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Crystallization and preliminary X-ray analysis of tubulin-folding cofactor A from *Arabidopsis thaliana*

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Tubulin-folding cofactor A (TFC A) is a molecular post-chaperonin that is involved in the β-tubulin-folding pathway. It has been identified in many organisms including yeasts, humans and plants. In this work, *Arabidopsis thaliana* TFC A was expressed in *Escherichia coli* and purified to homogeneity. After thrombin cleavage, a well diffracting crystal was obtained by the sitting-drop vapour-diffusion method at 289 K. The crystal diffracted to 1.6 Å resolution using synchrotron radiation and belonged to space group $I4_1$, with unit-cell parameters a = 55.0, b = 55.0, c = 67.4 Å.

1. Introduction

Microtubules, which are components of the cytoskeleton, are important in morphogenesis, cell division, intracellular transport and the establishment of cell polarity (Joshi, 1998; Mathur & Chua, 2000). The biogenesis of these microtubules plays an important role in the regulation of these functions. The microtubule polymers are made up of $\alpha\beta$-tubulin heterodimers. The formation of polymerization-competent $\alpha\beta$-tubulin heterodimers is a complex process that requires several molecular chaperones and tubulin-folding cofactors (TFCs; Lopez-Fanarraga *et al.*, 2001). The prefoldin (Vainberg *et al.*, 1998) first associates with the nascent α- and β-tubulin and transfers them to CCT (cytosolic containing TCP-1; Lewis *et al.*, 1996). TFC A then binds to β-tubulin and TFC B binds to α-tubulin. Subsequently, TFC D replaces TFC A to interact with β-tubulin, while TFC E replaces TFC B to interact with α-tubulin. Once the TFC D–β-tubulin–TFC E–α-tubulin complex has been formed, the tubulin heterodimers can be released in the presence of TFC C and GTP.

TFC A has been identified in many organisms, including yeasts, mammals and plants (Archer *et al.*, 1995; Kirik *et al.*, 2002; Campo *et al.*, 1994). Mammalian TFC A (CoA) was first identified as a molecular chaperone for β-tubulin (Gao *et al.*, 1994). *In vitro* experiments have shown that CoA binds noncovalently to β-tubulin and serves as a reservoir for excess β-tubulin (Llosa *et al.*, 1996; Fanarraga *et al.*, 1999). The budding yeast orthologue Rbl2p has been identified as a protein that is capable of rescuing cells from lethal β-tubulin overexpression (Archer *et al.*, 1995). The *Arabidopsis thaliana* TFC A orthologue encoded by *KIESEL* (*KIS*) was identified from morphogenesis-based mutation screens. $kis^{(A)}$ mutant plants exhibit cell-morphogenesis defects and abnormal cell-division phenotypes. *KIS* is also involved in maintaining the balance between α-tubulin and β-tubulin monomers (Steinborn *et al.*, 2002).

The structures of Rbl2p and human CoA have been reported (Steinbacher, 1999; Guasch *et al.*, 2002). Rbl2p forms a homodimer and is predicted to interact with β-tubulin *via* the loop regions of the homodimer (You *et al.*, 2004). However, human CoA crystallizes as a monomer and binds to β-tubulin *via* the helical region (Guasch *et al.*, 2002). The structure of TFC A in plants has not been reported and it is not clear how it interacts with β-tubulin. Here, we describe the preparation, crystallization and preliminary X-ray analysis of *A. thaliana* TFC A. The structure of this protein will further our understanding of the function of the *TFC A* gene in plants.

2. Materials and methods

2.1. Gene cloning and expression

The *KIS* gene (AT2G30410) was amplified by stepdown PCR using *A. thaliana* cDNA as the template. The forward (5′-GGTACTGCATATGGCAACGATAAGGAACT-3′) and reverse (5′-GTCGTCGACTCAGGCATCTTCAGTGGG-3′) primers used for PCR were designed to introduce an *Nde*I site at the N-terminus and a *Sal*I site at the C-terminus. The PCR product was digested and ligated into the *Nde*I and *Sal*I sites of pET-28a(+) (Novagen) vector with an N-terminal His_6-fusion tag. The construct was verified by DNA sequencing. The recombinant plasmid was transformed into *Escherichia coli* Rosetta (DE3) cells for protein expression.

Transformed cells were cultured at 310 K in LB medium containing 50 µg ml^{-1} kanamycin and 34 µg ml^{-1} chloramphenicol. When the cells reached an OD_{600} of 0.6, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M* and culturing for another 4 h.

2.2. Protein purification

The cells were harvested by centrifugation at 4000*g* for 15 min at 277 K and resuspended in buffer *A* (20 m*M* Tris–HCl pH 7.5, 500 m*M* NaCl). After sonication, the cellular debris was removed by centrifugation at 18 000*g* for 30 min at 277 K. The supernatant was loaded onto a 5 ml Ni^{2+}-affinity column (HiTrap Chelating HP, GE Healthcare) pre-equilibrated with buffer *A*. The column was washed with 40 ml buffer *A* containing 50 m*M* imidazole before the target protein was eluted with a linear gradient of 50–500 m*M* imidazole. The eluted His-tagged protein was pooled and loaded onto a 120 ml HiLoad Superdex 75 gel-filtration column (GE Healthcare, USA) equilibrated with buffer *B* (20 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl) for further purification. The fractions were concentrated to 15 mg ml^{-1} using an Amicon Ultra 5K concentrator (Millipore, USA). Subsequently, the first 17 amino acids of the N-terminus of the His-tagged recombinant TFC A protein were cleaved with thrombin by incubating 5 mg protein with 10 units of protease for 4 h at room temperature. Gel-filtration chromatography in buffer *B* was again applied to the recombinant TFC A protein without His tag and the fractions were concentrated to 10 mg ml^{-1} for crystallization trials

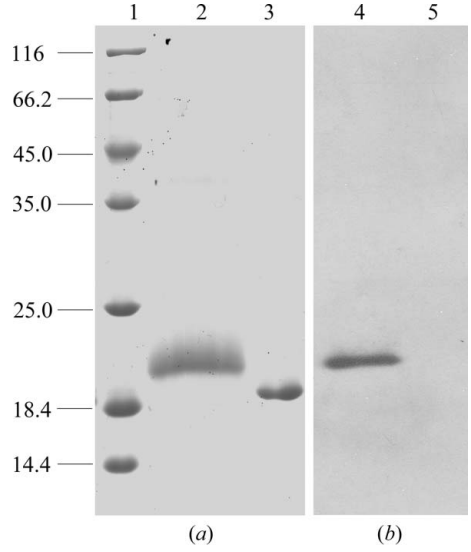

Figure 1
SDS–PAGE and Western blotting analyses of the proteins. (*a*) SDS–PAGE gel stained with Coomassie Brilliant Blue. Lane 1, molecular-weight markers (kDa); lane 2, His-tagged TFC A protein eluted from the Superdex 75 column; lane 3, dissolved TFC A crystals from protein that had been stored for three weeks at 277 K. (*b*) Western blotting analysis. The samples in lanes 4 and 5 correspond to those in lanes 2 and 3, respectively, but were diluted 100-fold.

2.3. Crystallization

The recombinant TFC A protein without His tag was crystallized at 289 K using the sitting-drop vapour-diffusion method with a 48-2 crystallization plate (XtalQuest Inc., Beijing, People's Republic of China). Hampton Research Crystal Screen, Crystal Screen 2 and Index kits (Hampton Research, USA) were used for initial crystallization screening. 1 µl protein solution was mixed with 1 µl reservoir solution and equilibrated against 100 µl reservoir solution.

2.4. X-ray diffraction data collection

X-ray diffraction data were collected using a MAR CCD system on beamline BW7A of the European Molecular Biology Laboratory (EMBL) at the DORIS storage ring, DESY, Hamburg. The crystal was flash-frozen to 100 K in a nitrogen-gas stream during data collection and the diffraction data were processed to 1.6 Å with the *HKL*-2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

The *A. thaliana* TFC A protein was expressed in *E. coli* in a soluble form and was purified by a series of chromatographic steps. The purity of the protein was estimated to be about 95% by SDS–PAGE (data not shown).

We initially used the recombinant His-tagged TFC A protein to perform crystal screening and clustered needle-shaped crystals were observed after two weeks in Crystal Screen condition No. 24 [30%(*w*/*v*) polyethylene glycol (PEG) 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* sodium acetate]. Starting from this result, about 300 crystallization conditions were tested, varying the pH, precipitant and salt concentration, for optimization. However, this did not improve the diffraction of the crystals. It was found that the protein formed crystals with better diffraction quality after storing the protein sample at 277 K for three weeks. The crystals were then checked by SDS–PAGE, which showed that the protein was still pure but that its molecular mass was smaller than that of the fresh protein that purified from the Superdex 75 column (Fig. 1*a*). Western blotting analysis with anti-His monoclonal antibody indicated that the N-terminal His

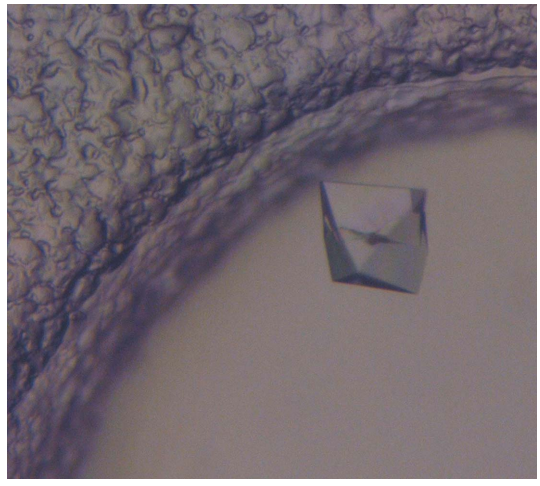

Figure 2
Crystal of TFC A grown by the sitting-drop method. The dimensions of the crystal are approximately 0.2 × 0.2 × 0.1 mm.

tag had been degraded (Fig. 1*b*). Therefore, the His-tagged TFC A protein was digested with thrombin and the N-terminally cleaved sample was used for further crystallization. Well diffracting crystals were obtained using the condition 35%(*w*/*v*) PEG 3350, 0.1 *M* sodium acetate pH 4.6 and 0.4 *M* sodium nitrate after three weeks using the recombinant TFC A protein without His tag (Fig. 2).

The crystal diffracted to 1.6 Å resolution (Fig. 3) and belonged to space group $I4_1$, with unit-cell parameters $a = b = 55.0$, $c = 67.4$ Å, $\alpha = \beta = \gamma = 90^\circ$. The crystal contains one molecule per asymmetric unit, giving a V_M value of 1.94 Å^3 Da^{-1} and corresponding to a solvent content of 36.6% (Matthews, 1968). The data-collection statistics are listed in Table 1.

The first structure from the cofactor A family to be reported was that of yeast Rbl2p (PDB code 1qsd; Steinbacher, 1999). Rbl2p crystallizes as a homodimer and each monomer forms a three-helix bundle. The sequence identity between Rbl2p and human CoA is 32%, but subsequent structure determination of human CoA failed when using Rbl2p as a search model for molecular replacement (Guasch *et al.*, 2002). The final structure of human CoA (PDB code 1h7c; Guasch *et al.*, 2002) solved by the multiwavelength anomalous diffraction method demonstrates that there are structural differences between these two proteins. The C-terminus of helix 1 and the middle part of helix 2 of the two proteins do not match. Meanwhile, two loops of Rbl2p and human CoA adopt different conformations. Moreover, the oligomeric state is also different: human CoA crystallizes as a monomer.

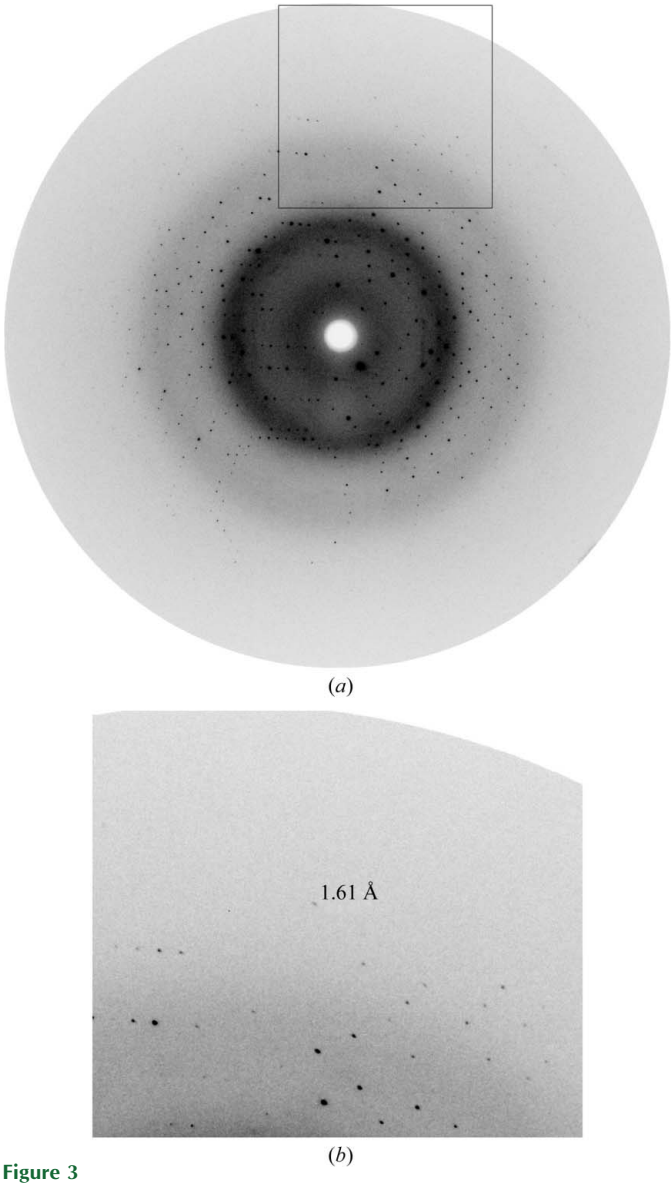

Figure 3
Diffraction pattern of the TFC A crystal. (*a*) A typical diffraction image. (*b*) An enlarged view showing the highest resolution spots.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.8015
Space group	$I4_1$
Unit-cell parameters (Å)	$a = 55.0$, $b = 55.0$, $c = 67.4$
Resolution (Å)	20.0–1.60 (1.66–1.60)
R_{merge}† (%)	8.2 (30.4)
$\langle I/\sigma(I)\rangle$	33.8 (4.2)
Completeness (%)	99.8 (98.5)
No. of observed reflections	95281
No. of unique reflections	13258
Redundancy	7.2 (5.5)
Molecules per asymmetric unit	1
V_M (Å^3 Da^{-1})	1.94

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

A. thaliana TFC A shares about 45% identity (65% similarity) to human CoA and 31% identity (56% similarity) to Rbl2p. Therefore, we attempted to solve the structure of *A. thaliana* TFC A using the molecular-replacement method. Structure determination was successful when the structure of Rbl2p was used as the search model. The coordinates and structure factors have been deposited in the Protein Data Bank (entry 3mxz).

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