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Purification, crystallization and preliminary X-ray diffraction analysis of the plant Rho protein ROP5

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The small G protein ROP5 from the model plant *Arabidopsis thaliana* was purified and crystallized using the hanging-drop vapour-diffusion method. ROP5 crystals were obtained using PEG 3000 as precipitant and belong to space group $P2_1$. A data set was collected to 1.53 Å resolution using synchrotron radiation at 100 K. A clear molecular-replacement solution was found using ROP4–GDP of the ROP4–GDP–PRONE8 complex as the search model.

1. Introduction

Members of the Ras superfamily of small G proteins are essential molecular switches in eukaryotes that regulate a diverse set of cellular events (Takai *et al.*, 2001). They bind guanine nucleotides with high affinity and toggle between an inactive GDP-bound ('OFF') and an active GTP-bound ('ON') state (Vetter & Wittinghofer, 2001). The activation status is tightly controlled by regulatory proteins: guanine nucleotide-exchange factors (GEFs) accelerate the intrinsically slow activating exchange of GDP for GTP, whereas GTPase-activating proteins (GAPs) catalyse the inactivating hydrolysis of GTP. The active form of small G proteins is able to instigate cellular processes by interacting with and activating effector proteins (Vetter & Wittinghofer, 2001).

The Ras superfamily comprises more than 150 members and can be divided into different subfamilies (Wennerberg *et al.*, 2005). The most prominent subfamilies are Ras, Rab, Rho, Arf and Ran. Ras is best known for its role in cell proliferation and differentiation, while Rab and Arf proteins control vesicular trafficking, Ran is indispensable for nucleo-cytoplasmic transport and the Rho proteins are key players in actin cytoskeleton dynamics (Takai *et al.*, 2001; Wennerberg *et al.*, 2005).

Plants lack the Ras family and the typical animal Rho-family members. Instead, plants contain the Rho-related ROP (Rho of plants) proteins. ROP proteins are involved in virtually every aspect of plant life: they regulate pollen-tube growth during pollination in flowering plants and root-hair development, they mediate cellular responses to phytohormones and are involved in plant pathogen-defence mechanisms and abiotic stress responses (Berken, 2006). In all plant species analysed to date, ROPs are encoded by a multigene family (Yang, 2002; Winge *et al.*, 2000). There are 11 members in the model plant *Arabidopsis thaliana* (ROP1–11), seven in rice and nine in maize (Christensen *et al.*, 2003).

Like other small G proteins, ROPs are post-translationally modified by prenyl and/or palmitoyl chains that anchor the proteins to the membrane where signal transduction takes place (Glomset & Farnsworth, 1994). The type of lipid moiety attached to the ROPs is determined by the so-called hypervariable region (HVR) at the C-terminus of the proteins (Ivanchenko *et al.*, 2000; Lavy *et al.*, 2002; Schultheiss *et al.*, 2003). Based on their mode of post-translational lipid modification, ROP proteins can be divided into two superordinate groups (I and II; Winge *et al.*, 2000). According to this

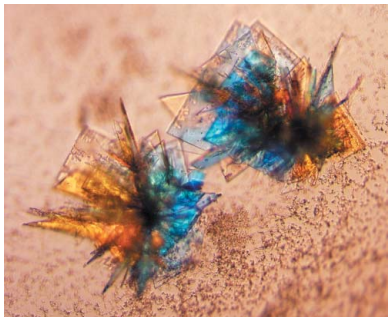

Table 1
Data-collection statistics.

Wavelength (Å)	0.9763
Resolution (Å)	50–1.53 (1.6–1.53)
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 36.4, b = 40.4, c = 57.2, β = 102.6
V_M (Å^3 Da^{-1})	2.1
Total measurements	68968
Unique reflections	23836
Average redundancy	2.9 (1.9)
$I/\sigma(I)$	12.7 (3.1)
Completeness (%)	96.5 (87.1)
Wilson B factor (Å^2)	24
R_{merge}†	5.8 (34.6)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$, where $\overline{I(hkl)}$ is the mean intensity of multiple $I_i(hkl)$ observations of symmetry-related reflections.

classification, ROP1–8 of the 11 ROP proteins of *A. thaliana* belong to group I, whereas group II comprises ROP9–11.

ROP proteins are activated by unique plant-specific guanine nucleotide-exchange factors (Berken *et al.*, 2005). The catalytic domain of these RopGEFs, named PRONE (plant-specific Rop nucleotide exchanger), has been crystallized in complex with the group I ROP protein ROP4 (Thomas *et al.*, 2006) and the three-dimensional structure has been determined (Thomas *et al.*, 2007).

Only one structure of uncomplexed ROP is known, namely that of the group II ROP9 (Sørmo *et al.*, 2006). In order to carry out structural comparisons between group I and II ROP proteins and to analyse the structural changes in ROP4 induced by the guanine nucleotide-exchange factor PRONE that lower nucleotide affinity and accelerate nucleotide dissociation, the type I ROPs ROP3, ROP4 and ROP5, which share 90% amino-acid sequence identity, have been overexpressed and purified and ROP5 has been crystallized in its free form.

2. Experimental

2.1. Overexpression and purification

The DNA constructs of ROP3 (residues 1–180; 19.6 kDa), ROP4 (Thomas *et al.*, 2006) and ROP5 (residues 1–180; 19.6 kDa) lacking the C-terminal prenylation motif were generated by PCR and cloned into the pGEX-4T-1 vector (Amersham Biosciences). Protein expression in *Escherichia coli* BL21-CodonPlus(DE3)-RIL (Stratagene) using Terrific Broth (TB) was induced at an optical density (OD_{600}) of 0.6 with 0.3 m*M* isopropyl β-D-1-thiogalactopyranoside and the culture was further grown overnight at 293 K and 180 rev min^{-1}. Harvested cells were resuspended in buffer *A* (30 m*M* HEPES pH 7.5, 100 m*M* NaCl, 5 m*M* DTE and 5 m*M* $MgCl_2$) containing 0.5%(*v*/*v*) Triton X-100 and 1 m*M* phenylmethylsulfonyl fluoride (PMSF). The suspension was subjected to sonication and the soluble cell extract after centrifugation (60 min at 95 000*g* and 277 K) was applied onto a glutathione Sepharose column equilibrated with buffer *A*. The column was washed with buffer *A* until the baseline was reached again and the glutathione *S*-transferase tag was removed by on-column digestion at 277 K overnight with 500 U thrombin (Serva). The cleaved protein was eluted, subjected to gel filtration using the same buffer as before on a Superdex 75 column (Amersham Biosciences) and finally concentrated by ultrafiltration (Amicon, Millipore). Cleaved ROP3, ROP4 and ROP5 contained additional residues at the N-terminus as a result of the cloning procedure (ROP3, Gly-Ser-Pro-Glu-Phe; ROP4, Gly-Ser; ROP5, Gly-Ser).

2.2. Crystallization

In order to crystallize a group I ROP protein, crystallization experiments with ROP3, ROP4 and ROP5 were carried out in parallel. Prior to crystallization, the protein solutions were supplemented with 5 m*M* $MgCl_2$ and 5 m*M* GDP. Thus, the protein solutions used in crystallization experiments had the following composition: 30 m*M* HEPES pH 7.5, 100 m*M* NaCl, 5 m*M* DTE, 10 m*M* $MgCl_2$ and 5 m*M* GDP. While ROP4 (5, 10 and 15 mg ml^{-1} were tested) only crystallized as small rods (0.2 *M* sodium citrate, 0.1 *M* sodium cacodylate pH 6.5, 30% 2-propanol; from 'The Classics' screen, Nextal Biotechnologies; 293 K) and needles [condition 1, 0.2 *M* $MgCl_2$, 0.1 *M* HEPES pH 7.5, 30% 2-propanol; condition 2, 0.1 *M* HEPES pH 7.5, 70%(*v*/*v*) MPD; both conditions from 'The Classics' screen, Nextal Biotechnologies; 293 K), ROP3 and ROP5 (5, 10 and 15 mg ml^{-1} were tested for both proteins) formed larger crystals under various conditions containing PEG [either 25%(*w*/*v*) PEG 1000, PEG 1500, PEG MME 2000, PEG 3000, PEG 4000, PEG 6000, PEG 8000 or 20%(*w*/*v*) PEG 10 000] as precipitant and with pH values of 4.0–7.5. For initial crystallization screening, three-well round V-bottom plates with 96 reservoir wells (Greiner Bio-One) were used. The largest and best-suited crystals for X-ray diffraction were obtained with ROP5 using 'The PEGs' crystallization screen (Nextal Biotechnologies). The initial condition was optimized to the following using the hanging-drop vapour-diffusion technique in Linbro 24-well tissue-culture plates: 1 µl of a 10 mg ml^{-1} protein solution was mixed with 1 µl reservoir solution [28%(*w*/*v*) PEG 3000, 100 m*M* MES pH 5.5] on a 22 mm circular glass cover slide and the reservoir was sealed with grease. The reservoir volume was 1 ml. ROP5 crystallized overnight at 293 K. ROP5 crystals consisted of intergrown plates (Fig. 1) that were separated from each other for subsequent diffraction experiments. The largest plates reached final dimensions of 0.3 × 0.3 × 0.005 mm. Crystals were collected, cryoprotected in reservoir solution containing 20%(*v*/*v*) glycerol and frozen in liquid nitrogen.

2.3. Data collection

The data set was collected at the Swiss Light Source (Villigen, Switzerland) at 100 K using a MAR 225 CCD detector. The crystal-to-detector distance for data collection was 150 mm, the oscillation width per frame was 1° and 147 frames were collected at 0.97634 Å (beamline X06SA). Data-collection statistics are shown in Table 1.

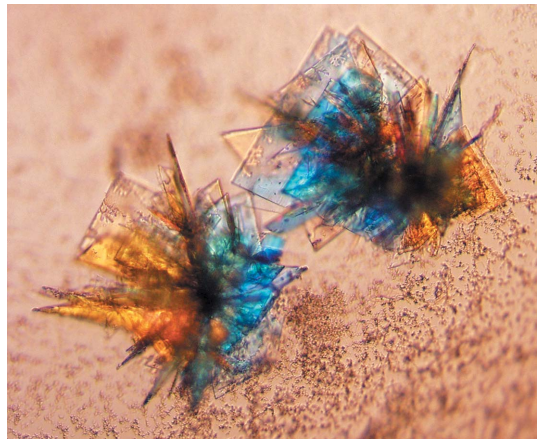

Figure 1
Photograph of ROP5 crystals (approximate dimensions 0.3 × 0.3 × 0.005 mm).

Data were indexed, integrated and scaled with the *XDS* package (Kabsch, 1993). The crystals of ROP5 belong to the monoclinic space group $P2_1$.

3. Results and discussion

Here, we describe the overexpression and purification of the type I Rho-family proteins ROP3, ROP4 and ROP5 from *A. thaliana* and the crystallization of ROP5. The proteins were overexpressed in *E. coli* and purified by affinity chromatography. These procedures yielded 10–20 mg pure protein per litre of expression culture, leading to protein crystals of ROP5 that were useful for X-ray data collection (statistics given in Table 1). ROP5 crystals diffracted to 1.53 Å resolution. The asymmetric unit of the ROP5 crystals contained one ROP5 molecule, corresponding to a Matthews coefficient V_M of 2.1 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 41%. Molecular replacement was performed using the coordinates of ROP4–GDP from the structure of ROP4–GDP–PRONE8 (Thomas *et al.*, 2007; PDB code 2nty) as a search model, with the P loop and the switch regions deleted. An unambiguous solution with a correlation coefficient of 55.3% and an *R* factor of 52.2% was found using the program *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 software package (Collaborative Computational Project, Number 4, 1994; the correlation coefficient and *R* factor for the second best solution were 38.2% and 60.1%, respectively). The output PDB file of *MOLREP* was refined using the program *REFMAC* (Murshudov *et al.*, 1997). During ten cycles of refinement, the free *R* factor dropped from 47.1% to 38.7% and the figure-of-merit increased from 40.3% to 67.1%, indicating that the molecular-replacement solution was correct.

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