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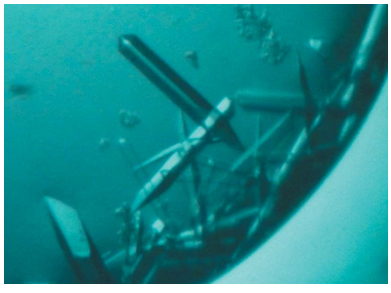

Cloning, purification, crystallization and preliminary X-ray diffraction crystallographic study of acyl-protein thioesterase 1 from *Saccharomyces cerevisiae*

Palmitoylation/depalmitoylation plays an important role in protein modification. yApt1 is the only enzyme in *Saccharomyces cerevisiae* that catalyses depalmitoylation. In the present study, recombinant full-length yApt1 was cloned, expressed, purified and crystallized. The crystals diffracted to 2.40 Å resolution and belonged to space group $P4_22_12$, with unit-cell parameters $a = b = 146.43$, $c = 93.29$ Å. A preliminary model of the three-dimensional structure has been built and further refinement is ongoing.

1. Introduction

Protein modification with fatty acids is a significant feature of many proteins in eukaryotic cells (Linder & Deschenes, 2007; Tsutsumi *et al.*, 2008). The major lipid modifications of eukaryotic proteins are generally divided into two types: *N*-acylation and *S*-acylation (or palmitoylation) (Magee & Courtneidge, 1985). *N*-Acylation functions as a co-translational irreversible process (Johnson *et al.*, 1994), while palmitoylation is the only reversible post-translational process in lipid modification (Smotrys & Linder, 2004; Linder & Deschenes, 2007). Similar to other dynamic reversible modifications such as acetylation, ubiquitination, phosphorylation *etc.*, palmitoylation plays important roles in dynamic subcellular localization, protein activity, stability and complex assembly (Hirano *et al.*, 2009; Yang *et al.*, 2010). Proteins that are involved in signal transduction, such as G_α subunits, which are regulators of G-protein signalling proteins (RGS proteins), Ras proteins and endothelial nitric oxide synthases (eNOSs), are the most common examples of palmitoylation (Linder & Deschenes, 2007; Resh, 2006).

Palmitoylation/depalmitoylation is defined as the addition/removal of palmitate to/from Cys residues through a thioester covalent linkage (Magee & Courtneidge, 1985; Linder & Deschenes, 2003). In eukaryotic cells, palmitoylation can take place by autoacylation or can be catalyzed by protein acyltransferase (PAT; Roth *et al.*, 2002; Valdez-Taubas & Pelham, 2005), while depalmitoylation is catalyzed by acyl-protein thioesterase (APT; Linder & Deschenes, 2007). In *Homo sapiens*, two kinds of APTs have been reported. One is acyl-protein thioesterase (hAPT), which removes palmitate from proteins on the cytosolic surface of membranes (Duncan & Gilman, 1998; Devedjiev *et al.*, 2000), and the other is protein palmitoylthioesterase (hPPT), a lipase that is localized in lysosomes (Verkruyse & Hofmann, 1996; Gupta *et al.*, 2001). In *Saccharomyces cerevisiae* there is only one enzyme, yApt1, which can catalyze depalmitoylation (Duncan & Gilman, 2002).

yApt1 shares 36% sequence identity with hAPT1 and 29% sequence identity with hAPT2. Like mammalian APT proteins, yApt1 exhibits both acyl-thioesterase activity (with palmitoyl proteins, palmitoyl peptides or palmitoyl-CoA as substrates) and low acyl-esterase activity (with lysophosphatidylcholine as a substrate) (Duncan & Gilman, 1998, 2002). However, yApt1 significantly prefers protein substrates (for example palmitoyl-$G_{i\alpha}$) with an at least several hundredfold higher apparent affinity compared with small-molecule substrates (palmitoyl-CoA and lysophosphatidylcholine) *in vitro* (Duncan & Gilman, 2002; Biel *et al.*, 2006), suggesting that palmitoyl proteins are better substrates for yAPT1. Furthermore, in comparison to mammalian APT enzymes, yApt1 displays a marked

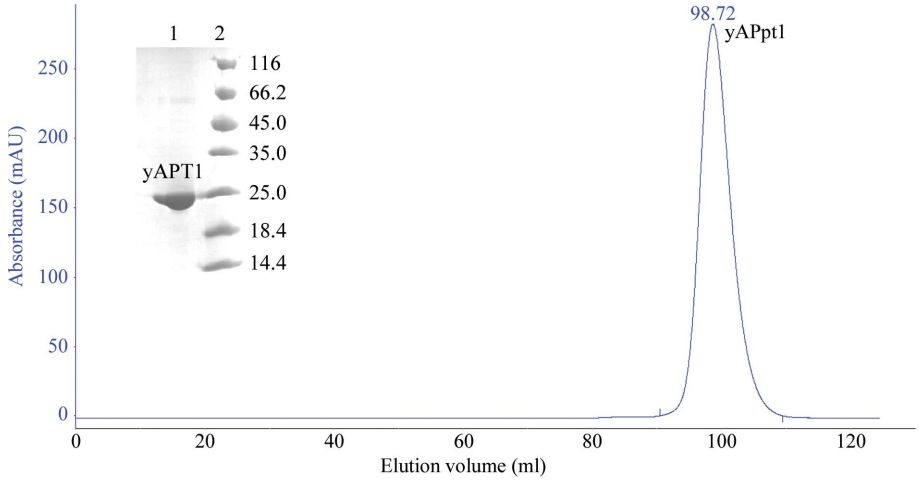

Figure 1
Gel-filtration chromatogram of purified yApt1 fractionated by Superdex 200 (GE Healthcare, USA). yApt1 elutes at a position consistent with a monomer. Inset: SDS–PAGE analysis of yApt1. The protein was analyzed on 12% SDS–PAGE stained with Coomassie Blue. Lane 1, full-length yApt1 after purification (24.8 kDa); lane 2, molecular-weight markers (labelled in kDa).

specificity for different protein substrates: yApt1 cannot depalmitoylate RGS4 or H-Ras, but has variable activity towards mammalian $G_{i\alpha}$, G_{α} subunits and eNOS (Duncan & Gilman, 2002). The structural relevance of this substrate preference remains unclear. In order to elucidate the structural differences and the details of the catalytic mechanism, recombinant full-length yApt1 was cloned, expressed, purified and crystallized and an X-ray diffraction data set was collected to 2.40 Å resolution.

2. Materials and methods

2.1. Cloning, expression and purification

The DNA of full-length yApt1 was obtained by PCR from the *S. cerevisiae* genome. The PCR product was digested and inserted into p22b vector (derived from pET22b; Novagen, USA) with *Nde*I and *Xho*I sites to create a recombinant yApt1 protein with a hexahistidine tag (LEHHHHHH) at the C-terminus (abbreviated as recombinant yApt1). All of the yApt1 constructs were confirmed by DNA sequencing. The plasmid containing recombinant yApt1 DNA was then transformed into *Escherichia coli* ArcticExpress (DE3) RIL (Agilent, USA) competent cells. The cells were cultured in 5 ml Luria–Bertani (LB) broth overnight and inoculated into 1.6 l LB medium containing 50 µg ml^{-1} ampicillin. The cells were grown at 310 K for 2.5 h until the OD_{600} reached 0.6–0.8; protein expression was then induced for 20 h with 0.30 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 289 K. The cells were harvested by centrifugation and resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 2 m*M* Triton X-100, 1 m*M* EDTA) and the suspension was lysed by sonication on ice. The cell lysates were centrifuged and the supernatant was purified on Ni–NTA agarose resin (GE Healthcare, USA) pre-equilibrated with buffer *A*. Further purification was achieved by gel filtration using a HiLoad 16/60 Superdex 200 size-exclusion column (GE Healthcare, USA) pre-equilibrated with buffer *B* (20 m*M* Tris–HCl pH 7.0, 200 m*M* NaCl, 1 m*M* *β*-mercaptoethanol). The retention volume corresponding to the target protein indicated that recombinant yApt1 was monomeric in solution (Fig. 1). Fractions of the peak were pooled and concentrated to 34–46 mg ml^{-1} using a 10 kDa cutoff centrifugal ultrafiltration concentrator (Millipore, USA) and kept in buffer *C* (20 m*M* Tris–HCl pH 7.0, 140 m*M* NaCl, 1 m*M* *β*-mercaptoethanol) at 193 K. Examination of the purified recombinant yApt1 by SDS–PAGE revealed a single band matching the expected molecular weight (24.8 kDa; Fig. 1). The protein concentration was measured using the BCA Protein Assay Kit (Pierce, USA).

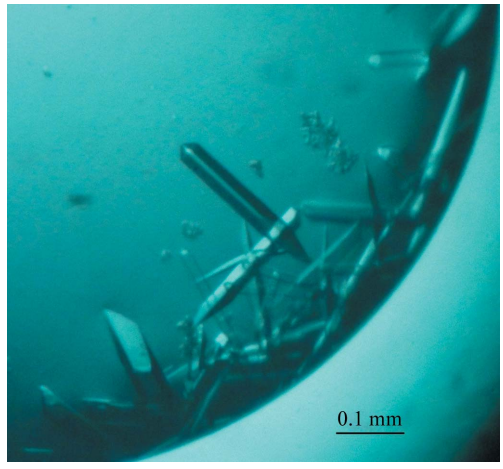

Figure 2
Typical crystals of yApt1 obtained as long three-dimensional rods from a condition consisting of 0.2 *M* ammonium acetate, 0.1 *M* sodium citrate pH 5.6, 28%(*w*/*v*) PEG 4000; these crystals were used in diffraction experiments.

2.2. Crystallization

Because of the high homogeneity of the recombinant yApt1, the final purified protein with a His6 tag was directly used for crystallization. Preliminary screening for initial crystallization conditions was performed with a Mosquito liquid-handling robot (TTP LabTech, UK) using f crystallization kits: Crystal Screen, Index, SaltRX, Grid Screen (Hampton Research, USA) and ProPlex (Molecular Dimensions, UK). Small crystals of yApt1 were observed in tens of conditions after 24 h. One condition, Crystal Screen condition No. 9, consisting of 0.2 *M* ammonium acetate, 0.1 *M* sodium citrate pH 5.6, 30%(*w*/*v*) PEG 4000, was selected for optimization using the hanging-drop vapour-diffusion method. Each hanging drop consisted of 1 µl 15–30 mg ml^{-1} recombinant yApt1 solution and 1 µl reservoir solution and was equilibrated against 100 µl reservoir solution.

Table 1
Data-collection statistics for recombinant full-length yApt1.

Values in parentheses are for the highest resolution shell.

Space group	$P4_22_12$
Wavelength (Å)	0.9793
Unit-cell parameters (Å, °)	$a = b = 146.43$, $c = 93.29$, $\alpha = \beta = \gamma = 90.00$
Resolution (Å)	57.60–2.40 (2.53–2.40)
No. of unique reflections	40145 (5757)
Multiplicity	7.8 (7.8)
Completeness (%)	99.9 (100.0)
Average $I/\sigma(I)$	15.1 (4.4)
$R_{\rm merge}$†	0.091 (0.506)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl.

Crystals with good diffraction quality grew as long three-dimensional rods in about 3 d from the optimized condition 0.2 *M* ammonium acetate, 0.1 *M* sodium citrate pH 5.6, 28%(*w*/*v*) PEG 4000 at 287 K (Fig. 2).

2.3. X-ray diffraction data collection and processing

Before data collection, crystals were quickly soaked in a cryoprotectant solution consisting of the corresponding reservoir solution supplemented with 25%(*v*/*v*) glycerol. X-ray diffraction data were collected on the BL17U1 beamline at Shanghai Synchrotron Radiation Facility (SSRF) using a Jupiter CCD detector at 100 K. The crystal-to-detector distance was maintained at 300 mm and a set of diffraction data consisting of 180 images was collected from a single crystal with an oscillation angle of 1° per image. The maximum resolution of the diffraction data was finally refined to 2.40 Å and the most likely space group was $P4_22_12$, for which the $R_{\rm merge}$ and the average $I/\sigma(I)$ in the outer (high-resolution) shell were both in an acceptable range. All data were integrated using *iMOSFLM* (Battye *et al.*, 2011) and scaled using *SCALA* (Evans, 2006) from the *CCP*4 Suite (Winn *et al.*, 2011). The final statistics of data collection and processing are tabulated in Table 1.

3. Results and discussion

Full-length yApt1 (acyl-protein thioesterase 1 from *S. cerevisiae*) was successfully cloned and expressed in *E. coli* and purified to homogeneity with a C-terminal His_6 tag. When purified by gel filtration, yApt1 showed an apparent molecular size of 25 kDa, suggesting that it is a monomer in solution. A yApt1 crystal was obtained from a reservoir solution consisting of 0.2 *M* ammonium acetate, 0.1 *M* sodium citrate pH 5.6, 28%(*w*/*v*) PEG 4000. X-ray diffraction data were collected to a maximum resolution of 2.40 Å and the crystal belonged to the tetragonal space group $P4_22_12$, with unit-cell parameters $a = b = 146.43$, $c = 93.29$ Å. The overall $R_{\rm merge}$ was 9.1% and that in the outer shell was 50.6%. Reasonable Matthews coefficients (Matthews, 1968) were 2.61 and 2.09 $Å^3\ Da^{-1}$, corresponding to solvent contents of 52.97 and 41.21%, respectively, and suggesting the presence of four or five yApt1 molecules per asymmetric unit. A preliminary structure model of yApt1 was constructed with *Phaser* (McCoy *et al.*, 2007) using the crystal structure of human APT1 (PDB entry 1fj2; Devedjiev *et al.*, 2000; 34.2% amino-acid sequence identity) as the initial model. The result showed that there were four molecules per asymmetric unit in the yApt1 crystal structure. After refinement, the *R* factor decreased to 28.17% and $R_{\rm free}$ was 33.23%. Final model building by restrained refinement using *REFMAC*5 (Murshudov *et al.*, 2011) alternating with manual refinement using *Coot* (Emsley & Cowtan, 2004) is in progress.

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