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A preliminary X-ray study of transketolase from *Burkholderia pseudomallei*

TktA is the most critical enzyme in the nonoxidative pentose phosphate pathway. It catalyzes the conversion of xylulose 5-phosphate and ribose 5-phosphate into sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate, and its products are used in the biosynthesis of acetyl-CoA, aromatic amino acids, nucleic acids and ADP-L-glycero- β -D-manno-heptose. TktA also has an unexpected role in chromosome structure that is independent of its metabolic responsibilities. Therefore, it is a new potent antibiotic target. In this study, TktA from *Burkholderia pseudomallei* has been cloned, expressed, purified and crystallized. Synchrotron X-ray data were also collected to 2.0 Å resolution. The crystal belonged to the monoclinic space group C2, with unit-cell parameters $a = 146.2$, $b = 74.6$, $c = 61.6$ Å, $\beta = 113.0^\circ$. A full structural determination is under way in order to provide insight into the structure–function relationship of this protein.

1. Introduction

The *tktA* gene from *Escherichia coli* encodes the major transketolase, which is a central component of the pentose phosphate pathway (Josephson & Fraenkel, 1969; Zhao & Winkler, 1994). The pentose phosphate pathway has two metabolic functions: (i) the oxidative pathway generates NADPH (Shi *et al.*, 1997) and (ii) the non-oxidative pathway supplements acetyl-CoA (Ikeda & Katsumata, 1999). TktA catalyzes the conversion of xylulose 5-phosphate (X5P) and ribose 5-phosphate (R5P) into sedoheptulose 7-phosphate (S7P) and glyceraldehyde 3-phosphate (G3P) in the nonoxidative pentose phosphate pathway (Stephanopoulos *et al.*, 1998). G3P mostly flows to acetyl-CoA and S7P flows to anabolic pathways for the biosynthesis of aromatic amino acids and nucleic acids and to the heptose-biosynthesis pathway for the production of ADP-L-glycero- β -D-manno-heptose. The latter pathway provides an alternative strategy for the development of new bactericidal agents that would weaken the outer membrane structure of Gram-negative pathogens (Eidels & Osborn, 1974). TktA also has an unexpected role in chromosome structure that is independent of its metabolic responsibilities (Hardy & Cozzarelli, 2005). The multiple functions of TktA in various biosynthetic pathways make it an interesting target for the development of novel antibiotics.

An open reading frame of *Burkholderia pseudomallei* codes for TktA (72.7 kDa), with 64% amino-acid sequence identity to *E. coli* TktA. The Gram-negative soil saprophyte *B. pseudomallei* is notorious for its pathogenicity; it causes melioidosis (Wiersinga *et al.*, 2006), which is an endemic tropical disease, and is considered to be a potential biological warfare agent owing to its highly infectious nature, especially by inhalation. Since mortality from melioidosis septic shock remains high despite appropriate antimicrobial therapy, the development of more effective antibiotics or antibiotic adjuvants is required. We initiated the determination of the three-dimensional structure of TktA from *B. pseudomallei* in order to provide a structural template for the development of a potential transketolase inhibitor with high specificity over human transketolase, which has a known structure (Mitschke *et al.*, 2010). Here, we report the cloning, overexpression, purification, crystallization and preliminary X-ray study of this enzyme.

2. Experimental methods

2.1. Cloning of *tktA* from *B. pseudomallei*

The cloning primers (Genotech, Daejeon, Republic of Korea) prepared for ligation-independent cloning (LIC) were 5'-GGCGG-TGGTGGCGGCATGACGACTTCGTCTCCCGCCTCC-3' for the forward strand and 5'-GTTCTTCTCCTTTGCGCCCCTACGCGA-GCACCGCCTTCG-3' for the reverse strand. The *tktA* gene was amplified by PCR using 200 ng *B. pseudomallei* genomic DNA template and 50 μ M of primers. PrimeSTAR HS DNA polymerase with GC buffer (Takara Bio Inc., Shiga, Japan) designed for high-GC-content genomic DNA was used. The amplified LIC expression vector pB2 (Kim *et al.*, 2005), a derivative of the pET21a vector (Novagen, Madison, Wisconsin, USA), was incubated with T4 DNA polymerase (New England Biolabs, Beverly, Massachusetts, USA) in the presence of 1 mM dATP at 310 K for 30 min followed by incubation at 343 K for 20 min. The amplified PCR product was prepared for vector insertion using the same protocol except for the presence of 1 mM dTTP instead of 1 mM dATP. The prepared insert was annealed into the pB2 vector, which expresses the cloned gene fused to a noncleavable N-terminal His₆ tag, and was transformed into DH5 α competent cells to obtain fusion clones. Clones were screened by plasmid DNA analysis and transformed into *E. coli* BL21 (DE3) cells for protein expression (Kim *et al.*, 1998).

2.2. Growth of cultures

E. coli BL21 (DE3) cells transformed with the cloned vector harbouring the *tktA* gene were grown on Luria-Bertani (LB) agar plates containing 150 μ g ml⁻¹ ampicillin. Several colonies were picked and grown in capped test tubes with 10 ml LB broth containing 150 μ g ml⁻¹ ampicillin. A cell stock of 0.85 ml culture and 0.15 ml glycerol was prepared and frozen at 193 K for use in a larger culture. The frozen cell stock was grown in 5 ml LB medium and diluted into 1000 ml fresh LB medium. The culture was incubated at 310 K with shaking until an OD₆₀₀ of 0.6–0.8 was reached. At this point, TktA was induced using isopropyl β -D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The culture was grown at 298 K for a further 16 h in a shaking incubator. Cells were harvested by centrifugation at 7650g (6500 rev min⁻¹) for 10 min in a high-speed refrigerated centrifuge at 277 K.

2.3. Protein purification

The cultured cell paste (2.5 g) was resuspended in 25 ml buffer consisting of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM imidazole, 1 mM PMSF, 10 μ g ml⁻¹ DNase I and Roche protease-inhibitor cocktail (Roche Applied Science, Indianapolis, Indiana, USA). The cell suspension was disrupted using a Digital Sonifier 450 (Branson Ultrasonics Co., Danbury, Connecticut, USA). Cell debris was pelleted by centrifugation at 24 900g (15 000 rev min⁻¹) for 30 min in a high-speed refrigerated centrifuge at 277 K. The supernatant was affinity-purified using a HisTrap column on an ÄKTAexplorer system (GE Healthcare, Piscataway, New Jersey, USA) at room temperature. The column was equilibrated with a buffer consisting of 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole. The target protein was eluted with a buffer consisting of 50 mM Tris-HCl pH 8.0, 100 mM NaCl with a gradient from 10 to 500 mM imidazole. TktA was further purified by ion-exchange chromatography using a 5 ml Hi-Trap Q column (GE Healthcare, Piscataway, New Jersey, USA) equilibrated with buffer consisting of 20 mM Tris-HCl pH 8.0. The protein was eluted at 0.33 M NaCl using a linear NaCl gradient. SDS-PAGE showed one band around 73.9 kDa corresponding to the molecular

weight of TktA with the tag. The purified protein contained a noncleavable N-terminal His₆ tag followed by five glycine residues (MHHHHHHGGGGG) and was concentrated to 10.7 mg ml⁻¹ (using an extinction coefficient of 1.42 M⁻¹ cm⁻¹ at 280 nm) for crystallization in a buffer consisting of 0.33 M NaCl, 20 mM Tris-HCl pH 8.0. Dynamic light scattering (DynaPro 99, Proterion Corporation, Piscataway, New Jersey, USA) showed a single monodisperse peak, indicating homogeneity of the protein.

2.4. Overexpression and purification of the selenomethionine derivative

A selenomethionine derivative of the protein was produced in a methionine auxotroph: *E. coli* strain B834 (DE3). There were a total of 15 methionine residues, including one in the tag. The cells were grown and induced in M9 minimal medium containing 50 mg l⁻¹ selenomethionine together with the other 19 amino acids (Ramakrishnan *et al.*, 1993). The purification of the selenomethionine derivative was performed as described above. The derivative protein was concentrated to 10.7 mg ml⁻¹ for crystallization in a buffer consisting of 0.33 M NaCl, 20 mM Tris-HCl pH 8.0.

2.5. Crystallization of selenomethionine-substituted TktA

Screening for crystallization conditions was performed using the sparse-matrix method (Jancarik & Kim, 1991) with several screens from Hampton Research (Laguna Niguel, California, USA). A Hydra Plus One crystallization robot (Matrix Technologies, Hudson, New Hampshire, USA) was used to set up the screens using the sitting-drop vapour-diffusion method at room temperature. A 96-well Intelli-Plate (Art Robbins Instruments, Salt Lake City, Utah, USA) was used; sitting drops were made by mixing 0.2 μ l protein solution (10.7 mg ml⁻¹) with 5 mM D-fructose 6-phosphate and 0.2 μ l reservoir solution and were equilibrated over 50 μ l reservoir solution. A VDX48 plate (Hampton Research, Laguna Niguel, California, USA) was used to optimize the crystallization conditions using hanging drops made by mixing 0.8 μ l protein solution and 0.8 μ l reservoir solution consisting of 20% PEG 1000, 0.1 M bis-tris pH 5.5 and equilibrated against 200 μ l reservoir solution.

Figure 1
Coomassie-stained SDS-PAGE (12%) of native and selenomethionine-substituted TktA (SeMet TktA). Lane 1, native TktA (3.7 μ g); lane 2, native TktA under reducing conditions (3.7 μ g); lane 3, SeMet TktA (3.3 μ g); lane 4, SeMet TktA under reducing conditions (3.3 μ g). Lanes M, molecular-mass marker (labelled in kDa).

Figure 2

Crystals of SeMet TktA from *B. pseudomallei*. These crystals appeared in the condition 20% PEG 1000, 0.1 M bis-tris pH 5.5. The crystals grew to approximate dimensions of $0.15 \times 0.05 \times 0.03$ mm in a week.

2.6. Data collection and reduction

Before being flash-cooled in liquid nitrogen, crystals were soaked in LV CryoOil (MiTeGen, Ithaca, New York, USA). X-ray diffraction data were collected at a single wavelength on beamline 4A at Pohang Light Source using a Quantum 4 CCD detector (Area Detector Systems Corp., Poway, California, USA) placed 300 mm from the sample. A fluorescence scan was performed on a selenomethionine-labelled protein crystal and data were collected at the peak wavelength ($\lambda = 0.97933$ Å). The oscillation range per image was 1.0° , with 1 s exposures. 360 oscillation images were collected with no overlap between contiguous images. X-ray diffraction data were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL-2000* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

The yield of purified selenomethionine-substituted TktA was 18.2 mg per litre of *E. coli* culture, which was comparable to that of the wild type (20.8 mg per litre). After anion-exchange chromatography, TktA appeared to be approximately 99% pure, with a prominent protein band at around 73.9 kDa on SDS-PAGE (Fig. 1). Diffraction-quality crystals were obtained using a reservoir solution consisting of 20% PEG 1000, 0.1 M bis-tris pH 5.5. Crystals grew to dimensions of $0.15 \times 0.05 \times 0.03$ mm within a week at 296 K (Fig. 2). Synchrotron data were collected to 2.0 Å resolution. The crystal belonged to the monoclinic space group *C2*, with unit-cell parameters $a = 146.2$, $b = 74.6$, $c = 61.6$ Å, $\beta = 113.0^\circ$. Based on the Matthews coefficient (Matthews, 1968), the asymmetric unit contained one TktA molecule, with a solvent content of 41.3%. Details of the data-collection statistics are presented in Table 1. Molecular replacement was performed using *EPMR* (Kissinger *et al.*, 2001) with the monomeric form of the X-ray crystal structure of TktA from *E. coli* (PDB entry 2r8p; Asztalos *et al.*, 2007) as a search model. The best solution was obtained with an *EPMR* score and an *EPMR* *R* factor of 0.347 and 52.4, respectively. A relatively good electron-density map was obtained after automated model building and refinement with the *PHENIX AutoBuild* wizard (Adams *et al.*, 2010). However, the N-terminal domain and its nearby regions (~200 amino acids) was not traceable owing to invisible electron density. Therefore, single-wavelength anomalous dispersion phases were calculated and a

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Pohang Light Source
X-ray wavelength (Å)	0.97933
Temperature (K)	100
Space group	<i>C2</i>
Unit-cell parameters (Å, °)	$a = 146.2$, $b = 74.6$, $c = 61.6$, $\beta = 113.0$
Solvent content (%)	41.3
V_M (Å ³ Da ⁻¹)	2.09
Resolution range (Å)	50.00–2.03 (2.07–2.03)
Multiplicity	3.8 (3.4)
Unique reflections	76934 (3844)
$R_{\text{merge}}^\dagger$ (%)	8.0 (54.0)
Data completeness (%)	99.9 (99.2)
Average $I/\sigma(I)$	14.7 (2.3)

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of the unique reflection hkl and $\langle I(hkl) \rangle$ is the mean of the intensities of all observations of reflection hkl .

reasonable electron-density map was obtained after finding seven clear Se-atom positions using the *PHENIX AutoSol* wizard. The current model has an *R* factor of 23.8% and an R_{free} of 27.9% and consists of 675 amino acids. The structure of TktA from *B. pseudomallei* will be reported in due course.

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