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Archaeal ADP-dependent phosphofructokinase: expression, purification, crystallization and preliminary crystallographic analysis

Jong-Jin Jeong, Shinya Fushinobu,* Sohei Ito, Hirofumi Shoun and Takayoshi Wakagi

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Correspondence e-mail: asfushi@mail.ecc.u-tokyo.ac.jp

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Thermococcus litoralis uses a modified Embden–Meyerhof pathway. Its phosphofructokinase (TLPFK) catalyses the phosphorylation of fructose-6-phosphate by ADP, but not by ATP, in the presence of Mg^{2+}, yielding fructose-1,6-bisphosphate. The gene encoding TLPFK was cloned and overexpressed in *Escherichia coli*. Recombinant TLPFK consists of a dimer with a 52 kDa subunit. The native crystals belong to space group $P4_12_12$, with unit-cell parameters $a = b = 85.41$, $c = 163.93$ Å, and diffract to beyond 2.6 Å resolution. The TLPFK structure was preliminarily analyzed by means of multiple isomorphous replacement with four heavy-atom derivatives.

1. Introduction

ATP is considered to be the universal energy carrier in all living cells and the preferred high-energy phosphate donor in most kinase reactions. ADP-dependent kinases were found for the first time by Kengen and coworkers in the hyperthermophilic archaeon *Pyrococcus furious* (Kengen *et al.*, 1994). The catabolism of sugars, such as starch, laminarin, maltose and cellobiose, has been investigated in detail. This led to the discovery of a modified Embden–Meyerhof pathway in this organism, involving several enzymatic steps that are different from classical ones (Kengen *et al.*, 2001; Sakuraba *et al.*, 2002). Glucokinases and phosphofructokinases (PFK) involved in the modified Embden–Meyerhof pathway require ADP as the phosphoryl group donor instead of ATP. The occurrence of ADP-dependent kinases is limited to members of the order *Thermococcales*, including *P. furiosus* and *Thermococcus litoralis*. The amino-acid sequences of ADP-dependent glucokinases and PFKs exhibit significant similarity and form a novel kinase family, which has been named the PFKC family (Ronimus & Morgan, 2001). Crystal structures of the ADP-dependent glucokinases from *T. litoralis* (Ito *et al.*, 2001) and *P. horikoshii* OT3 (Tsuge *et al.*, 2002) have been reported and shown to have basically similar folds to those of ATP-dependent ribokinases and adenosine kinases. Here, we report the overexpression, purification, crystallization and preliminary crystallographic analysis of the ADP-dependent PFK from *T. litoralis* (TLPFK).

2. Materials and methods

2.1. Cloning, overexpression and purification of recombinant TLPFK

The ADP-dependent phosphofructokinase activity was measured according to the method of Tuininga *et al.* (1999). *T. litoralis* genomic DNA was prepared as described previously (Jeon *et al.*, 1997). Based on the strictly conserved amino-acid sequences of known ADP-dependent PFKs, the following primer pair was designed for PCR: 5′-AGG MTC GAA TAT CCV AG-3′ and 5′-TGA GAT TGT GTC TCC GAT TCC TAC KGT-3′. A PCR product of about 1.2 kbp was amplified from the genomic DNA as a template and subsequently used as a probe by labelling with DIG (digoxigenin). Chromosomal DNA of *T. litoralis* was partially digested with *Bam*HI. DNA fragments ranging in size from 2.0 to 4.0 kbp were purified from agarose gels and subsequently ligated into the *Bam*HI site of pUC119. *Escherichia coli* JM109 was transformed with each ligation mixture. Positive clones with a 3.1 kbp *Bam*HI insert were selected and sequenced. Sequence analysis was performed with a CEQ 2000XL DNA Analysis System (Beckman Coulter). The sequence data are available in the GenBank database under accession Nos. AB050016 (DNA) and BAB69952 (protein). For expression of the gene, the following primer pair was designed to amplify the ORF by PCR: 5′-ACT GAG GGT GAT **CAT ATG** ATG GAG TTC CTC-3′ (sense; *Nde*I site in bold) and 5′-GCT TTG TTG ATG **CTC GAG** TGA AAG AGT AG-3′ (antisense; *Xho*I site in bold). The PCR

Table 1
Data-collection statistics.

Data statistics for the indicated resolution shells are given in parentheses.

Data set	Native	K_2PtCl_4	$HAuCl_4$	PHMB	PHMBS
Wavelength (Å)	1.0723	1.0720	1.0375	1.0080	1.0080
Unit-cell parameters					
$a = b$ (Å)	85.4	86.1	85.1	85.5	85.7
c (Å)	163.8	163.6	163.2	164.3	164.4
Resolution (Å)	20.0–2.4 (2.70–2.59/ 2.49–2.40)	46.1–3.0 (3.16–3.00)	19.9–2.6 (2.93–2.80/ 2.69–2.60)	20.0–2.6 (2.93–2.80/ 2.69–2.60)	20.0–2.6 (2.93–2.80/ 2.69–2.60)
Total reflections	533428	619622	575495	435637	435283
Unique reflections	24454	13002	19112	19404	19441
Completeness (%)	99.5 (100.0/100.0)	99.9 (99.9)	99.3 (100.0/100.0)	99.5 (100.0/100.0)	99.2 (100.0/100.0)
R_{merge}† (%)	4.8 (19.5/36.0)	8.8 (34.8)	6.5 (23.6/42.3)	5.8 (25.2/46.7)	6.1 (22.6/42.8)
Mean $\langle I/\sigma(I)\rangle$	60.2 (16.2/8.3)	6.8 (2.2)	38.2 (12.8/6.8)	46.2 (12.3/6.5)	42.4 (13.6/7.3)
Phasing power, acentric/centric‡		0.70/0.53	0.51/0.38	1.57/1.05	1.59/1.08
R_{cullis}, acentric/centric‡		0.93/0.91	0.96/0.96	0.72/0.77	0.72/0.76
No. of heavy-atom sites		3	1	1	1

† $R_{merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of $I(h,i)$ for all i measurements. ‡ Calculated with *MLPHARE* from the *CCP*4 program suite.

products were digested with *Nde*I and *Xho*I and then cloned into a *Nde*I/*Xho*I-digested pET21d vector. *E. coli* BL21 (DE3) CodonPlus RIL (Stratagene, La Jolla, CA) cells harbouring this TLPFK expression vector were grown at 310 K in 1 l Luria–Bertani (LB) medium containing 100 µg ml^{-1} of ampicillin to an optical density of 0.5 at 600 nm before induction with 0.5 m*M* isopropyl-1-thio-β-D-galactopyranoside for 6 h. Cells were harvested by centrifugation (15 000*g* for 30 min), washed with 50 m*M* Tris–HCl pH 7.5 containing 100 m*M* NaCl and then sonicated for 15 min on ice. Cell debris was removed by centrifugation (15 000*g* for 30 min) and the supernatant was treated at 348 K for 20 min. The precipitated protein was removed by centrifugation (15 000*g* for 30 min). The supernatant was filtered through a 0.22 µm filter and then brought to 40% ammonium sulfate saturation. After centrifugation, the supernatant was loaded on a phenyl-Toyopearl column (Tosoh) equilibrated with 20 m*M* Tris–HCl pH 7.5 (buffer *A*) containing 30% ammonium sulfate and then eluted with a linear gradient of 30–0% saturation of ammonium sulfate. The active fractions were combined, desalted and concentrated with a Centriprep (Millipore). The concentrated protein was loaded onto a Mono-Q HR 5/5 column (Amersham Biosciences) equilibrated with buffer *A* and then eluted with a linear gradient of NaCl (0–0.5 *M* in buffer *A*). The concentrated protein was then loaded onto a Superdex 200 HiLoad 26/60 column (Amersham Biosciences) equilibrated with 150 m*M* NaCl in buffer *A* and eluted with the same buffer.

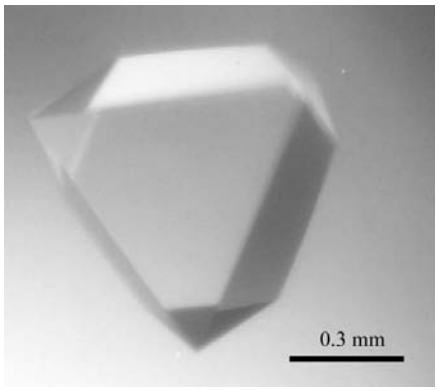

Figure 1
A crystal of TLPFK.

2.2. Crystallization, diffraction data collection, processing and initial phasing

The crystallization conditions were initially screened by the sitting-drop vapour-diffusion method using the sparse-matrix kits Crystal Screen 1 and 2 from Hampton Research at 277 and 298 K. 5 µl of a 10 mg ml^{-1} solution of TLPFK in 150 m*M* sodium chloride and 5 m*M* Tris–HCl pH 7.5 was mixed with an equal volume of reservoir solution. The best crystallization condition was obtained with a reservoir solution containing 4 *M* sodium chloride and 100 m*M* Tris–HCl pH 7.5. After incubation for about one week at 298 K, suitable crystals were obtained. Four derivative data sets were obtained from crystals soaked for 2 d in 2.85 m*M* K_2PtCl_4, 2.8 m*M* $HAuCl_4$ and 2 m*M* *p*-hydroxymercuribenzoic acid (PHMB) or 0.4 m*M* *p*-hydroxymercuribenzenesulfonic acid (PHMBS). To prepare heavy-atom derivatives, crystals were transferred to a reservoir solution containing the heavy-atom reagent. Prior to data collection, the crystals were briefly immersed in a cryoprotectant solution comprising 20%(*w*/*v*) glycerol in the mother liquor and flash-cooled by plunging into liquid nitrogen. All data were collected at 100 K. Data from a native crystal and from the $HAuCl_4$, PHMB and PHMBS derivatives were collected with an ADSC Quantum 4R CCD camera at beamline BL40B2 of SPring-8 (Hyogo, Japan). A total of 180 frames were collected using the oscillation method with 1° oscillations and 10 s exposures at a crystal-to-detector distance of 180 mm. Data for K_2PtCl_4 derivatives were collected with an ADSC Quantum 4R CCD camera at beamline BL18B of the Photon Factory (Tsukuba, Japan). A total of 258 frames were collected using the oscillation method with 0.7° oscillations and 30 s exposures at a crystal-to-detector distance of 220 mm. The data collected at BL40B2 and BL18B were processed using *HKL*2000 (Otwinowski & Minor, 1997) and *MOSFLM* (Powell, 1999), respectively. Isomorphous difference Patterson maps were calculated with the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The program *SOLVE* (Terwilliger & Berendzen, 1999) was used for phase calculation of the MIRAS data sets.

3. Results and discussion

The recombinant TLPFK protein was overexpressed and purified to homogeneity. On SDS–PAGE, the purified TLPFK protein migrates as a single band corresponding to approximately 52 kDa, which corresponds to the calculated molecular mass of the gene product (52 851 Da). The relative molecular size determined on gel-filtration chromatography showed that the recombinant

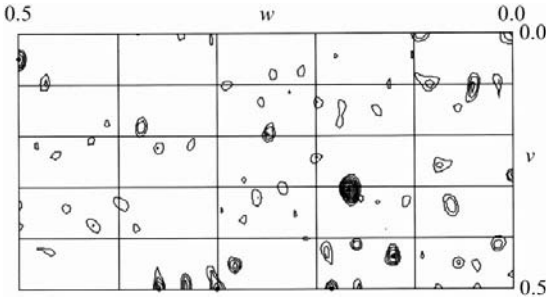

Figure 2
Harker section (0.5, v, w) of the difference Patterson map for the K_2PtCl_4 derivative.

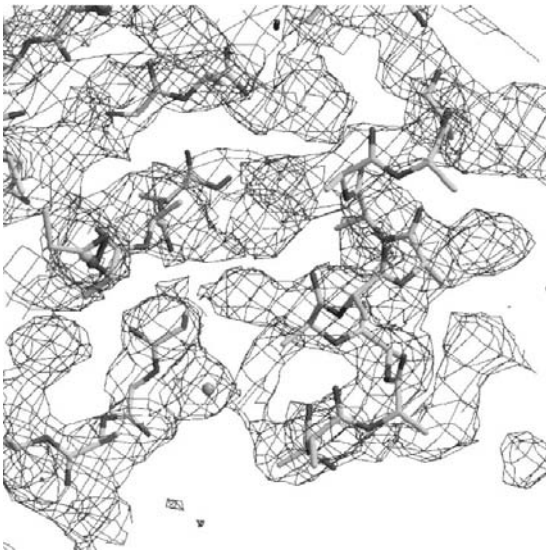

Figure 3
Electron-density map and the main-chain model generated by *RESOLVE*. One of the Pt-atom sites is shown.

TLPFK elutes as a homodimer of 125 kDa. The expression and purification protocols described here allowed the recovery of approximately 15.3 mg of pure recombinant TLPFK enzyme from 1 l of culture, with an enzymatic specific activity of 157 $\mu M\ min^{-1}\ mg^{-1}$. The kinetic parameters of TLPFK were analyzed, with a K_M value of 2.56 mM for fructose-6-phosphate and a V_{max} value of 344 $\mu M\ min^{-1}\ mg^{-1}$ being obtained, which indicated that the recombinant TLPFK enzyme is appropriate for crystallographic analysis and represents the wild-type enzyme well.

The optimized conditions resulted in the formation of crystals of approximately 0.5 × 0.9 × 1.1 mm in size (Fig. 1). The crystals belong to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 85.4$, $c = 163.8$ Å. The native crystals diffracted to about 2.6–2.4 Å resolution with a synchrotron X-ray source. The statistics for the native and derivative data are presented in Table 1. Assuming one monomer per asymmetric unit, the calculated V_M value (Matthews, 1968) and solvent content are 2.83 $Å^3\ Da^{-1}$ and 56.5%, respectively. Inspection of a Harker section of the isomorphous difference Patterson synthesis map of K_2PtCl_4 revealed the position of at least one Pt atom with high occupancy (Fig. 2). Structure solution was performed using the program *SOLVE* (Terwilliger & Berendzen, 1999), resulting in an overall figure of merit of 0.51 at 2.8 Å resolution (Z score = 22.7). Density modification and automated main-chain building were performed with *RESOLVE* (Terwilliger, 2000), yielding a figure of merit of 0.65 and a preliminary model comprising 305 amino-acid residues (Fig. 3). The overall structure of the model resembles those of ADP-dependent glucokinases (Ito *et al.*, 2001; Tsuge *et al.*, 2002), which consist of large and small domains. Characteristic β-sheets and α-helices in both domains can be seen clearly, but the main-chain trace around the hinge region remains ambiguous. The crystal structure of TLPFK seems to have an open conformation, as observed for the apo structure of the ADP-dependent glucokinase from *P. horikoshii* OT3 (Tsuge *et al.*, 2002). Crystallographic refinement of the TLPFK structure is currently under way.

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