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Molecular cloning, expression, purification and crystallographic analysis of zebrafish THEM2

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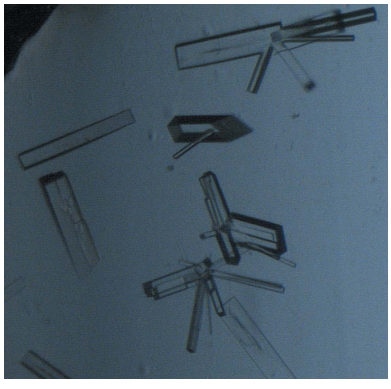

Thioesterase superfamily member 2 (THEM2) is essential for cell proliferation of mammalian cells. It belongs to the hotdog-fold thioesterase superfamily and catalyzes the hydrolysis of the thioester bonds of acyl-CoA *in vitro*. In this study, THEM2 protein from zebrafish (fTHEM2) was expressed in *Escherichia coli* and purified by Ni-affinity and gel-filtration chromatography. fTHEM2 crystals were obtained using the sitting-drop vapour-diffusion method with PEG 10 000 as precipitant. X-ray diffraction data were collected to 1.80 Å resolution using a synchrotron-radiation source. The crystals belonged to the monoclinic space group $C2$, with unit-cell parameters a = 77.1, b = 74.4, c = 96.6 Å, β = 93.7°.

1. Introduction

Thioesterase superfamily member 2 (THEM2), also known as acyl-CoA thioesterase 13 (ACOT13), is considered to be essential for the cell proliferation of mammalian cells (Cheng, Bao *et al.*, 2006). THEM2, which has a single hotdog domain, catalyzes the hydrolysis of the thioester bonds of a variety of CoA esters *in vitro* (Wei *et al.*, 2009). Long-chain fatty-acyl-CoAs, especially palmitoyl-CoA, were found to be the most favourable substrates of THEM2. Moreover, the thioesterase activity of THEM2 requires the formation of its homodimer, since the catalytic centre is composed of highly conserved residues from two monomers. In both solution and in crystals the THEM2 protein forms a stable tetramer in a back-to-back mode (Cheng, Song *et al.*, 2006). In addition, the interaction between THEM2 and phosphatidylcholine-transfer protein (PC-TP) may contribute to the key role of THEM2 in the regulation of hepatic metabolism (Kang *et al.*, 2009, 2010; Kanno *et al.*, 2007). Recent research results also showed both an increased concentration of fatty-acyl-CoA and a decreased concentration of free fatty acid in the liver of THEM2 knock-down mice (Kang *et al.*, 2012).

Although the three-dimensional structure of human THEM2 (hTHEM2) has been determined and the mechanisms of its substrate recognition and catalysis have also been studied (Cao *et al.*, 2009), the biological function of THEM2 is still not entirely clear. In a study on the biological function of THEM2 in cell proliferation using zebrafish as a model organism system, zebrafish THEM2 (fTHEM2) was found to play an important role in the development of zebrafish embryos by interacting with microtubules (unpublished work). Although full-length fTHEM2 (residues 1–141) shares about 70% sequence identity with hTHEM2, the N-terminal region (residues 1–35) of fTHEM2 differs from that of hTHEM2 (with a sequence identity of 34%). In view of this fact, we hope to obtain structural information on fTHEM2 and to lay a solid foundation for the study of its biological function in embryogenesis. Here, we report the expression, purification, crystallization and preliminary X-ray analysis of fTHEM2.

2. Materials and methods

2.1. Plasmid construction

The gene encoding full-length fTHEM2 (residues 1–141; UniProt accession No. Q1LU96) was amplified from a zebrafish (*Danio rerio*) cDNA library, a gift from Dr Shilai Bao, using the primers fTHEM2-F, 5′-GGAATTC*CATATG*GCTTCATTAACGCTGAAT-3′ (*Nde*I site in italics), and fTHEM2-R, 5′-CCG*CTCGAG*AATGAATAC-

CCAGAGACA-3′ (*Xho*I site in italics). The PCR mixture (50 µl) consisted of 0.2 µ*M* forward and reverse primers, 25 µ*M* dNTP, 1 µl cDNA library and 1.0 U KOD-Plus-Neo polymerase (Toyobo). The purified PCR product was double-digested with *Nde*I and *Xho*I (NEB) and then inserted into the expression vector pET-22b(+) (Novagen) using a DNA Ligation Kit (Takara).

2.2. Protein expression and purification

Escherichia coli Rosetta (DE3) cells (Stratagene) were transformed with the expression vector constructed above and recombinant cells were cultured in 1 l Luria–Bertani medium containing 50 µg ml^{-1} ampicillin at 310 K until the OD_{600} reached 0.6. Protein expression was induced by the addition of 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma). Cell growth was continued at 310 K for 4 h after initiating IPTG induction.

The cells were harvested by centrifugation at 6500*g* for 30 min and resuspended in lysis buffer (50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 1 m*M* phenylmethanesulfonyl fluoride) at 277 K. The protein was extracted from the cells by sonication and was separated from insoluble materials by centrifugation at 30 000*g* for 30 min at 277 K. The supernatant containing 6×His-tagged fTHEM2 was loaded onto a 2 ml Ni–NTA column (Qiagen). After washing the resin with lysis buffer, His-tagged fTHEM2 was obtained by elution with increasing concentrations of imidazole (20, 50, 100, 200 and 500 m*M*; Fig. 1*a*). Elution fractions 9 and 10 of the recombinant fTHEM2 protein were collected and concentrated by ultrafiltration (3 kDa cutoff) for gel-filtration chromatography on a Superdex 75 column (GE Healthcare) in low-ionic-strength buffer (50 m*M* Tris–HCl pH 7.5, 50 m*M* NaCl). The fractions containing the protein were analyzed using 15% SDS–PAGE. A Gel Filtration Calibration Kit LMW (GE Healthcare) was employed to produce the standard curve for molecular-mass calculation.

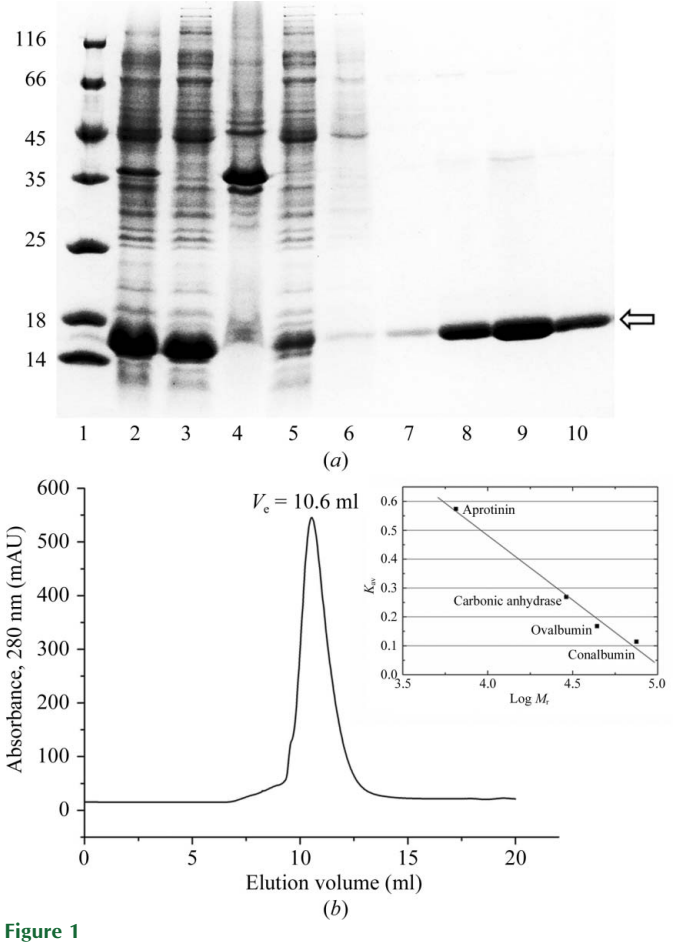

Figure 1
Results of the purification of tetrameric fTHEM2. (*a*) Purification of fTHEM2 by Ni-affinity chromatography. Lane 1, molecular-mass marker proteins (labelled on the left in kDa); lane 2, total cell lysate after IPTG induction; lanes 3 and 4, soluble and insoluble fractions of lysate, respectively; lane 5, flowthrough; lanes 6, 7, 8, 9 and 10, elution fractions with 20, 50, 100, 200 and 500 m*M* imidazole, respectively. The open arrow represents fTHEM2 with a C-terminal 6×His tag. (*b*) Purification and molecular-mass calibration of tetrameric fTHEM2 by gel-filtration chromatography on Superdex 75 10/300. Aprotinin (6.5 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa) and conalbumin (75 kDa) from the Gel Filtration Calibration Kit LMW were used to obtain the standard curve shown on the top right.

2.3. Crystallization and X-ray data collection

Crystallization trials were performed with Crystal Screen, Crystal Screen 2 and Index kits (Hampton Research) at 289 K using the

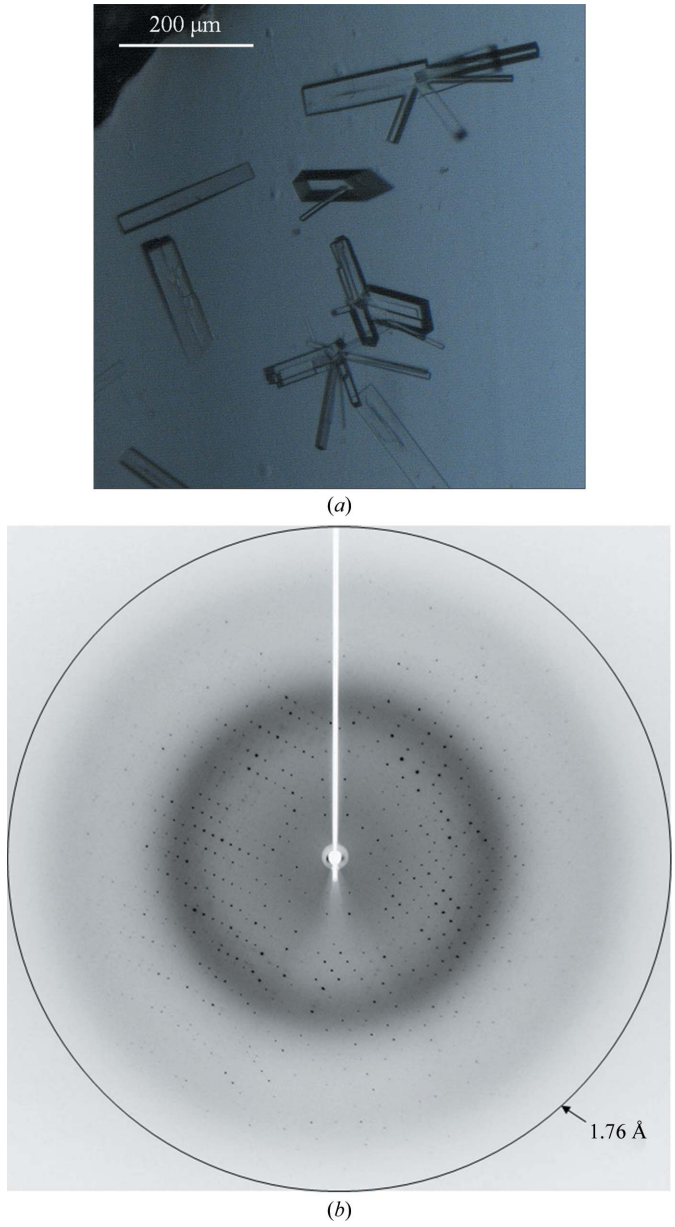

Figure 2
(*a*) Typical crystals of fTHEM2 with a C-terminal 6×His tag. (*b*) X-ray diffraction image of an fTHEM2 crystal. The black ring represents 1.76 Å resolution.

sitting-drop vapour-diffusion method. 1 µl protein solution in gel-filtration buffer (at a concentration of 10 mg ml^{-1} as determined using a Bio-Rad Protein Assay Kit) was mixed with 1 µl reservoir solution and equilibrated against 100 µl reservoir solution. The crystals that gave the best X-ray diffraction pattern were obtained using solution No. 65 of the Index kit [0.1 *M* ammonium acetate, 0.1 *M* bis-tris pH 5.5, 17%(*w*/*v*) PEG 10 000] after 3 d. Fig. 2(*a*) shows the crystals that were obtained.

Data collection was performed on beamline BL17U of Shanghai Synchrotron Radiation Facility (SSRF) using a MAR CCD 225 detector (MAR Research). After the addition of 15%(*v*/*v*) glycerol to the mother liquor, the crystals were mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystem. A complete data set was collected to 1.8 Å resolution (Fig. 2*b*). The diffraction images were indexed, integrated and scaled using *DENZO* and *SCALEPACK* as implemented in *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

The expression plasmid encoded a fusion protein consisting of the full-length fTHEM2 and a C-terminal 6×His tag with a Leu-Glu linker (translated from the *Xho*I site) between them. The fusion protein contained a total of 149 residues with a molecular mass of 16.1 kDa; 10 mg pure recombinant protein was obtained from 4 g *E. coli* cell pellets. The space group of the fTHEM2 crystal was determined to be *C*2, with unit-cell parameters a = 77.1, b = 74.4, c = 96.6 Å, β = 93.7°. X-ray data-collection statistics are given in Table 1. The self-rotation function calculated using *MOLREP* from the *CCP*4 suite (Winn *et al.*, 2011) is shown in Fig. 3. The fTHEM2 crystal contained four fTHEM2 molecules per asymmetric unit, based on a Matthews coefficient of 2.16 Å^3 Da^{-1} and a solvent content of 43.09% (Matthews, 1968). There are no peaks higher than 15% of the height of the origin peak in the native Patterson. There appear to be three mutually perpendicular noncrystallographic (NCS) twofold axes at θ, φ, χ angles of (15, 0, 180°), (80, 135, 180°) and (80, 225, 180°). The crystallographic axis appears at (90, 90, 180°) and (90, 270, 180°). The combination of the crystallographic axis and the NCS axes generate peaks at (15, 0, 90°) and (75, 180, 180°). The self-rotation function is thus consistent with a tetramer with 222 point-group symmetry.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Source	BL17U, SSRF
Wavelength (Å)	0.9716
Oscillation angle (°)	1
Space group	*C*2
Unit-cell parameters (Å, °)	a = 77.1, b = 74.4, c = 96.6, β = 93.7
Resolution range (Å)	50.0–1.80 (1.86–1.80)
Total No. of reflections	193229 (18737)
No. of unique reflections	50707 (5064)
Multiplicity	3.8 (3.7)
Data completeness (%)	99.8 (100.0)
$\langle I\rangle/\langle\sigma(I)\rangle$	15.5 (2.5)
$R_{\rm merge}$† (%)	8.7 (58.8)

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

As fTHEM2 shares ∼70% sequence identity with hTHEM2, there were considerable similarities from recombinant protein purification to crystallization, as predicted. The apparent molecular weight of fTHEM2 was 66.1 kDa, which indicated that fTHEM2 oligomerizes as a tetramer in solution, as did hTHEM2 (Fig. 1*b*). In addition, the catalytic residues, including Asn50, Gly57, Asp65 and Ser83, were highly conserved. This evidence indicates the possibility that a common catalytic mechanism may be shared between hTHEM2 and fTHEM2, and structural similarity will provide further evidence to support this conclusion.

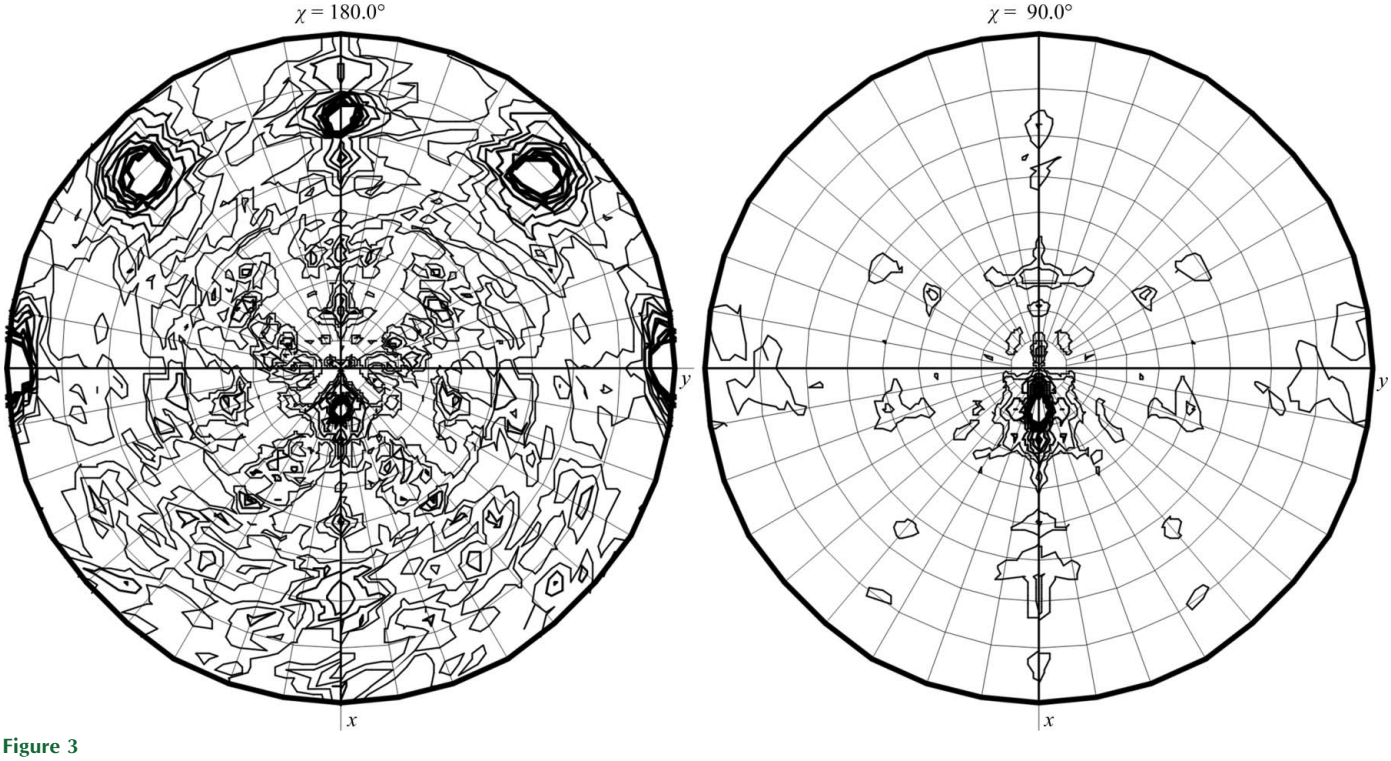

Figure 3
Self-rotation function for the crystal of fTHEM2.

However, the variability of the N-terminal region may have an impact on the biochemical properties of THEM2; for example, on its selectivity for substrates and its interaction with other functional partners. Related experiments are in progress.

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