Crystallization and preliminary X-ray characterization of the dephospho-CoA kinase from *Legionella pneumophila*

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Dephospho-CoA kinases (DPCKs) are members of the kinase family that catalyze the final step in CoA biosynthesis. Their function is phosphorylation of the 3-hydroxyl group of the ribose using ATP as a phosphate donor. Structural changes induced by ATP binding play an important role during the DPCK catalytic cycle. In this work, DPCK from *Legionella pneumophila* was overexpressed in *Escherichia coli*. The purification, crystallization and preliminary X-ray analysis of crystals of this protein are described. The protein was crystallized in space group $P2_12_12$, with unit-cell parameters $a = 36.29$, $b = 82.20$, $c = 81.80$ Å, using the hanging-drop vapour-diffusion method. Diffraction data were collected at 100 K and the phases were determined using the molecular-replacement method.

1. Introduction

As an essential cofactor, coenzyme A (CoA) plays an important role in cellular metabolism. It functions as an acyl-group carrier and mediates a wide variety of biochemical reactions in numerous biochemical pathways (Begley *et al.*, 2001). In the final two steps of CoA biosynthesis, 4'-phosphopantetheine is adenylylated to form dephosphocoenzyme A (dephospho-CoA) by the enzyme phosphopantetheine adenylyltransferase (PPAT), followed by phosphorylation of the 3'-hydroxyl group of the ribose moiety of dephospho-CoA to form CoA, which is catalyzed by dephospho-CoA kinase (DPCK) (Geerlof *et al.*, 1999; Mishra *et al.*, 2001). In mammalian cells, a single bifunctional enzyme which contains PPAT and DPCK domains performs the final two reactions (Zhyvoloup *et al.*, 2002).

DPCK contains the highly conserved P-loop or Walker A sequence motif GXXXXGKT/S (where *X* represents any residue) responsible for nucleotide binding (Walker *et al.*, 1982). This motif is preceded by a β -strand and is followed by an α -helix. It interacts with the phosphate groups of the nucleotide (Seto *et al.*, 2005; Obmolova *et al.*, 2001).

Kinases have to protect the active site from the omnipresent water to avoid becoming ATPases. In many kinase structures, the ligand(s) are thought to induce structural changes to shield their catalytic centres (Schulz *et al.*, 1990). According to the previous studies of DPCK structures from *Escherichia coli* (O'Toole *et al.*, 2003), *Haemophilus influenzae* (Obmolova *et al.*, 2001) and *Thermus thermophilus* HB8 (Seto *et al.*, 2005), this type of structural change also plays an important role in DPCKs. Thus, revealing the domain movement will contribute to understanding the DPCK phosphorylation mechanism.

DPCK from *Legionella pneumophila* is 207 amino acids long, with a molecular mass of 23.6 kDa. It has 40%, 38% and 31% sequence identity to the DPCK proteins from *E. coli*, *H. influenzae* and *T. thermophilus* HB8, respectively. In order to better understand the details of the biological functions and the domain actions of DPCKs, we purified and crystallized *L. pneumophila* dephospho-CoA kinase expressed in *E. coli* and performed preliminary X-ray analysis of the crystal.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

SSRF beamline	BL17U
Wavelength (Å)	0.97907
Space group	$P2_12_12$
Molecules in asymmetric unit	1
Unit-cell parameters (Å)	$a = 36.29, b = 82.20, c = 81.80$
Resolution range (Å)	22.76–2.10 (2.21–2.10)
No. of measured reflections	103914
No. of unique reflections	12710 (1754)
$R_{p.i.m.}^\dagger$ (%)	4.1 (17.6)
$R_{merge}^\ddagger$ (%)	11.3 (46.7)
Average $I/\sigma(I)$	13.9 (4.6)
Multiplicity	8.2 (7.5)
Completeness (%)	84.9 (82.4)
Matthews coefficient (Å ³ Da ⁻¹)	2.48
Solvent content (%)	50.5

$^\dagger R_{p.i.m.} = \sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $N(hkl)$ is the data multiplicity, $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity of multiple observations from symmetry-related reflections. It is an indicator of the precision of the final merged and averaged data set. $^\ddagger R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the average intensity of multiple observations from symmetry-related reflections.

2. Materials and methods

2.1. Cloning, expression and purification

DNA for *L. pneumophila* DPCK (NCBI entry code YP_095496.1) was amplified by PCR from *L. pneumophila* genomic DNA using the following oligonucleotide primers containing artificial *NdeI* and *XhoI* sites (in bold): 5'-GGAATTCC**ATATGA**AGGAGCCCACTCAGATGG-3' and 5'-CCGCTC**GAGT**TACTGTTTGATTTTGCTTCCTAAG-3'. The PCR product was digested with *NdeI* and *XhoI* and was ligated into the *NdeI* and *XhoI* sites of the bacterial expression vector p28 derived from pET-28a (Novagen) by deleting the sequence AGCAGCGGCCTGGTGGCGCGCGGCAGC between the *NcoI* and *NdeI* restriction sites. The resultant N-terminal amino-acid sequence of recombinant *L. pneumophila* DPCK is MGHHHHHHMKKE, where the residue in bold is the initiation methionine of native DPCK. The cloning junctions were confirmed by DNA sequencing. This recombinant plasmid was transformed into

E. coli strain Rosetta. Cells were grown at 310 K in Luria–Bertani medium containing 100 µg ml⁻¹ kanamycin. Expression of the DPCK was induced at an OD₆₀₀ of 0.8–1.0 by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 289 K for 16 h. The cells were harvested and sonicated in 20 mM Tris–HCl buffer pH 8.0 containing 200 mM NaCl in an ice–water bath. The lysate was clarified by centrifugation at 15 000g for 30 min at 277 K. The soluble fraction was loaded onto a nickel-chelating resin (Amersham Biosciences) pre-equilibrated with buffer A (20 mM Tris–HCl pH 8.0, 200 mM NaCl). The resin was washed with ice-cold buffer B (20 mM Tris–HCl pH 8.0, 100 mM imidazole, 200 mM NaCl) and the target protein was eluted with ice-cold buffer C (20 mM Tris–HCl pH 8.0, 300 mM imidazole, 200 mM NaCl).

The eluate was loaded onto a gel-filtration column (Superdex 200 16/60, GE Healthcare) at 285 K in 20 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl. The eluted recombinant DPCK was buffer-exchanged with buffer D (20 mM Tris–HCl pH 8.0, 20 mM NaCl) and concentrated to 30 mg ml⁻¹ by centrifugal ultrafiltration (Millipore) for crystallization assays. The protein concentration was determined by the Bradford method (Bio-Rad protein assay) using bovine serum albumin as standard. The presence and purity of recombinant DPCK were then analyzed on SDS–PAGE (greater than 95% purity), and it was judged to be suitable for crystallization. The yield of the soluble recombinant protein was about 6.5 mg per litre of induced culture.

2.2. Crystallization

The enzyme was crystallized in a substrate-free form. Initial crystallization trials were set up with Crystal Screen, Crystal Screen 2 and PEG/Ion Screen reagent kits (Hampton Research) at 285 K using the hanging-drop vapour-diffusion method. Each drop, consisting of 1 µl protein solution (5–20 mg ml⁻¹) and an equal volume of reservoir solution, was equilibrated against 200 µl reservoir solution. Further crystal optimization experiments were performed by systematic variation of the precipitant concentration and protein concentration and by testing the effects of additives. The crystals grew to their full dimensions in the final conditions after three weeks.

The crystals were harvested using cryoloops and immersed briefly in a cryoprotectant solution consisting of 80% (v/v) reservoir solution and 20% (v/v) glycerol. The crystals were subsequently flash-cooled and stored in liquid nitrogen and transferred to beamline BL17U of the SSRF (Shanghai Synchrotron Radiation Facility) for X-ray diffraction analysis and data collection.

2.3. Data collection and processing

After screening for diffraction quality, X-ray data were collected at 100 K from a single crystal to 2.1 Å resolution at a wavelength of 0.97907 Å using an ADSC Quantum 315r CCD detector. A total of 250 images were recorded with 0.8 s exposure at a crystal-to-detector distance of 250 mm using an oscillation range of 1°. The diffraction data were indexed and integrated by *iMosflm* (Battye *et al.*, 2011). The data were then scaled and merged using *SCALA* from the *CCP4* program suite (Winn *et al.*, 2011). In order to solve the phase problem, the molecular-replacement method was applied. Homologous structures (PDB entries 1jjv, 1n3b and 1uf9; Obmolova *et al.*, 2001; O'Toole *et al.*, 2003; Seto *et al.*, 2005) were used as the search models. The program *Phaser* (McCoy *et al.*, 2007) was used for molecular-replacement calculations. Table 1 gives a summary of the data-collection statistics.

Figure 1

L. pneumophila DPCK crystal of approximately 0.03 × 0.03 × 1.0 mm in size.

Figure 2
X-ray diffraction image of the *L. pneumophila* DPCK crystal. The resolution circles are at approximately 3.1 and 2.1 Å resolution.

3. Results and discussion

Recombinant *L. pneumophila* DPCK was expressed in *E. coli* strain Rosetta and purified to homogeneity. The enzyme was crystallized in the substrate-free form. Within one week, PEG/Ion condition 48 [0.2 M ammonium citrate dibasic, 20% (w/v) PEG 3350] gave small needle-like crystals. After initial crystal optimization experiments, crystals with rod-like morphology were obtained from the reservoir solution consisting of 0.23 M ammonium citrate dibasic, 17.5% (w/v) PEG 3350. However, the crystals diffracted X-rays to below 4 Å resolution. Therefore, various different additives were tested to increase the quality of the crystal diffraction. Crystals obtained in the presence of 1,3-butanediol and ATP produced diffraction of decent quality. The final crystals (approximately 0.03 × 0.03 × 1.0 mm in size; Fig. 1) were produced by mixing 2.0 µl protein solution (7 mg ml⁻¹) and an equal volume of reservoir solution consisting of 0.23 M ammonium citrate dibasic, 17.5% (w/v) PEG 3350, 0.01 M ATP, 5% (v/v) 1,3-butanediol and equilibrating against 200 µl reservoir solution at 285 K.

The diffraction data were collected at 100 K using synchrotron X-rays (Fig. 2). The *L. pneumophila* DPCK crystal belonged to space group *P*₂₁₂₁₂, with unit-cell parameters *a* = 36.29, *b* = 82.20, *c* = 81.80 Å. From the 103 914 accepted observations to 2.1 Å resolution, the data set contains 12 710 unique reflections and scaled with an overall *R*_{merge} of 11.3%. The reason for the low completeness of the diffraction data (84.9%) seems to be that reflections are missing in resolution shells where ice rings can occur (3.90, 3.67, 3.44, 2.67 and 2.25 Å). Crystal parameters, data-collection and data-processing statistics are summarized in Table 1.

A search of the Protein Data Bank using the *L. pneumophila* DPCK sequence yielded a number of potential structural homologues that could serve as molecular-replacement templates. However, molecular replacement using any of these templates with the full-length protein did not give any convincing solutions. There-

fore, in order to generate the best possible search model, the sequence of the *H. influenzae* DPCK structure (PDB entry 1jjv; Obmolova *et al.*, 2001) was 'mutated' to that of *L. pneumophila* DPCK using the program CHAINSAW (Stein, 2008). The flexible loops and several secondary-structure elements, which were thought to be involved in the structural change during the catalytic cycle, were deleted from the structure. In addition, all solvent molecules and heteroatoms (Hg, sulfate ion and ATP) were stripped from the model. Finally, using the improved model which contains 100 residues, a single solution obtained using Phaser (McCoy *et al.*, 2007) showed a log-likelihood gain (LLG) of 79 and Z-scores for the rotation function (RFZ) of 5.5 and translation function (TFZ) of 10.4. This initial model was subjected to 20 cycles of rigid-body refinement and ten cycles of restrained refinement using REFMAC5 (Murshudov *et al.*, 2011), which resulted in *R*-factor and *R*_{free} values of 44.5% and 47.9%, respectively.

The solution shows one *L. pneumophila* DPCK molecule in the crystallographic asymmetric unit. This is in agreement with the Matthews coefficient calculation (Matthews, 1968), with the best result giving a *V*_M of 2.48 Å³ Da⁻¹ and a solvent content of 50.5%.

At present we are completing the refinement of the structure. Structural analysis and attempts to obtain crystals of complexes of DPCK with substrate(s) by either co-crystallization or crystal soaking are both currently under way.

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