Li Zhang,^a Zheng Guo,^a Jing Huang,^a Meiruo Liu,^a Yuandong Wang^a and Chaoneng Ji^{a,b*}

^aState Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai 200433, People's Republic of China, and ^bShanghai Engineering Research Center of Industrial Microorganisms, Shanghai 200433, People's Republic of China

Correspondence e-mail: chnji@fudan.edu.cn

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Expression, purification, crystallization and preliminary X-ray crystallographic analysis of fructose-1,6-bisphosphate aldolase from *Escherichia coli*

Fructose-1,6-bisphosphate aldolase is one of the most important enzymes in the glycolytic pathway and catalyzes the reversible cleavage of fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The full-length *fbaB* gene encoding fructose-1,6-bisphosphate aldolase class I (FBPA I) was cloned from *Escherichia coli* strain BL21. FBPA I was overexpressed in *E. coli* and purified. Biochemical analysis found that the optimum reaction temperature of FBPA I is 330.5 K and that the enzyme has a high temperature tolerance. Crystals of recombinant FBPA I were obtained by the sitting-drop vapour-diffusion technique in a condition consisting of 19 mg ml⁻¹ FBPA I in 0.1 M Tris pH 9.0, 10% (w/v) polyethylene glycol 8000 and diffracted to 2.0 Å resolution. The crystals belonged to the monoclinic space group C2, with unit-cell parameters $a = 217.7$, $b = 114.9$, $c = 183.9$ Å, $\beta = 124.6^\circ$. The asymmetric unit of these crystals may contain ten molecules, giving a Matthews coefficient of 2.48 Å³ Da⁻¹ and a solvent content of 50.5%.

1. Introduction

The glycolytic enzyme fructose-1,6-bisphosphate aldolase (FBPA; EC 4.1.2.13) catalyzes the reversible aldol cleavage of D-fructose 1,6-bisphosphate (D-FBP) to dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (D-GAP). According to the methods of enzyme catalysis (Rutter, 1964; Lebherz & Rutter, 1969; Alefounder *et al.*, 1989), FBPA can be divided into two classes: FBPA I and FBPA II. The FBPA I enzymes form a Schiff-base intermediate between the ϵ -amino group of the active-site lysine residue and the carbonyl of the substrate, whereas the FBPA II enzymes are metal-dependent enzymes, the substrate of which is coordinated to divalent metal ions such as magnesium, iron or zinc (Verlinde & Quigley, 1999). The classical FBPA I enzyme is mostly found in eukaryotic organisms and is only observed in a few bacteria (Galperin *et al.*, 2000; Thomson *et al.*, 1998), while FBPA II is mainly found in bacteria (Marsh & Lebherz, 1992). The FBPA I enzymes can be further subdivided into two families according to sequence. FBPA Is from eukaryotes are homomeric tetramers, while those from bacteria and archaea vary in their oligomeric arrangements from monomers to decamers (Siebers *et al.*, 2001; Thomson *et al.*, 1998). The crystallographic study of FBPA I can be traced back to 1987 (Sygusch *et al.*, 1987), since when more than 30 FBPA I structures have been deposited in the PDB. Several structures of FBPA I from eukaryotes and archaea (Hester *et al.*, 1991; Lorentzen *et al.*, 2003, 2005; Galkin *et al.*, 2009; Blom & Sygusch, 1997) have been solved, but there is no information about the three-dimensional structures of bacterial FBPA I enzymes. *Escherichia coli* is one of the few organisms which contain both types of FBPA (Thomson *et al.*, 1998). The FBPA I from *E. coli* (*Ec*-FBPA I) has a maximum of only 27% amino-acid sequence identity to FBPA I enzymes of known structure (Lorentzen *et al.*, 2003). In this communication, the 2.0 Å resolution crystal structure of *Ec*-FBPA I is reported, which will enable detailed structural analysis of its key components. This structure may also assist in determining the structure–function relationship of FBPA I in

Table 1

Macromolecule-production information.

Source organism	<i>E. coli</i> strain BL21
DNA source	<i>E. coli</i> strain BL21 complete genome
Forward primer	5'-CACGCTAGCATGACAGATATTGCACAGTTGC-3'
Reverse primer	5'-CACCTCGAGTCAGGCGATAGTAATTTTGTCTAT-3'
Cloning vector	pET-28b
Expression vector	pET-28b
Expression host	<i>E. coli</i> strain BL21
Complete amino-acid sequence of the construct produced	MTDIAQLLGKADNLLQHRCTIPSDQLYLPGHYVDRVMID- NNRPPAVLRNMQTLTYNTGRLAGTGYLSILPVDQGVHSAG- ASFAANPLYFDPKNIVELAEAGCNVASTYGVLASVSRR- YAHRIPLVLKLNHNETLSYPNTYDQTLTYASVEQAFNMGAV- AVGATIFYGSEESRRQIEEISAAFERAHELGMVTVLWAYL- RNSAFKKGVDVYHVSADLTGQANHLAATIGADIVKQKMAE- NNGGYKAINGYTDDRVSYSKLTSENPIDLVRVQLANCYMG- RAGLINSGGAAGGETDLSDAVRTAVINKRAGGMGLILGRK- AFKKSMDAGVKLINAVQDVYLDISKITIA

related pathogens including *Shigella*, *Salmonella*, *Klebsiella oxytoca*, *Yokenella regensburgei* and *Enterobacter cancerogenus* (95–99% sequence identity) owing to their high sequence similarity.

2. Materials and methods

2.1. Cloning, expression and purification

The gene coding for FBPA I was amplified from the *E. coli* strain BL21 complete genome using the polymerase chain reaction (PCR) with the following primers: sense, 5'-CACGCTAGCATGACAGAT-ATTGCACAGTTGC-3' (the *NheI* restriction-enzyme site is underlined); antisense, 5'-CACCTCGAGTCAGGCGATAGTAATTTT-GCTAT-3' (the *XhoI* restriction-enzyme site is underlined). The amplified DNA fragment and pET-28b vector (Novagen, USA) were digested by *NheI/XhoI* restriction endonucleases and then ligated by T4 DNA ligase (New England Biolabs, USA). After confirmation of the inserted fragment through sequencing, the recombinant pET-28b-*fbpA* vector was transformed into *E. coli* strain BL21 (DE3). The bacteria were grown at 310 K in 500 ml LB medium with 34 mg l⁻¹ chloramphenicol and 50 mg l⁻¹ kanamycin until the OD₆₀₀ reached 0.7. Protein overexpression was initiated by adding 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). After 10 h induction at 298 K, the cells were harvested by centrifugation at 5000g for 5 min at 277 K.

The cell pellet was resuspended in lysis buffer (300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole pH 8.0) and the cells were crushed at 277 K in a JN-3000 Plus cell disruptor at a pressure of 12 kg cm⁻². Cell debris was removed by centrifugation at 13 000g for 40 min at 277 K. The supernatant was passed through a HisTrap HP 5 ml column (GE Healthcare) pre-equilibrated with lysis buffer. After washing the resin with buffer (300 mM NaCl, 50 mM sodium phosphate, 50 mM imidazole pH 8.0), the six-His-tagged FBPA I was eluted with a linear elution gradient (50–250 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate pH 8.0). The protein was further purified by size-exclusion chromatography on a Superdex 75 column (GE Healthcare) with 200 mM NaCl, 20 mM Tris-HCl pH 8.0 as the mobile phase. *Ec*-FBPA I was concentrated by centrifugation using a 30 kDa centrifugal filter (Millipore). The concentrated *Ec*-FBPA I was stored in buffer consisting of 4 mM Tris pH 8.0. The concentration of FBPA I was determined by the Bradford assay and the purity of the protein was analyzed by 12% SDS-PAGE. The concentrated FBPA I was flash-frozen in liquid nitrogen and stored at 193 K.

The His-tag was not removed for the enzymatic assay or crystallization steps. Macromolecule-production information is summarized in Table 1.

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	96-well CrystalQuick Plus
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	19
Buffer composition of protein solution	4 mM Tris-HCl pH 8.0
Composition of reservoir solution	0.1 M Tris-HCl pH 9.0, 10%(w/v) PEG 8000
Volume and ratio of drop	2.4 μ l, 1:1
Volume of reservoir (μ l)	100

2.2. Enzymatic assay of recombinant FBPA I

The cleavage activity of FBPA I was determined in a coupled assay using triosephosphate isomerase from baker's yeast and glycerol-phosphate dehydrogenase from rabbit muscle (Sigma-Aldrich) as auxiliary enzymes. The rate of substrate cleavage was measured by monitoring the decrease in absorbance per minute at 340 nm [$\epsilon_{340\text{ nm}}(\text{NADH}) = 6.2\text{ mM}^{-1}\text{ cm}^{-1}$; Say & Fuchs, 2010] in a single cuvette with a 1 mm optical path using a NanoDrop 8000 (Thermo Scientific). The standard assay mixture consisted of 100 μ l 100 mM Tris-HCl pH 8.0, 20 mM MgCl₂, 20 mM DTE, 0.55 mM NADH, 5 mM FBP, 5 U triosephosphate isomerase, 0.25 U glycerolphosphate dehydrogenase with 0.65–0.78 mg FBPA I. All reagents apart from FBPA I were dispensed into a PCR tube and pre-warmed at the assay temperature for 3 min. The reaction was started by the addition of FBPA I. After 10 min, the assay mixture was placed on ice for 2 min and then monitored at 340 nm. To determine the optimum temperature of *Ec*-FBPA I, the enzyme activity was measured at temperatures from 273 to 348 K at pH 8.0 (Thomson *et al.*, 1998). The optimum pH was determined at 330.5 K in solutions containing 100 mM Tris-HCl pH 7.0–9.0. The result shows that this enzyme has over 90% of the maximal activity (Supplementary Fig. S1[†]) in the pH range 7.75–9.0. Within this range, *Ec*-FBPA I showed maximum activity at pH 8.75, so the pH value was changed to 8.75 in the subsequent enzymatic experiments. The kinetic parameters of *Ec*-FBPA I are given in the Supporting Information. The thermostability of *Ec*-FBPA I was estimated by measuring the enzymatic residual activity at pH 8.75 (330.5 K) after incubating the enzyme for different time periods at 318, 323, 328, 333, 338 and 343 K. All assays were performed in triplicate.

2.3. Crystallization and data collection

The appropriate FBPA I concentration for crystallization was screened using the PCT kit (Hampton Research). The preliminary crystallization conditions were screened by the sitting-drop vapour-diffusion method at 293 K in 96-well plates using commercial crystallization kits (Index HT and Crystal Screen HT from Hampton Research). Crystals were obtained by mixing 1.2 μ l protein solution (19 mg ml⁻¹) with 1.2 μ l reservoir solution and equilibrating against 100 μ l reservoir solution. Multiple needle-like crystals were observed within a week in a condition consisting of 0.1 M Tris-HCl pH 8.5, 8%(w/v) polyethylene glycol (PEG) 8000. The optimized crystallization condition for FBPA I consisting of 0.1 M Tris-HCl pH 9.0, 10%(w/v) PEG 8000 was obtained by varying the PEG concentration and pH value over wide ranges. These crystals were soaked for ~10 s in cryoprotectant solution, which consisted of 70% reservoir solution and 30% glycerol, before being flash-cooled in a nitrogen stream at 100 K. Diffraction data were collected at a wavelength of 0.9793 Å at 100 K on beamline BL17U-MX at Shanghai Synchrotron Radiation

[†] Supporting information has been deposited in the IUCr electronic archive (Reference: NJ5194).

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline BL17U, SSRF
Wavelength (Å)	0.97930
Temperature (K)	100
Detector	MAR DTB
Crystal-to-detector distance (mm)	280
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	C2
Unit-cell parameters (Å, °)	$a = 217.7, b = 114.9, c = 183.9,$ $\alpha = \gamma = 90, \beta = 124.6$
Mosaicity (°)	0.625
Resolution range (Å)	50–2.0 (2.03–2.00)
Total No. of reflections	869862
No. of unique reflections	248532
Completeness (%)	99.0 (98.1)
Multiplicity	3.5
$\langle I/\sigma(I) \rangle$	18.8 (2.3)
$R_{\text{merge}}^{\dagger}$	0.078 (0.53)
Overall B factor from Wilson plot (Å ²)	23.6

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections, $\sum_i$ is the sum over i measurements of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity of all observations i of reflection hkl .

Facility (SSRF) using a MAR DTB detector. The diffraction data were indexed, integrated and scaled with the *HKL-2000* package (Otwinowski & Minor, 1997). Crystallization information is summarized in Table 2 and data-collection statistics are summarized in Table 3.

3. Results and discussion

The recombinant *Ec*-FBPA I was successfully cloned, overexpressed and purified. The protein was concentrated to 38 mg ml^{−1} with 97% purity (Fig. 1) as estimated using *Quantity One* (Bio-Rad). The FBPA I shows maximum activity at 330.5 K (Fig. 2a) and reveals high thermostability as it retains almost 100% activity after incubating the enzyme at 328 K for 12 h (Fig. 2b). The relative activity of *Ec*-FBPA I was close to zero at 338 K in the optimum temperature experiment,

Figure 1

12% SDS-PAGE showing the purity of the *Ec*-FBPA I used for crystallization. Lane 1, molecular-weight marker (labelled in kDa). lane 2, *Ec*-FBPA I sample used in crystallization trials.

but it has over 85% activity after incubating the enzyme at 338 K for 30 min in the thermostability experiments (Fig. 2b). This phenomenon may be caused by a reversible conformational change of *Ec*-FBPA I which can be partially recovered when the temperature is decreased. The conformational change may hinder substrate binding to the enzyme or result in improper positioning of the catalytic amino acids. FBPA I from *E. coli* is more stable at high temperature than FBPA I from rabbit muscle and FBPA II from *Magnaporthe grisea* and *Pseudomonas aeruginosa* (Labbe *et al.*, 2011).

Monoclinic crystals were obtained at 293 K by the sitting-drop vapour-diffusion technique from a reservoir solution consisting of 0.1 M Tris pH 9.0, 10% PEG 8000. The crystals grew to maximal dimensions of 0.26 × 0.17 × 0.05 mm within two weeks (Fig. 3). The preliminary crystallographic data of the FBPA I crystal are shown in Table 3. The FBPA I crystals belonged to space group C2, with unit-cell parameters $a = 217.7, b = 114.9, c = 183.9$ Å, $\alpha = \gamma = 90, \beta = 124.6^\circ$ and diffracted to 2.0 Å resolution. As the molecular weight of the *Ec*-FBPA I precursor is 38 kDa, the Matthews coefficients calculated for ten molecules in the asymmetric unit for the C2 crystals was 2.48 Å³ Da^{−1}, corresponding to a solvent content of 50.5%. More-

Figure 2

Enzymatic assay of recombinant *Ec*-FBPA I. (a) The optimal reaction temperature of *Ec*-FBPA I. (b) The thermostability of *Ec*-FBPA I. All data were average values of triplicate independent experiments. The highest activities were defined as 100%.

Figure 3

Crystal of *Ec*-FBPA I. Crystals were obtained using 0.1 M Tris pH 9.0, 10% (w/v) polyethylene glycol (PEG) 8000. The dimensions of the crystal were about $0.26 \times 0.17 \times 0.05$ mm after two weeks.

over, the asymmetric unit of FBPA I from known archaeal enzyme structures contains ten molecules (Lorentzen *et al.*, 2003, 2005; Siebers *et al.*, 2001). Preliminary crystallographic research on *Ec*-FBPA I will facilitate future structural and functional studies. The three-dimensional structure of *Ec*-FBPA I may provide insight into its high thermostability.

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