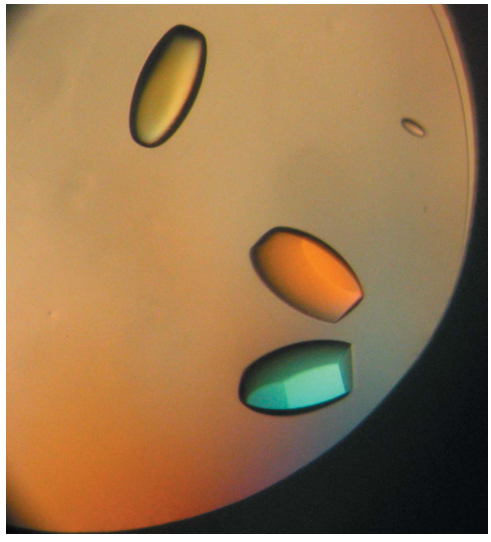

Figure 2
Crystals of the calmodulin–ACA8(40–95) complex from *A. thaliana*. Crystals were obtained by the sitting-drop vapour-diffusion method and grew to dimensions of 0.6 × 0.3 × 0.15 mm.

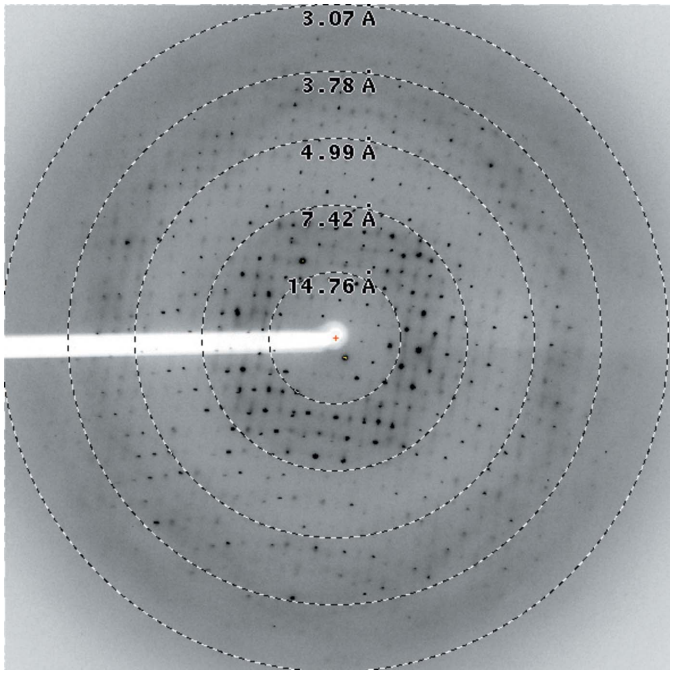

Figure 3
X-ray diffraction pattern of the CaM7–ACA8(40–95) complex. Dashed circles indicate diffraction resolution limits. See Table 1 for details.

CaM7–ACA8(40–95) complex and excluded major degradation as a reason for the unusual slow crystal growth (data not shown).

3.2. Data collection and X-ray diffraction analysis

Diffraction data were collected to a resolution limit of 3.0 Å (Fig. 3). A full data set consisting of 400 oscillation images with an interval of 0.5° and an exposure time of 3 s was collected at a wavelength of 1.0064 Å using a crystal-to-detector distance of 330 mm on the PX3 beamline of the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland). Reflections were indexed and scaled with *XDS* (Kabsch, 1993). The crystals belonged to the monoclinic space group *C*2, with unit-cell parameters a = 176.8, b = 70.0, c = 69.8 Å, β = 113.2°. A summary of the data statistics is given in Table 1. Structure determination is in progress and will be reported elsewhere.

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Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell (3.1–3.0 Å).

Beamline	PX3, Swiss Light Source
Space group	*C*2
Wavelength (Å)	1.0064
Resolution range (Å)	30–3.0
Unit-cell parameters (Å, °)	a = 176.8, b = 70.0, c = 69.8, β = 113.2
Total No. of reflections	46964
No. of unique reflections	15350
Completeness (%)	97.8
$R_{\rm meas}$†‡ (%)	7.7 (48.7)
$R_{\rm mrgd\text{-}F}$†§ (%)	9.5 (47.4)
$\langle I/\sigma(I)\rangle$	10.2 (2.6)
Molecules per ASU (Z)	3¶
Matthews coefficient (Å^3 Da^{-1})	2.79¶
Solvent content (%)	56.0¶

† $R_{\rm meas}$ and $R_{\rm mrgd\text{-}F}$ are quality measures of the individual intensity observations and the reduced structure-factor amplitudes, respectively (Diederichs & Karplus, 1997). ‡ $R_{\rm meas}$ is defined as $\sum_h [n_h/(n_h-1)]^{1/2} \sum_i^{n_h} |\langle I_h\rangle - I_{h,i}| / \sum_h \sum_i^{n_h} I_{h,i}$. § $R_{\rm mrgd\text{-}F}$ is defined as $\sum |A_{Ih,P} = A_{Ih,Q}| / 0.5 \sum (A_{Ih,P} + A_{Ih,Q})$ (Diederichs & Karplus, 1997). ¶ Following the most probable solution according to statistical sampling (Kantardjieff & Rupp, 2003).

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