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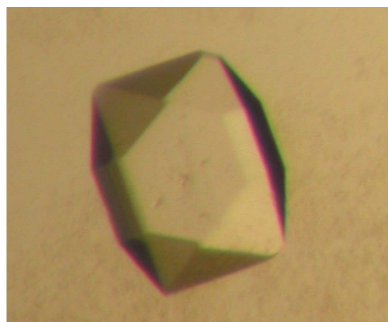

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Expression, purification, crystallization and preliminary crystallographic analysis of the phosphoglycerate kinase from *Acinetobacter baumannii*

Acinetobacter baumannii is a common multidrug-resistant clinical pathogen that is often found in hospitals. The *A. baumannii* phosphoglycerate kinase (*Ab*PGK) is involved in the key energy-producing pathway of glycolysis and presents a potential target for antibiotic development. *Ab*PGK has been expressed and purified; it was crystallized using lithium sulfate as the precipitant. The *Ab*PGK crystals belonged to space group $P222_1$. They diffracted to a resolution of 2.5 Å using synchrotron radiation at the Canadian Light Source.

1. Introduction

Acinetobacter baumannii infections are increasingly prevalent and pose not only a troublesome clinical risk but also a serious risk to long-term care facilities (Peleg *et al.*, 2008; Sengstock *et al.*, 2010). These Gram-negative aerobic bacteria have been implicated in ventilator-associated pneumonia, bloodstream infections, urinary-tract infections, meningitis and wound infections, contributing to prolonged intensive-care unit stays and increased mortality in patients (Boucher *et al.*, 2009; Peleg *et al.*, 2008; Lockhart *et al.*, 2007). The ability of *A. baumannii* to form biofilms and resist removal from an environment adds to the difficulty in its eradication (Vila & Pachón, 2011). Strains of *A. baumannii* now exist that are resistant to all known antibiotics, and the ability of *A. baumannii* to readily incorporate genetic elements from other bacteria suggests that resistance to new antibiotics will be easily acquired by this pathogen (Peleg *et al.*, 2008; Vila & Pachón, 2011). It is evident that new methods and approaches are urgently required to eradicate this debilitating pathogen (Peleg *et al.*, 2008; Boucher *et al.*, 2009; Vila & Pachón, 2011).

Phosphoglycerate kinase (PGK; EC 2.7.2.3) catalyzes an ATP-producing step in glycolysis by transferring a phosphate group from 1,3-diphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate (Banks *et al.*, 1979; Watson *et al.*, 1982; Roychowdhury *et al.*, 2011). There are a large number of phosphoglycerate kinase structures in the Protein Data Bank (PDB), but only two structures from pathogenic bacteria have been reported. Owing to its essential role in glycolysis, inhibition of PGK could possibly decrease the viability and growth of this organism (Verlinde *et al.*, 2001).

This paper describes the expression, purification and crystallization of the phosphoglycerate kinase from *A. baumannii* (*Ab*PGK) for structural determination. The structure of *Ab*PGK will provide valuable information on a potential target for antibiotic development.

2. Materials and methods

The *A. baumannii* PGK gene (gi:260555211) from strain ATCC19606 was amplified using the polymerase chain reaction (PCR). The sequences of the PCR primers (Integrated DNA Technologies) used were *Ab*PGKf, GGG ACA AGT TTG TAC AAA AAA GCA GGC TCC GAA AAC CTG TAT TTT CAG GGT GGT TCC GGT ATG GTA TTT TCA CAT TTA GGT CGT CC, and *Ab*PGKr, GGG ACC ACT TTG TAC AAG AAA GCT GGG TCT TAA GCA CGT TCA AGT AAA ACG GC. The primers contained ends that allowed the

incorporation of the *Ab*PGK coding sequence into Gateway cloning vectors (Invitrogen). The length of the PCR product was 1288 base pairs.

The PCR product was gel-purified and inserted into the plasmid pDONR221 (Invitrogen) by homologous recombination. This coding region was then cloned into the expression vector pET-57 DEST (EMD4 Biosciences Canada). This vector encodes amino-terminal hexahistidine and NusA tags to facilitate target-protein solubilization and purification.

The expression plasmid was used to transform *Escherichia coli* expression strain BL2-AI (arabinose-inducible). A single colony was picked and used to inoculate 50 ml 2× YT medium (Sambrook *et al.*, 1989) containing 0.2%(*w*/*v*) glucose and 1 mg ml^{-1} ampicillin (Sigma), which was grown on a shaker at 250 rev min^{-1} and 310 K for 16 h. About 5 ml of this inoculum was transferred into 1 l of 2× YT medium plus 0.2%(*w*/*v*) glucose and 2 mg ml^{-1} ampicillin and incubated at 310 K in a shaker until an $OD_{600\,nm}$ of 0.6 was obtained. At this point, 0.2%(*w*/*v*) arabinose was added and the temperature was lowered to 301 K for 16 h.

The bacterial culture was harvested by centrifugation at 11 000*g* for 15 min and resuspended in 50 ml lysis buffer consisting of 50 m*M* HEPES pH 7.5, 500 m*M* NaCl, 5%(*v*/*v*) glycerol, 70 m*M* imidazole, 0.1 *M* benzamidine (Sigma), 200 µ*M* PMSF (ICN Biomedical), one Complete protease-inhibitor tablet (Roche) and 0.4 mg ml^{-1} lysozyme (Sigma). The bacterial resuspension was stored at 193 K.

On the day of purification, the bacterial pellets were thawed and then ultrasonicated on ice. Ultrasonication was performed in three rounds of 10 s at 90% with 30 s between each sonication round. The resulting lysate was cleared by centrifugation at 30 000*g* for 45 min at 277 K. The clarified supernatant was loaded onto a 5 ml HisTrap FF column (GE Healthcare) pre-equilibrated with buffer *A* [50 m*M* HEPES pH 7.5, 500 m*M* NaCl, 5%(*v*/*v*) glycerol, 70 m*M* imidazole] on an ÄKTAexplorer FPLC (Amersham Pharmacia Biotech). The *Ab*PGK fusion protein was eluted with an increasing gradient of buffer *B* [50 m*M* HEPES pH 7.5, 500 m*M* NaCl, 5%(*v*/*v*) glycerol, 500 m*M* imidazole]. The amino-terminal tag composed of His_6 and NusA was proteolytically cleaved from the fusion protein using recombinant tobacco etch virus protease (rTEV; 1:100 rTEV:protein ratio; Fig. 1*a*). After incubation at 298 K for 16 h in dialysis buffer to remove imidazole, complete proteolysis yielded native *Ab*PGK with an extra glycine residue at the amino-terminus. The dialysis buffer consisted of 4 l 500 m*M* NaCl, 50 m*M* HEPES pH 7.5, 5% glycerol, 1 m*M* TCEP. The digested protein was loaded onto two 5 ml HisTrap FF columns in tandem pre-equilibrated with buffer *A* and the flow-through was collected and dialysed for 16 h at 277 K to remove imidazole and glycerol. The dialysis buffer consisted of 4 l 5 m*M* HEPES pH 7.5, 200 m*M* NaCl. The flowthrough was spin-concentrated to ~10 mg ml^{-1} and a final volume of ~500 µl using Amicon Ultra concentrators with a 10K molecular-weight cutoff (Millipore). The protein purity was tested on a 15% SDS–PAGE gel (Fig. 1*b*).

Crystallization conditions were initially screened using the sitting-drop vapour-diffusion technique employing a Hydra-Plus-One crystallization robot (Robbins Scientific) in a 96-well format with the 96 conditions of Index Screen and the first 48 conditions of both Crystal Screen and Crystal Screen 2 (Hampton Research). The volume of the reservoir-solution droplet was 0.3 µl and the volume of the protein-containing droplet was 0.3 µl; the total reservoir volume was 80 µl.

The condition that seemed most promising, based on the presence of microcrystals of protein, was verified by SDS–PAGE. This condition was optimized using a manual setup of the hanging-drop method with 500 µl reservoir solution in the well. The drop was composed of 1.6 µl *Ab*PGK solution and 0.8 µl reservoir solution containing 3.3 m*M* AMPPNP, an ATP analogue. The final drop volume was 2.4 µl. Crystals were grown at 298 K for 3 d (Fig. 2). The mother liquor consisted of 0.2 *M* potassium sodium tartrate, 0.1 *M* sodium citrate pH 6.0, 1.8 *M* lithium sulfate.

Table 1
Crystallographic statistics of *Ab*PGK data collected at the Canadian Light Source.

Values in parentheses are for the highest resolution shell.

Space group	$P222_1$
Unit-cell parameters (Å)	*a* = 73.73, *b* = 177.85, *c* = 237.49
Matthews coefficient (Å^3 Da^{-1})	2.36
Solvent content (%)	47.85
Data collection	
Temperature (K)	100
Detector	MAR 325 CCD
Wavelength (Å)	0.9795
Resolution (Å)	20.00–2.50 (2.59–2.50)
Unique reflections	99669
Multiplicity	3.9 (3.5)
$\langle I/\sigma(I)\rangle$	20.99 (1.72)
Completeness (%)	93.4 (89.7)
R_{merge}† (%)	8.4 (81.6)

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

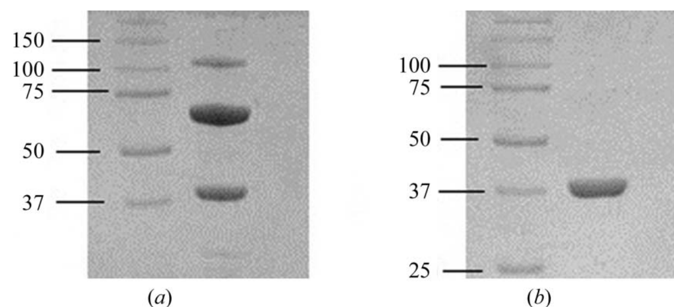

Figure 1
Purification of *Ab*PGK as monitored by SDS–PAGE. (*a*) Sample during rTEV cleavage. The bands correspond to the full construct and the cleavage products NusA and *Ab*PGK. (*b*) Purified sample of *Ab*PGK. Molecular-weight markers are labelled in kDa.

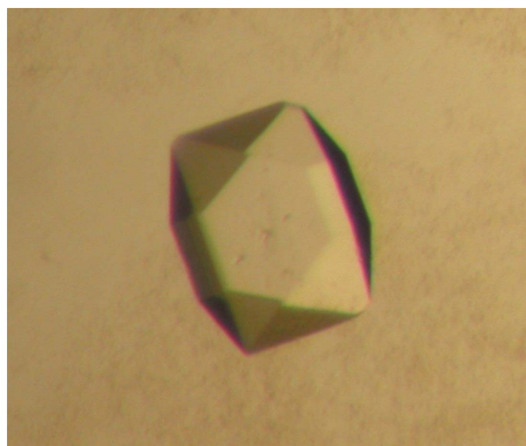

Figure 2
Morphology of the *Ab*PGK crystals. The crystals were grown in 0.2 *M* potassium sodium tartrate, 0.1 *M* sodium citrate pH 6.0, 1.8 *M* lithium sulfate for 3 d. The crystals were approximately 400 × 400 × 400 µm in size.

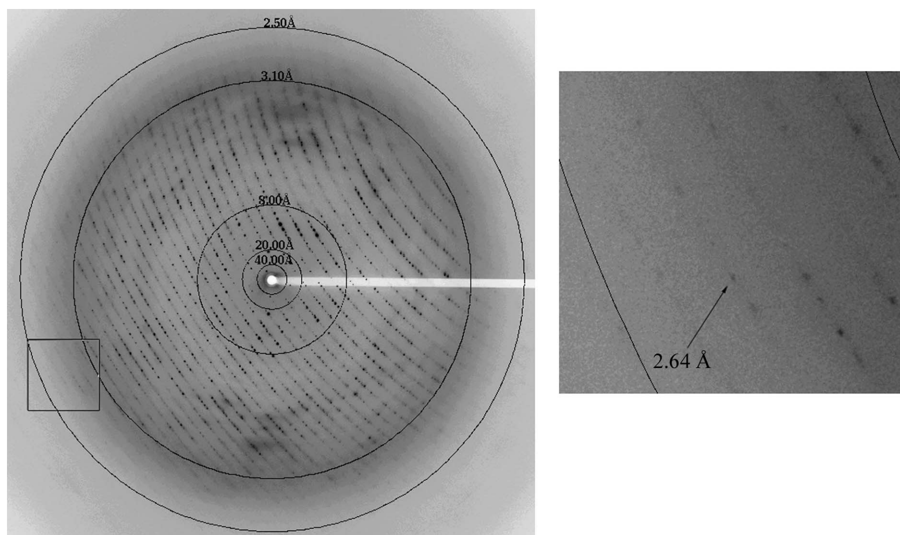

Figure 3
A sample frame from the diffraction data set of an *Ab*PGK crystal obtained at the Canadian Light Source. Resolution values are indicated.

The crystals were briefly cryoprotected in 2 *M* lithium sulfate and mother liquor in a 3:7 ratio by transferring the crystal into a drop of cryoprotectant for approximately 5 s prior to flash-cooling in liquid nitrogen. X-ray diffraction data were collected on both a Rigaku R-AXIS IV^{++} rotating-anode X-ray generator and at the Canadian Light Source in Saskatoon, Canada (beamline CMCF1). The data were indexed, integrated and scaled (Table 1, Fig. 3) using the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results

We have expressed, purified and crystallized the phosphoglycerate kinase from *A. baumannii* and collected data both at a home source and on a synchrotron beamline. The crystals belonged to space group $P222_1$ and diffracted to a maximum resolution of 2.5 Å at the synchrotron. The unit-cell parameters are a = 73.73, b = 177.85, c = 237.49 Å. Using the molecular mass of *Ab*PGK of 41 260 Da, we calculated that there may be as many as eight *Ab*PGK molecules per asymmetric unit, giving a calculated Matthews coefficient of 2.36 $Å^3\ Da^{-1}$ and a solvent content of 47.85% (Table 1). This molecular mass does not account for the extra glycine added at the amino-terminus during rTEV cleavage. The structural solution of *Ab*PGK is currently under way and will be determined *via* molecular replacement. Our initial attempts at solving the phase problem will use the structure of *E. coli* PGK (PDB entry 1zmr; Young *et al.*, 2007) as the search model.

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