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Overexpression, crystallization and preliminary X-ray crystallographic analysis of phosphopantetheine adenylyltransferase from *Enterococcus faecalis*

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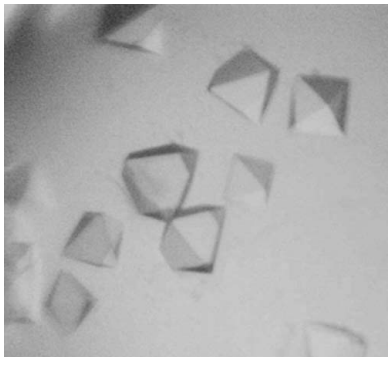
Phosphopantetheine adenylyltransferase, an essential enzyme in the coenzyme A biosynthetic pathway, catalyzes the reversible transfer of an adenylyl group from ATP to 4′-phosphopantetheine, yielding 3′-dephospho-CoA and pyrophosphate. *Enterococcus faecalis* PPAT has been overexpressed in *Escherichia coli* as a fusion with a C-terminal purification tag and crystallized at 297 K using a reservoir solution consisting of 0.1 *M* sodium HEPES pH 7.5, 0.8 *M* sodium dihydrogen phosphate and 0.8 *M* potassium dihydrogen phosphate. X-ray diffraction data were collected to 2.70 Å at 100 K. The crystals belong to the primitive tetragonal space group $P4_1$ (or $P4_3$), with unit-cell parameters $a = b = 160.81$, $c = 225.68$ Å. Four copies of the hexameric molecule are likely to be present in the asymmetric unit, giving a crystal volume per protein weight (V_M) of 3.08 Å^3 Da^{-1} and a solvent content of 60.1%.

1. Introduction

Coenzyme A (CoA), the principal acyl-group carrier in all living cells, is required for numerous synthetic and degradative reactions in intermediary metabolism (Robishaw & Neely, 1985). CoA biosynthesis proceeds in five steps, utilizing pantothenate (vitamin B5), cysteine and ATP (Robishaw & Neely, 1985). Phosphopantetheine adenylyltransferase (PPAT) catalyzes the penultimate step, the reversible transfer of an adenylyl group from ATP to 4′-phosphopantetheine (Ppant), yielding 3′-dephospho-CoA (dPCoA) and pyrophosphate (Martin & Drueckhammer, 1993). Subsequent phosphorylation at the 3′-hydroxyl of the ribose ring by dephospho-CoA kinase (dPCoAK) produces the acyl-group carrier CoA (Izard & Geerlof, 1999). The rate of CoA biosynthesis is regulated by feedback inhibition of the first enzyme of the biosynthetic pathway, pantothenate kinase (Halvorsen & Skrede, 1982). Studies on the CoA-biosynthetic intermediates in *Escherichia coli* have shown that PPAT catalyzes an additional rate-limiting step in the pathway (Jackowski & Rock, 1984).

In contrast to bacteria, PPAT and dPCoAK occur in mammals as a bifunctional enzyme called CoA synthase (Suzuki *et al.*, 1967; Worrall & Tubbs, 1983). Sequence alignment indicates that human PPAT and bacterial PPATs are highly dissimilar in their primary structures. This makes bacterial PPATs an attractive target for the discovery of novel antibiotics. Crystal structures of PPATs from *Escherichia coli* have been reported in complex with a variety of ligands (Izard & Geerlof, 1999; Izard, 2002, 2003). In addition, the structures of PPAT from *Mycobacterium tuberculosis* (Morris & Izard, 2004) in the apo form and PPAT from *Thermus thermophilus* in the Ppant-bound form (Takahashi *et al.*, 2004) have been reported. They are hexamers with 32 point-group symmetry.

Enterococcus faecalis is a Gram-positive pathogen and causes many of the same problems as other members of the intestinal flora, which include opportunistic urinary tract infections and wound infections. It is capable of causing life-threatening infections in humans, especially in the nosocomial environment. *En. faecalis* PPAT shows a sequence identity of 44, 39 and 45% with PPATs from *E. coli*,



M. tuberculosis and *T. thermophilus*, respectively. Structural data on *En. faecalis* PPAT will be useful in structure-based drug discovery. As a first step towards the structure determination of *En. faecalis* PPAT, we overexpressed it in *E. coli* and crystallized it. Its crystallization conditions and preliminary X-ray crystallographic data are reported here.

2. Experimental

2.1. Protein expression and purification

The PPAT gene (EF2451) was amplified by the polymerase chain reaction using the genomic DNA of *En. faecalis* strain V583 as a template. The forward and reverse oligonucleotide primers were designed using the published genome sequence (Paulsen *et al.*, 2003) as 5′-CGC **GGA TCC** ATG CGT AAA ATT GCT CTG TTT CCC G-3′ and 5′-CCG CCG **CTC GAG** GCT CCA GTC ATT CTT CTT TTG TTT T- 3′, respectively. The bases in bold represent the *Bam*HI and *Xho*I restriction-enzyme cleavage sites. The amplified DNA was inserted into the *Bam*HI/*Xho*I-digested expression vector pET-21a(+) (Novagen). This vector construction adds an eight-residue tag (LEHHHHHH) to the carboxyl-terminus of the gene product to facilitate protein purification. The protein was overexpressed in *E. coli* BL21(DE3) cells. The cells were grown at 310 K to an OD_{600} of 0.5 in Terrific Broth medium containing 50 µg ml^{-1} ampicillin and protein expression was induced with 0.5 m*M* isopropyl *β*-D-thio-galactopyranoside (IPTG). Cell growth continued at 298 K for 24 h after IPTG induction and cells were harvested by centrifugation at 5600*g* (8000 rev min^{-1}; Sorvall GSA rotor) for 10 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer *A* [20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl, 5 m*M* imidazole, 5%(*v*/*v*) glycerol and 1 m*M* phenylmethylsulfonylfluoride] and was then homogenized with an ultrasonic processor.

The crude cell extract was centrifuged at 36 000*g* (18 000 rev min^{-1}; Hanil Supra 21K rotor) for 1 h at 277 K and the recombinant protein in the supernatant fraction was purified in three chromatographic steps. The supernatant was subject to affinity chromatography on Ni–NTA resin (Qiagen), which was previously charged with Ni^{2+} and equilibrated with buffer *A*. After washing the column with buffer *A* containing 60 m*M* imidazole, the bound protein was eluted with a stepwise gradient of 0.2, 0.4, 0.6, 0.8 and 1.0 *M* imidazole. Tenfold-diluted supernatant fraction was then applied onto a Source-Q Sepharose prep-grade column (6 ml; Amersham Biosciences), which was previously equilibrated with 50 m*M* Tris–HCl pH 7.9 and 50 m*M* NaCl. Upon eluting with a gradient of NaCl in the same buffer, PPAT was eluted at 300–450 m*M* NaCl. Next, gel filtration was performed on a HiLoad XK-16 Superdex 200 prep-grade column (Amersham Biosciences), which was previously equilibrated with 20 m*M* Tris–HCl pH 7.5 and 200 m*M* NaCl. The homogeneity of the purified protein was assessed by SDS–PAGE (Laemmli, 1970). The protein solution was concentrated using a YM10 ultrafiltration membrane (Millipore-Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated extinction coefficient of 14 440 M^{-1} cm^{-1} (SWISS-PROT; http://www.expasy.ch/).

2.2. Crystallization and dynamic light scattering

Crystallization was performed at 297 K by the hanging-drop vapour-diffusion method using 24-well VDX plates (Hampton Research). Initial crystallization conditions were established using screening kits from Hampton Research (Crystal Screens I and II, SaltRX, Index I and II, PEG/Ion and MembFac) and from deCODE Biostructures Group (Wizard I and II). To grow the best crystals, each hanging drop was prepared on a siliconized cover slip by mixing 2 µl each of protein solution (at 20 mg ml^{-1} concentration in a buffer consisting of 20 m*M* Tris–HCl pH 7.5 and 200 m*M* NaCl) and reservoir solution (0.1 *M* sodium HEPES pH 7.5, 0.8 *M* sodium dihydrogen phosphate and 0.8 *M* potassium dihydrogen phosphate). Each hanging drop was placed over 0.48 ml reservoir solution. Dynamic light-scattering experiments were performed using a DynaPro-801 instrument from Wyatt (Santa Barbara, California). The data were measured at 297 K with the protein at 1 mg ml^{-1} concentration in 20 m*M* Tris–HCl pH 7.5 and 200 m*M* NaCl.

2.3. X-ray diffraction experiment

The crystals were flash-cooled using a cryoprotectant solution consisting of 0.1 *M* sodium HEPES pH 7.5, 0.8 *M* sodium dihydrogen phosphate, 0.8 *M* potassium dihydrogen phosphate and 30%(*v*/*v*) glycerol. Crystals were soaked in 5 µl cryoprotectant solution for 10 s before being flash-cooled in liquid nitrogen. The crystal was annealed *in situ* by blocking the cold nitrogen-gas stream briefly (∼10–15 s), during which procedure the crystal was thawed before being refrozen (Ellis *et al.*, 2002). X-ray diffraction data were collected at 100 K on a Bruker CCD area-detector system at the BL-6B experimental station of Pohang Light Source, South Korea. The crystal was rotated through a total of 300° with a 1.0° oscillation range per frame. The raw data were processed and scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997).

3. Results

PPAT from *En. faecalis* was overexpressed in *E. coli* in soluble form, with a yield of ∼14 mg of homogeneous protein per litre of culture. The native molecular weight (∼120 kDa) of the recombinant *En. faecalis* PPAT, as estimated by dynamic light-scattering analysis, indicates that it exists as a homohexamer in solution (the calculated monomer weight including the C-terminal tag is 19 741 Da). This result is in agreement with the hexameric structures of the PPATs from *E. coli*, *M. tuberculosis* and *T. thermophilus* (Izard & Geerlof, 1999; Morris & Izard, 2004; Takahashi *et al.*, 2004). Despite the presence of the eight-residue tag at its C-terminus, the recombinant enzyme readily formed reasonably well diffracting crystals. The best crystals were grown with a reservoir solution consisting of 0.1 *M* sodium HEPES pH 7.5, 0.8 *M* sodium dihydrogen phosphate and 0.8 *M* potassium dihydrogen phosphate. Tetragonal bipyramidal crystals grew to approximate dimensions of 0.1 × 0.1 × 0.1 mm within a few days (Fig. 1).

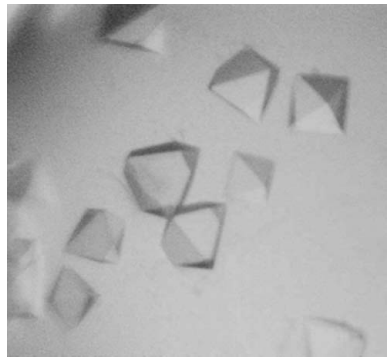


Figure 1
Crystals of PPAT from *En. faecalis*. Their approximate dimensions are 0.10 × 0.10 × 0.10 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Pohang Light Source BL-6B
X-ray wavelength (Å)	1.1271
Temperature (K)	100
Space group	$P4_1$ (or $P4_3$)
Unit-cell parameters (Å)	a = 160.81, b = 160.81, c = 225.68
Resolution range (Å)	50.0–2.70 (2.80–2.70)
Total/unique reflections	2105335/218514
$R_{\rm merge}$† (%)	7.8 (33.2)
Data completeness (%)	99.3 (97.9)
Average $I/\sigma(I)$	8.9 (2.3)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)_i$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h)$ for all i measurements.

A set of X-ray diffraction data was collected to 2.70 Å resolution at 100 K. A total of 2 105 335 measured reflections were merged into 218 514 unique reflections, giving an $R_{\rm merge}$ of 7.8% and a completeness of 99.3%. The space group was determined to be $P4_1$ (or $P4_3$) on the basis of systematic absences and symmetry of the diffraction intensities. The unit-cell parameters are $a = b = 160.81$, $c = 225.68$ Å. Table 1 summarizes the statistics of data collection. If it is assumed that the crystallographic asymmetric unit contains four copies of the hexameric molecule, the crystal volume per protein weight ($V_{\rm M}$) is 3.08 Å^3 Da^{-1} and the solvent content is 60.1% (Matthews, 1968).

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