Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

SOS3 (salt overly sensitive 3) from *Arabidopsis thaliana*: expression, purification, crystallization and preliminary X-ray analysis

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Received 12 March 2004
Accepted 13 April 2004

The salt-tolerance gene *SOS3* (salt overly sensitive 3) of *Arabidopsis thaliana* encodes a calcium-binding protein that is able to sense the cytosolic calcium signal elicited by salt stress. SOS3 activates the SOS2 protein kinase, which activates various ion transporters. SOS3 was cloned into a plasmid and expressed in *Escherichia coli*, allowing purification of the protein to homogeneity. Two crystals with different additive contents were grown. Both diffract to 3.2 Å resolution and belong to space group $I4_1$, with unit-cell parameters a = 93.65, c = 80.08 Å and a = 91.79, c = 85.78 Å, respectively. A promising molecular-replacement solution has been found using neuronal calcium-sensor 1 as the search model. Interestingly, no solution was found using AtCBL2 (*A. thaliana* calcineurin B-like protein) structure as a search model, although this protein belongs to the same family and displays 50% sequence identity.

1. Introduction

The role of calcium as a second messenger controlling many cellular processes has been reviewed (Klee & Means, 2002; Lewit-Bentley & Rety, 2000; Skelton *et al.*, 1994). In plants, many extracellular stimuli, including hormones and environmental signals such as gravity, light, salinity, drought, cold, oxidative stress, anoxia and mechanical perturbation, cause changes in the cytosolic free Ca^{2+} concentration (Poovaiah & Reddy, 1987; Trewavas & Malho, 1998; Bush, 1993).

Cytosolic calcium changes can vary in magnitude, duration, frequency and spatial arrangement within the cell, depending on the nature of the stimulus.

Despite this variety of stimuli, the most common architecture for calcium binding is the EF-hand motif (Lewit-Bentley & Rety, 2000). This motif is highly conserved and involves two helices and a loop of 12 residues that participate in calcium coordination. However, the structures of EF-hand proteins in the calcium-bound or free states or in complex with target peptides or proteins show a variety of conformations, each unique to the particular activation mechanism in which calcium is involved (Klee & Means, 2002).

In *Arabidopsis thaliana*, SOS3 is a myristoylated calcium-binding protein that is capable of sensing the cytosolic calcium signal elicited by salt stress (Liu & Zhu, 1998; Ishitani *et al.*, 2000). SOS3 is able to complex with and to activate the serine/threonine protein kinase SOS2, which then activates various ion transporters, such as the Na^+/H^+ antiporter SOS1.

SOS3 is predicted to share the same fold as the homologous AtCBL2 protein, which consists of two domains of pairs of adjacent EF-hand motifs. However, AtCBL2 interacts with a different target, has no *N*-myristoylation motif and its expression profile suggests a role in light-signal transduction rather than in salt stress (Ishitani *et al.*, 2000; Nagae *et al.*, 2003). In this work, we describe the expression, purification, crystallization and preliminary diffraction data of SOS3. Unexpectedly, it is shown that molecular replacement using the structure of AtCBL2 as a search model fails, indicating significant structural differences between SOS3 and AtCBL2. Comparison of their molecular structures would be of biological significance for the understanding of the different methods of sensing calcium by these proteins.

2. Experimental

2.1. Protein expression and purification

The *SOS3* gene from *A. thaliana* was cloned into a pET14b (Novagen, Madison, WI, USA) expression plasmid to yield pET-His-*SOS3*. The plasmid was transformed into *Escherichia coli* strain BL21(DE3) for protein expression (Ishitani *et al.*, 2000).

5 ml aliquots of an overnight culture were subcultured into 500 ml fresh LB (Luria–Bertani) medium (10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl per litre of solution) plus ampicillin (50 µg ml^{-1}) and allowed to grow to A_{600} = 0.7 at 310 K. Protein expression was then induced for 3 h with 0.3 m*M* isopropyl-β-D-thiogalactoside and cells were harvested by centrifugation (10 min, 9000 rev min^{-1}).

The cell pellet was then resuspended in buffer 1 [20 m*M* sodium phosphate pH 7.4, 0.5 *M* NaCl, 5 m*M* imidazole, 0.05%(*w*/*v*) NaN_3] and cells were disrupted by sonication. After centrifugation (30 min, 17 000 rev min^{-1}) at 277 K, the clear supernatant was filtered (pore diameter 0.45 µm; Millipore Corporation, Bedford, MA, USA) and applied onto a nickel-Sepharose column (5 ml; Amersham Biosciences Limited, UK) equilibrated with buffer 1. A washing step with five column volumes of buffer 1 followed by five volumes of buffer containing 20 m*M* sodium phosphate pH 7.4, 0.5 *M* NaCl, 30 m*M* imidazole, 0.05%(*w*/*v*) NaN_3 was performed.

The column was re-equilibrated with buffer 1 and thrombin protease (Amersham Biosciences Limited, UK) was loaded onto the column for an overnight thrombin protease digestion at 293 K (7.5 thrombin units per milligram of SOS3). Cleaved SOS3 was eluted with buffer 1. The time courses of the chromatography and enzymatic digestion were monitored by SDS–PAGE (see Fig. 1). The sample buffer was changed to 20 m*M* Tris–HCl pH 7.5, 0.05 *M* NaCl, 0.05%(*w*/*v*) NaN_3 (buffer 2) by running through a PD-10 column (Amersham Biosciences Limited, UK). A final polishing step was performed using an anion-exchange Resource Q column (6 ml; Amersham Biosciences Limited, UK) with a salt gradient from 0.05 to 0.5 *M* NaCl. The protein eluted at 0.1 *M* NaCl (see Fig. 2).

The protein buffer was changed to buffer 2 using a PD-10 column (Amersham Biosciences Limited, UK). SOS3 was concentrated to a final concentration of 20 mg ml^{-1} with a 10 kDa cutoff Amicon protein concentrator (YM-10; Millipore Corporation, Bedford, MA, USA). The final protein concentration was determined spectrophotometrically using a molar absorption coefficient of 10 930 M^{-1} cm^{-1} at 280 nm. The sample was aliquoted and immediately frozen at 253 K. The final sample purity was examined by SDS–PAGE and mass spectrometry; the measured mass [$(M + H) = 25\,975$] was coincident with the expected value (25 974.7).

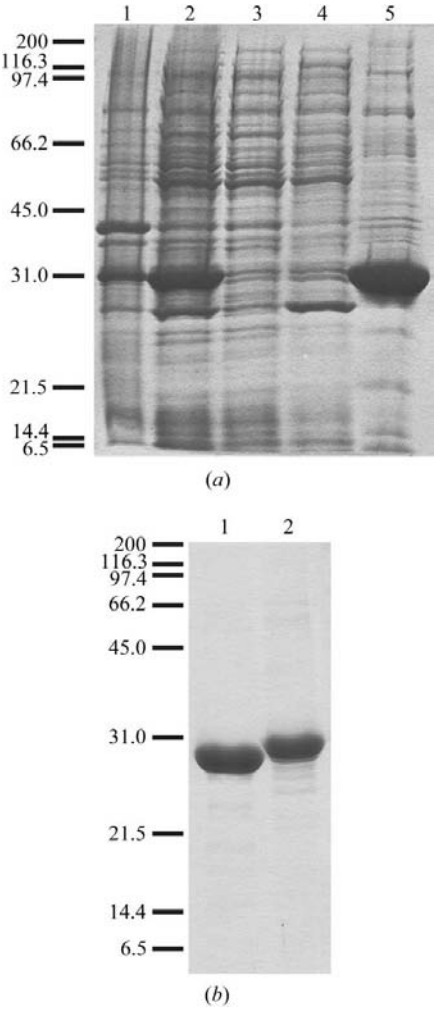

Figure 1
(*a*) SOS3 expression and purification by nickel-affinity chromatography. Proteins were analyzed by 12% SDS–PAGE gels. Lanes 1 and 2, precipitated and soluble fractions after cell disruption. Lane 3, flowthrough from the affinity column. Lane 4, protein fractions eluted after washing with 30 m*M* imidazole buffer. Lane 5, protein fractions eluted with 500 m*M* imidazole. (*b*) On-column cleavage of the tagged protein. 15% SDS–PAGE gel analysis. Lane 1, eluted cleaved protein after digestion with thrombin protease. Lane 2, the remaining uncleaved protein eluted with 500 m*M* imidazole. Samples were loaded under reducing conditions. The position of the molecular-weight markers (MW in kDa; BioRad Laboratories Inc., USA) is indicated. Gels were stained using Coomassie Brilliant Blue.

2.2. Crystallization and preliminary data collection

Preliminary crystallization conditions were established using the sparse-matrix sampling technique (Jancarik & Kim, 1991) with the hanging-drop vapour-diffusion method at 293 K using Crystal Screens I, II, Lite and Index (Hampton Research, CA, USA). Drops containing equal volumes (1 µl) of protein (10 mg ml^{-1}) and reservoir solution were equilibrated against 500 µl reservoir solution. Condition No. 40 [(0.1 *M* trisodium citrate dihydrate pH 5.6, 10%(*v*/*v*) 2-propanol] from Crystal Screen Lite (Hampton Research, CA, USA) produced needle-like microcrystals.

Subsequent optimization yielded thin clustered plate-like and needle-like crystals that were not usable for diffraction experiments (see Fig. 3*a*). Drops containing 2 µl protein (10 mg ml^{-1}, 3.85 m*M* $MnCl_2$) and 1 µl reservoir solution were equilibrated against 500 µl reservoir solution [20%(*w*/*v*)

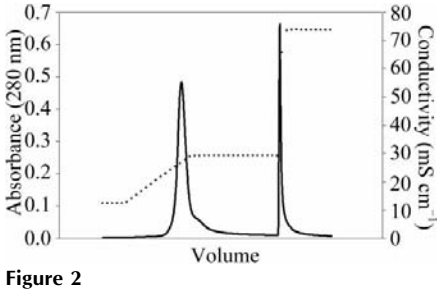

Figure 2
Anion-exchange chromatography of SOS3. The dotted line represents the conductivity of the solution. The black line represents the absorbance recorded at 280 nm.

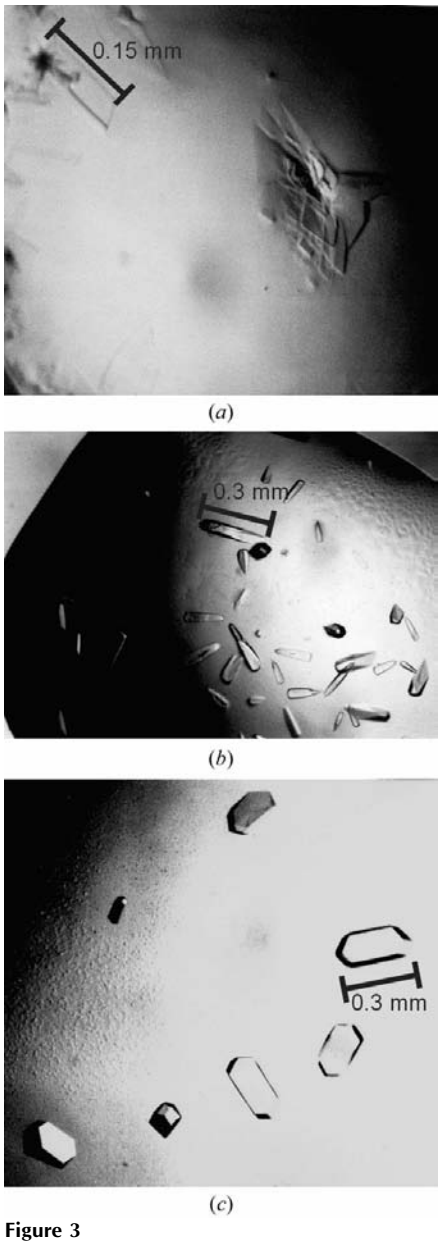

Figure 3
Effect of additives on SOS3 crystals. (*a*) Crystals grown without additives. (*b*) Crystals grown in the presence of sucrose and ethanol. (*c*) Crystals grown with $MnCl_2$ and NaI.

Table 1
Data-collection and processing statistics of tetragonal crystals of SOS3.

Values in parentheses are for the highest resolution shell (3.20–3.31 Å).

	SOS3 (additives: sucrose and EtOH)	SOS3 (additives: $MnCl_2$ and NaI)
Space group	$I4_1$	$I4_1$
Unit-cell parameters (Å)	$a = b = 93.65$, $c = 80.08$	$a = b = 91.79$, $c = 85.78$
Resolution limit (Å)	3.2	3.2
Observations	18957	19633
Unique reflections	5736	5888
Completeness (%)	98.9 (99.5)	99.2 (100)
Multiplicity	3.3 (3.3)	3.3 (3.3)
$R_{\rm merge}$† (%)	0.09 (0.42)	0.09 (0.43)
$I/\sigma(I)$	15.3 (5.2)	14.6 (2.8)

† $R_{\rm merge} = \sum_{hkl} |I_{hkl} - \langle I_{hkl}\rangle| / \sum_{hkl} I_{hkl}$.

PEG 4000, 30%(*v*/*v*) MPD, 0.1 *M* sodium citrate buffer pH 4.9]. Different additives were tested around these conditions.

A new rod-shaped crystal form (see Fig. 3*b*) was grown from drops containing 10.7 mg ml^{-1} SOS3, reservoir solution [20%(*w*/*v*) PEG 4000, 30%(*v*/*v*) MPD, sodium citrate pH 5.2] and 30%(*w*/*v*) D-(+)-sucrose in the ratio 2:0.75:1. The quality of these crystals was improved by adding 0.75 µl 30%(*v*/*v*) ethanol to these conditions. Preliminary diffraction data were collected on an in-house Kappa 2000 CCD detector with Cu $K\alpha$ X-rays generated by a Nonius FR-591 rotating-anode generator with Montel mirrors operated at 45 kV and 100 mA. It was not necessary to use a cryoprotectant solution before flash-freezing the crystal to 120 K as the crystals were grown under cryoprotectant conditions. The crystal-to-detector distance was set to 70 mm with $\Delta\varphi = 0.5^\circ$ and 10 min exposure per image. The maximum resolution reached was 3.2 Å.

The quality of the crystals was improved by treating the protein sample with a chelating resin (Hampton Research, CA, USA) that preferentially complexes trace divalent metal ions (Dunn *et al.*, 1980) prior to crystallization experiments and by using NaI as additive. These crystals (see Fig. 3*c*) grew from drops containing SOS3 (10 mg ml^{-1}, 3.85 m*M* $MnCl_2$), 1 *M* NaI and reservoir solution [20%(*w*/*v*) PEG 4000, 26%(*v*/*v*) MPD, sodium citrate buffer pH 4.8] in the ratio 2:0.75:1. A complete 3.2 Å data set was collected using an in-house X-ray source at 120 K. The crystal-to-detector distance was set to 70 mm with $\Delta\varphi = 0.5^\circ$ and 10 min exposure per image. All data sets were processed with the *HKL*2000 suite of programs (Otwinowski & Minor, 1997).

3. Results and discussion

Recombinant SOS3 protein was purified to homogeneity using a two-step procedure. The nickel-affinity column facilitated the purification by providing relatively high purity in one step. After enzymatic cleavage of the His tag, the purity of the protein was further improved by anion-exchange chromatography. The sample purity at each purification step was monitored by SDS–PAGE (see Fig. 1). The purity of the protein sample used for crystallization was examined by mass spectrometry and the measured mass was the same as the expected value.

A 3.2 Å resolution data set for SOS3 crystals grown in the presence of sucrose and ethanol as additives was collected. The space group was tetragonal $I4_1$, with unit-cell parameters $a = b = 93.65$, $c = 80.08$ Å. Specific volume calculations (Matthews, 1968; Kantardjieff & Rupp, 2003) yielded eight molecules of SOS3 in the unit cell, with a solvent content of 63.6% ($V_{\rm M}$ = 3.38 Å^3 Da^{-1}), corresponding to one monomer per asymmetric unit. Data-collection and processing statistics are summarized in Table 1.

Crystals grown in the presence of NaI and $MnCl_2$ diffracted to 3.2 Å. The space group was also tetragonal $I4_1$, with unit-cell parameters $a = b = 91.79$, $c = 85.78$ Å. The asymmetric unit contains one SOS3 molecule and has 64.6% solvent content ($V_{\rm M}$ = 3.48 Å^3 Da^{-1}; Matthews, 1968; Kantardjieff & Rupp, 2003). The statistics for this data set are shown in Table 1.

Molecular replacement with the program *AMoRe* (Navaza, 1994), using coordinates from the neuronal calcium sensor 1 (NCS1; PDB code 1g8i; Bourne *et al.*, 2001) as the search model (sequence identity of 26.6%), has yielded a promising but not conclusive solution that allows unambiguous determination of the space group (correlation coefficient = 32.1%, *R* factor = 50.3%, resolution range 15–3.5 Å). However, the electron-density map calculated with this model was extremely noisy and was clearly biased by the NCS1 structure. Interestingly, no molecular-replacement solution has been found using the AtCBL2 structure as a search model, although AtCBL2 belongs to the SOS3 family and has a high percentage of sequence identity (50.4%) with SOS3. This suggests large structural dissimilarities between them that are probably motivated by different calcium substitution, as their sequence differences are located at the calcium-binding EF-hand motifs (Nagae *et al.*, 2003). Synchrotron diffraction measurements at longer wavelength (from 1.5 to 1.7 Å) are being prepared to phase the structure using the anomalous signal from the I atoms. Further heavy-atom derivative and selenomethionine-substituted protein preparations are also projected.

MJS-B was supported by a FPU studentship from Ministerio de Educación, Cultura y Deporte. This work was funded by grant BMC2002-04011-C05-03 from the Spanish 'Plan Nacional' (MCYT).

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