A preliminary X-ray study of 3-deoxy-D-manno-oct-2-ulosonic acid 8-phosphate phosphatase (YrbI) from *Burkholderia pseudomallei*

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3-Deoxy-D-manno-oct-2-ulosonic acid 8-phosphate phosphatase (YrbI), the third enzyme in the pathway for the biosynthesis of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO), hydrolyzes KDO 8-phosphate to KDO and inorganic phosphate. YrbI belongs to the haloacid dehalogenase (HAD) superfamily, which is a large family of magnesium-dependent phosphatase/phosphotransferase enzymes. In this study, YrbI from *Burkholderia pseudomallei*, the causative agent of melioidosis, has been cloned, expressed, purified and crystallized. Synchrotron X-ray data were also collected to 2.25 Å resolution. The crystal belonged to the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 63.7$, $b = 97.5$, $c = 98.0$ Å. A full structural determination is in progress to elucidate the structure–function relationship of this protein.

1. Introduction

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, plays an important role in maintaining the structural integrity of the bacterial outer membrane (Kneidinger *et al.*, 2002) and provides a barrier against the entry of toxic hydrophobic compounds into the bacterial cell (Nikaido & Vaara, 1985). LPS can be divided into three distinct structures known as lipid A, a conserved core oligosaccharide region and an O-specific polysaccharide chain. In the majority of Gram-negative bacteria, the core oligosaccharide region can be subdivided into an outer core, typically composed of hexoses and hexosamines, and an inner core made of L,D-heptose units and 3-deoxy-D-manno-oct-2-ulosonic acid (KDO; Kneidinger *et al.*, 2002). The synthesis of LPS critically requires KDO, which links the core oligosaccharide to the lipid A moiety. It has been shown that disruption of the biosynthesis of KDO causes the accumulation of lipid A precursors and subsequent interruption of cell growth (Wu & Woodard, 2003). Thus, the enzymes involved in the biosynthetic pathway of KDO may be an alternative target for the development of new antibiotics or antibiotic adjuvants.

KDO 8-phosphate phosphatase (YrbI), the third enzyme in the pathway for the biosynthesis of KDO, hydrolyzes KDO 8-phosphate to KDO and inorganic phosphate (Wu & Woodard, 2003). YrbI belongs to the haloacid dehalogenase (HAD) superfamily, which is a large family of magnesium-dependent phosphatase/phosphotransferase enzymes. The members of the HAD superfamily share three conserved sequence motifs (Koonin & Tatusov, 1994) and a mixed α/β Rossmann fold (Morais *et al.*, 2000). Motif I contains a DXDX(T/V) sequence, and the first aspartate of the sequence forms a phosphoaspartate intermediate with the substrate.

Motif II, a conserved serine or threonine residue, is responsible for the binding of the substrate phosphate. Motif III, a (G/S)(D/S)XXX(D/N) sequence, also contains elements of the active site that bind the metal ligands and hold the substrate (Koonin & Tatusov, 1994). Although the enzymes belonging to the HAD superfamily have various biological functions, only a few HAD enzymes have been well characterized in bacteria, *e.g.* *Escherichia coli* (Kuznetsova *et al.*, 2006), and eukaryotes, *e.g.* *Homo sapiens* (Seifried *et al.*, 2013).

The Gram-negative soil saprophyte *Burkholderia pseudomallei* is notorious for its pathogenicity and causes melioidosis (Wiersinga *et al.*, 2006). It is also classified as a potential bioterrorism agent because of its rareness in Western countries, its high accessibility, its environmental persistence, the high prevalence of severe sepsis and septic shock mortality and the fact that no vaccine is available (Gilad *et al.*, 2007). Therefore, the development of antibiotics against melioidosis is essential to cope with a potentially disastrous situation. Since biosynthesis of KDO is essential for synthesis of LPS, YrbI of *B. pseudomallei* (*BpYrbI*) could be a good target for the development of antibiotics against melioidosis. In order to understand the structure–function relationship of *BpYrbI*, its crystallographic study has been performed. Here, we report the cloning, overexpression, purification, crystallization and preliminary X-ray study of this enzyme.

2. Experimental methods

2.1. Cloning of YrbI from *B. pseudomallei*

The cloning primers (Genotech, Daejeon, Republic of Korea) prepared for ligation-independent cloning (LIC) were 5'-GGCGGTGGTGGCGGCATGTCCGCGCCCCCTGC-3' for the forward strand and 5'-GTTCTTCTCCTTTGCGCCCTAGGCTCCGCAGGCGGCC-3' for the reverse strand. The *yrbI* gene was amplified by PCR using 200 ng *B. pseudomallei* genomic DNA template and 50 µM 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 pET-21a 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 apart from 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 DH5α competent cells to obtain fusion clones. Clones were screened by plasmid DNA analysis and transformed into *E. coli* BL21 (DE3) for protein expression (Kim *et al.*, 1998).

2.2. Growth of cultures

E. coli BL21 (DE3) cells transformed with the cloned vector harbouring the *yrbI* gene were grown on Luria–Bertani (LB)

agar plates containing 150 µg ml⁻¹ ampicillin. Several colonies were picked and grown in capped test tubes with 10 ml LB broth containing 150 µg 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, expression of *BpYrbI* was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The culture was further grown at 298 K for 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 (4.09 g) was resuspended in 25 ml buffer consisting of 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg ml⁻¹ DNase I. The cell suspension was disrupted using a Digital Sonifier 450 (Branson Ultrasonics, Danbury, Connecticut, USA). Cell debris was pelleted by centrifugation at 24 900g (15 000 rev min⁻¹) for 1 h in a high-speed refrigerated ultracentrifuge 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. *BpYrbI* was further purified by ion-exchange chromatography using a 5 ml HiTrap 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 0.54 M NaCl using a linear NaCl gradient. SDS–PAGE showed one band around 20 kDa corresponding to the molecular weight of *BpYrbI* with the tag. The purified protein contained a non-cleavable N-terminal His₆ tag followed by five glycine residues (MHHHHHHGGGGG) and was concentrated to 5.3 mg ml⁻¹ (using an extinction coefficient of 0.712 M⁻¹ cm⁻¹ at 280 nm) for crystallization in a buffer consisting of 0.54 M NaCl, 20 mM Tris–HCl pH 8.0.

2.4. Dynamic light-scattering experiment

Dynamic light scattering (DLS) was used to determine the hydrodynamic radius of native *BpYrbI*. It was performed using a DynaPro Titan instrument (Wyatt, Santa Barbara, California, USA). A protein concentration of 1.0 mg ml⁻¹ was used. A micro cuvette with a volume of 12 µl and a path length of 0.15 cm was used for the measurements. The purified *BpYrbI* showed a single monodisperse peak corresponding to the approximate size of tetrameric *BpYrbI*, indicating homogeneity of the protein.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Beamline 7A, PLS
X-ray wavelength (Å)	0.97933
Temperature (K)	100
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 63.7, b = 97.5, c = 98.0$
Volume fraction of solvent (%)	35.0
V_M (Å ³ Da ⁻¹)	1.89
Resolution range (Å)	50.0–2.25 (2.31–2.25)
Multiplicity	10.1 (5.9)
Unique reflections	27107 (1188)
R_{merge} † (%)	6.3 (16.1)
Data completeness (%)	90.1 (56.4)
Average $I/\sigma(I)$	25.5 (7.2)

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, 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 .

Figure 1

Coomassie-stained SDS-PAGE (12%) of *BpYrbI*. Lane 1, native *BpYrbI* (1.5 mg); lane 2, flowthrough; lane 3, *BpYrbI* under reducing conditions (1.5 mg); lane M, molecular-mass marker (labelled in kDa).

Figure 2

Crystals of *BpYrbI*. These crystals appeared in the condition 23% PEG 3350, 0.1 M Tris pH 8.5. The crystals grew to approximate dimensions of $0.12 \times 0.10 \times 0.04$ mm in 5 d.

2.5. Crystallization of *BpYrbI*

Screening for crystallization conditions was performed at room temperature 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 in a 96-well Intelli-Plate (Art Robbins Instruments, Salt Lake City, Utah, USA). Sitting drops were made by mixing 0.2 µl protein solution (5.3 mg ml⁻¹) with 0.2 µl reservoir solution and were equilibrated against 50 µl 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 µl protein solution and 0.8 µl reservoir solution consisting of 23% (w/v) PEG 3350, 0.1 M Tris pH 8.5 and equilibrated against 200 µl reservoir solution.

2.6. Data collection and reduction

Before being flash-cooled in liquid nitrogen, crystals were soaked in the reservoir solution with an additional 15% (w/v) PEG 3350 as a cryoprotectant. X-ray diffraction data were collected at a single wavelength on beamline 7A at Pohang Light Source (PLS) using an ADSC Quantum 4 CCD detector (Area Detector Systems Corporation, Poway, California, USA) placed 300 mm from the sample. X-ray data were collected at a single wavelength (0.97933 Å) from one crystal. The oscillation range per image was 0.5°, with 1 s exposures. 240 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 *BpYrbI* was 26.3 mg per litre of *E. coli* culture. After anion-exchange chromatography, *BpYrbI* appeared to be approximately 99% pure, with a prominent protein band at around 20 kDa on SDS-PAGE (Fig. 1). Diffraction-quality crystals were obtained using a reservoir solution consisting of 23% (w/v) PEG 3350, 0.1 M Tris pH 8.5. The crystals grew to dimensions of $0.12 \times 0.10 \times 0.04$ mm within 5 d at 296 K (Fig. 2). Synchrotron data were collected to 2.25 Å resolution. The crystal belonged to the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 63.7, b = 97.5, c = 98.0$ Å. Based on the Matthews coefficient (Matthews, 1968), the asymmetric unit could contain four *BpYrbI* protomers with a solvent content of 35.0%. The details of the data-collection statistics are presented in Table 1. Molecular replacement (MR) was performed using *PHENIX* (Adams *et al.*, 2010), with the tetrameric form of the X-ray crystal structure of the probable YrbI family phosphatase from *Pseudomonas syringae* pv. *phaseolica* 1448a (*PsyYrbI*; PDB entry 3mn1), which has a sequence identity of 44.2% to *BpYrbI*, as a search model (Daughtry *et al.*, 2013). Unfortunately, it failed to give an MR solution. Therefore, homology

models of *BpYrbI* were built using *MODELLER* 9.11 (Eswar *et al.*, 2006). A molecular-replacement solution was found using the top model. A relatively good electron-density map was obtained after automated model building and refinement with the *PHENIX AutoBuild* wizard (Terwilliger *et al.*, 2008). The electron-density map clearly showed that there are four protomers of *BpYrbI* in the asymmetric unit and that they form a tetramer with a similar pattern to that of *PsyYrbI*. The presence of a tetrameric form with an approximate size of 90 kDa was confirmed by the DLS experiment. The square-planar shape of tetrameric *BpYrbI* instead of a globular shape in the electron-density map explains the slightly larger size of ~90 kDa instead of the expected ~80 kDa in solution. The model has an *R* factor of 17.3% and an *R*_{free} of 22.5%. Full structure determination is in progress.

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