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Crystallization and preliminary X-ray analysis of pyruvate kinase from *Bacillus stearothermophilus*

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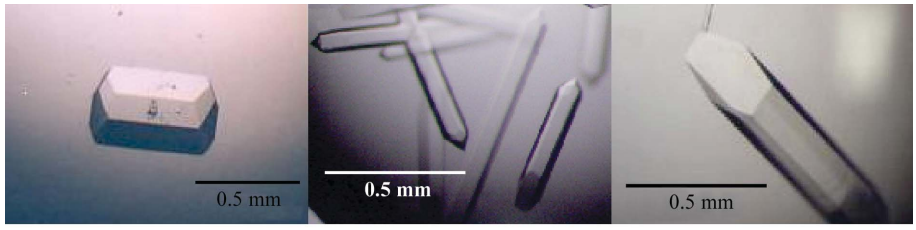
Pyruvate kinase (PK) from a moderate thermophile, *Bacillus stearothermophilus* (BstPK), is an allosteric enzyme activated by AMP and ribose 5-phosphate but not by fructose 1,6-bisphosphate (FBP). However, almost all other PKs are activated by FBP. The wild-type and W416F/V435W mutant BstPKs were crystallized by the hanging-drop vapour-diffusion method. However, they were unsuitable for structural analysis because their data sets exhibited low completeness. A crystal suitable for structural analysis was obtained using C9S/C268S enzyme. The crystal belonged to space group $P6_222$, with unit-cell parameters $a = b = 145.97$, $c = 118.03$ Å.

1. Introduction

Pyruvate kinase (PK; EC 2.7.1.40) plays a major role in the regulation of glycolysis. This enzyme catalyzes the transphosphorylation from phosphoenolpyruvate (PEP) to ADP, yielding pyruvate and ATP (Kayne, 1973; Valentini *et al.*, 2000). The reaction is essentially irreversible and is critical for control of the metabolic flux of glycolysis. Moreover, the substrate and the product are involved in a variety of metabolic pathways. Almost all PKs are activated by fructose 1,6-bisphosphate (FBP). However, the pyruvate kinase from *Bacillus stearothermophilus* (BstPK) is allosterically activated by AMP and ribose 5-phosphate (R5P), but not by FBP. It is very stable at high temperature, in contrast to the instability of other microbial pyruvate kinases (Sakai *et al.*, 1986). PKs from a number of prokaryotes and eukaryotes have been isolated and characterized. Most of them exist as tetramers of identical subunits (Kayne, 1973; Schramm *et al.*, 2000; Fothergill *et al.*, 2000). The crystal structures of cat, rabbit muscle, yeast, *Escherichia coli* and *Leishmania mexicana* PKs have been reported and their overall structures are similar (Stuart *et al.*, 1979; Larsen *et al.*, 1994; Jurica *et al.*, 1998; Speranza *et al.*, 1989; Rigden *et al.*, 1999). PKs from the genus *Bacillus* and some other bacteria have a long extra C-terminal sequence (ECTS) composed of about 110 amino-acid residues (Sakai & Ohta, 1993; Tanaka *et al.*, 1995; Kaneko *et al.*, 1996; Muñoz *et al.*, 1997, 2003). Part of the sequence is homologous to the PEP-binding motif of maize pyruvate phosphate dikinase, with 75% sequence identity (Pocalyko *et al.*, 1990; Sakai & Ohta, 1993; Nguyen & Saier, 1995). Furthermore, it has been suggested that the ETCS has a weak interaction between the A-domain and C-domain (Sakai, 2004). The ECTS may play some role in the structural stability of the enzyme.

In this report, we describe the crystallization of three types (wild type, W416F/V435W and C9S/C268S) of BstPK and preliminary X-ray diffraction data collection. Trp416 and Val435, which are located in the allosteric effector-binding site of BstPK, were mutated to Phe and Trp, respectively. The W416F/V435W mutant exhibited high affinity for PEP compared with that of wild-type enzyme, but its saturation curve was still sigmoidal and became hyperbolic in the presence of allosteric activators. Thus, the mutant seems to be a partially activated form. This BstPK has two cysteine residues (Cys9 and Cys268). These two residues were mutated to serine and the resultant mutated enzyme was designated C9S/C268S (Sakai, 2005). There is no information on the X-ray structures of PKs containing an ECTS that are allosterically activated by AMP or R5P. This is the first report of the crystallization of a PK having an ECTS and that is allosterically activated by AMP or R5P.



(*a*) (*b*) (*c*)

Figure 1
BstPK crystals. (*a*) Wild type, (*b*) W416F/V435W, (*c*) C9S/C268S.

2. Experimental procedures

2.1. Protein expression and purification

The wild-type BstPK and two mutants were composed of 587 amino acids and were expressed and purified as described previously (Sakai, 2004). Pyruvate kinase-deficient *E. coli* mutant PB25 (*pyrA*::*kan pyrF*::*cat*; Ponce *et al.*, 1995) was used as the host in this experiment. Plasmid pKH 510 was employed for pyruvate kinase production (Sakai, 2005). The enzymes were purified by means of Butyl-Toyopearl 650S (Tosoh, Japan) and Resource Q column (Amersham Biosciences) chromatography. The purified BstPKs were dialyzed against 5 m*M* Tris–HCl buffer pH 7.5 and then concentrated to 10–15 mg ml^{-1} with a Centricon YM-30 (Millipore). About 60 mg of purified BstPK was obtained from 1 l LB culture.

2.2. Crystallization

The protein was crystallized by the hanging-drop and sitting-drop vapour-diffusion and oil-batch methods at 285 and 293 K. 3 µl protein solution (5–20 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5) and 3 µl reservoir solution were mixed and equilibrated against 700 µl of the same reservoir solution. The initial crystallization conditions were examined using screening kits from Hampton Research (Crystal Screens 1 and 2, Additive Screens 1 and 2, Crystal Screen Cryo, Quick Screen and Index HT). The conditions were further refined by changing the protein concentration, pH and buffer concentration to produce larger and better crystals.

2.3. X-ray diffraction experiments

Diffraction data for the wild type, W416F/V435W mutant and C9S/C268S mutant were collected at beamline BL-18B of the High Energy Accelerator Research Organization (KEK, Tsukuba, Japan), BL44XU of SPring-8, (Harima, Japan) and NW12, Photon Factory, Advanced Ring (PF-AR), Tsukuba, Japan, respectively. The crystals were soaked for a few minutes in mother liquor containing 20%(*v*/*v*) glycerol and flash-frozen in liquid nitrogen. Diffraction data were collected with a Quantum 210 CCD X-ray detector (Area Detector Systems Corporation, Poway, CA, USA). The data set was processed and scaled using programs *DENZO* and *SCALEPACK* from the *HKL*2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

Small and thin needle-shaped crystals of wild-type and W416F/V435W mutant BstPK were obtained using several buffer conditions involving ammonium sulfate in the Crystal Screen 1 and 2 crystallization kits. Extensive fine-screening by varying the protein concentration and the amounts of precipitant, salt and buffer pH did not significantly improve the wild-type and W416F/V435W mutant BstPK crystal quality. Typical conditions for the crystallization of BstPKs were at 285 K with 10–15 mg ml^{-1} protein solution and the following reservoir solutions: 2.0 *M* ammonium sulfate, 0.1 *M* MES pH 6.5 for the wild type and 2.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 7.5, 2% PEG 200 for W416F/V435W. Crystals grew within two weeks (Figs. 1*a* and 1*b*). Although no apparent crystal decay was observed, the diffraction quality of orthorhombic crystals of the wild type and W416F/V435W mutant was poor. Attempts to improve the crystal quality using Additive Screens, Crystal Screen Cryo, Quick Screen and Index HT (Hampton Research) with various methods were unsuccessful. Finally, we tried to crystallize the C9S/C268S mutant. Good crystals were obtained with a reservoir solution comprising 2.1 *M* ammonium sulfate, 0.1 *M* MES pH 6.5 at 285 K. These crystals grew in one or two weeks (Fig. 1*c*). The V_M values (Matthews, 1968) and solvent contents of the wild type, W416F/V435W mutant and C9S/C268S mutant were determined to be 3.43, 3.37 and 2.91 Å^3 Da^{-1} and 64.2, 63.5 and 57.8%, respectively, assuming the presence of four, four and one molecule per asymmetric unit, respectively. The data-collection statistics for these crystals are presented in Table 1.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

	Wild type	W416F/V435W	C9S/C268S
X-ray wavelength (Å)	1.000	0.900	1.000
Space group	$P2_12_12_1$	$P2_12_12_1$	$P6_222$
Unit-cell parameters			
a (Å)	95.70	94.87	145.97
b (Å)	97.00	95.41	145.97
c (Å)	368.80	371.77	118.03
α (°)	90.0	90.0	90.0
β (°)	90.0	90.0	90.0
γ (°)	90.0	90.0	120.0
Resolution (Å)	43–2.7 (2.80–2.70)	54–2.8 (2.89–2.80)	50–2.4 (2.49–2.40)
No. of observations	383177	708015	821411
No. of unique reflections	93288	68647	28179
Completeness (%)	78.0 (74.2)	81.0 (76.3)	97.7 (79.9)
R_{merge} (%)	15.1 (35.8)	14.4 (35.1)	5.5 (32.2)
Mean $I/\sigma(I)$	6.5 (2.8)	6.7 (2.9)	16.7 (6.1)

The mutated Cys-free enzyme was prepared with the aim of improving crystal diffraction quality.

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