Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Amlan Roychowdhury, Somnath Mukherjee and Amit Kumar Das*

Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur 721 302, India

Correspondence e-mail: amitk@hijli.iitkgp.ernet.in

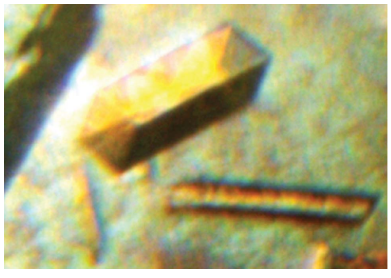
Received 2 January 2011
Accepted 26 February 2011

Expression, purification, crystallization and preliminary X-ray diffraction studies of phosphoglycerate kinase from methicillin-resistant *Staphylococcus aureus* MRSA252

Phosphoglycerate kinase (PGK) from methicillin-resistant *Staphylococcus aureus* MRSA252 has been cloned in pQE30 expression vector, overexpressed in *Escherichia coli* SG13009 (pREP4) cells and purified to homogeneity. The protein was crystallized from 0.15 *M* $CaCl_2$, 0.1 *M* HEPES–NaOH pH 6.8, 20%(*w*/*v*) polyethylene glycol 2000 at 298 K by the hanging-drop vapour-diffusion method. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 45.14$, $b = 74.75$, $c = 58.67$ Å, $\beta = 95.72^\circ$. X-ray diffraction data have been collected and processed to a maximum resolution of 2.3 Å. The presence of one molecule in the asymmetric unit gives a Matthews coefficient (V_M) of 2.26 $Å^3$ Da^{-1} with a solvent content of 46%. The structure has been solved by molecular replacement and structure refinement is now in progress.

1. Introduction

The conversion of glucose to pyruvate in the glycolytic pathway results in the generation of ATP, which meets the primary energy demands of the cell. Amongst many other glycolytic enzymes, the ubiquitous enzyme phosphoglycerate kinase (PGK; EC 2.7.2.3) catalyzes the transfer of a phosphoryl group from 1,3-bisphosphoglycerate (1,3-BPG) to ADP and thus plays a crucial role in energy metabolism. This bilobal enzyme generates ATP by substrate-level phosphorylation together with 3-phosphoglycerate (3-PG) as a product. Like all other kinases, it also creates an internal water-free environment for successful phosphoryl transfer. The enzyme usually consists of two domains: the N-domain, which binds the substrate, and the C-domain, which binds the nucleotide. The relative distance and orientation of these two domains as prerequisites for efficient catalysis has been the subject of intensive investigation and it has been proposed that the two domains are connected by a narrow hinge, the bending movement of which is solely responsible for the open and closed conformations (Banks *et al.*, 1979; Blake & Rice, 1981). There is also evidence of a novel mechanism in which a synergistic combination of substrate-induced effects produces major conformational changes in the enzyme (Bernstein *et al.*, 1997). Some of the structures of PGK solved to date by X-ray crystallography and solution small-angle X-ray scattering have provided new insights into the role of the nucleotide in domain closure (Szilágyi *et al.*, 2001), the hydrogen-bonding network (Varga *et al.*, 2006) and the different interdomain interactions, together with the residues involved in the stabilization of the transition state of the phosphoryl group (Auerbach *et al.*, 1997). Comparison of the enzyme structure in the open and closed conformations between different species reveals that conformational change reflects a change in the interactions between the different domains (Lee *et al.*, 2006). The importance of the balancing of the charges within the transition state which dominates the enzyme-catalyzed phosphoryl transfer has also been pointed out recently (Cliff *et al.*, 2010). Despite extensive elaboration and continuing investigation, the mechanism of conformational change varies between species and no unique or absolute mechanism has yet been established. This definitely provides an ample reason to continue the structural investigation of this important glycolytic enzyme in order to delineate the hinge-closure mechanism.



Staphylococcus aureus is one of the most dreaded opportunistic nosocomial human pathogens and is responsible for ailments from minor skin infections to life-threatening diseases such as meningitis, pneumonia, osteomyelitis, endocarditis, septic arthritis, toxic shock syndrome and septicaemia (Archer, 1998). The resistance of this bacterium to antibiotics such as methicillin and vancomycin has further added to its already growing menace. The methicillin-resistant *S. aureus* MRSA252 possesses a single phosphoglycerate kinase (SaPGK; SAR0829) comprised of 396 amino acids. Little is known about the importance of this protein in this deadly coccus, but as the glycolytic enzymes are responsible for the production of ATP, a steady level of which is required for sustenance of the pathogen in the host, this enzyme may be a suitable target for inhibitor design against the glycolytic pathway. Moreover, it has been shown that during biofilm formation the upregulation of glycolytic enzymes is stimulated by factors other than oxygen limitation in the cell which may reflect their putative role in complex microbial population and reduced antimicrobial susceptibility (Becker *et al.*, 2001). It is also evident that glycolytic enzymes interact among themselves to synchronize processivity and regulation in eukaryotic cells (Weber & Bernhard, 1982). There is strong evidence of a complex between aldolase and fructose 1,6-bisphosphatase (MacGregor *et al.*, 1980), while interaction between PGK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *in vitro* has also been studied biochemically (Weber & Bernhard, 1982). No such interaction has been reported in prokaryotes to date. Structures of GAPDH (Mukherjee *et al.*, 2010) and triosephosphate isomerase (TIM; Mukherjee *et al.*, 2009) from MRSA252 have already been solved. Therefore, structural analysis of SaPGK and its complexes will certainly aid in elucidating its mode of interaction. Hence, we have focused our attention on structural and mechanistic studies of this important enzyme and the present work reports the cloning, overexpression, purification, crystallization and preliminary X-ray diffraction analysis of SaPGK from *S. aureus* MRSA252.

2. Materials and methods

2.1. Cloning

The sequences corresponding to the open reading frame of SaPGK were amplified by PCR from *S. aureus* MRSA252 genomic DNA as the template using the primer pair 5′-CGGGATCCATGGCTAAAAAAATTGTTTCTG-3′ (forward primer with a *Bam*HI recognition site) and 5′-GCGGGGTACCTTATTTATTATTGATTGCTTTG-3′ (reverse primer with a *Kpn*I recognition site). The purified PCR product was subsequently cloned into the *Bam*HI and *Kpn*I sites of the expression vector pQE30 (Qiagen, USA) which contains six consecutive histidines to the immediate upstream of the multiple cloning sites. The recombinant DNA was then transformed into chemically competent *Escherichia coli* SG13009 (pREP4) cells and was subsequently selected on ampicillin/kanamycin plates. The positive clones were verified by DNA sequencing.

2.2. Overexpression and purification

The positive clone harbouring the desired construct of SaPGK was grown in LB medium supplemented with 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} kanamycin at 310 K for 3 h, during which the A_{600} reached 0.6, induced with 100 µ*M* IPTG and grown for a further 16 h at 288 K to maximize the overexpression of the recombinant protein in the cytosolic fraction. The harvested cell mass was resuspended in buffer *A* (10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 10 m*M* imidazole) containing 0.1 m*M* each of leupeptin, pepstatin and aprotinin, and 0.02 m*M* phenylmethylsulfonyl fluoride (PMSF). The suspension was lysed by ultrasonication on ice and the lysate was centrifuged at 22 000*g* for 40 min. The supernatant was loaded onto Ni-Sepharose High Performance affinity matrix (GE Healthcare Biosciences) pre-equilibrated with buffer *A*. The column was then washed extensively with buffer *A* to remove bound contaminants. Recombinant His_6-tagged SaPGK was finally eluted with buffer *B* (10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 50 m*M* imidazole). The eluted protein was dialyzed against buffer *C* (50 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl and 2 m*M* DTT). The dialyzed protein was subjected to anion-exchange chromatography to enhance the level of purity. It was loaded onto a C 16/20 column packed with Q-Sepharose Fast Flow matrix (GE Healthcare Biosciences) pre-equilibrated with buffer *C*. The column was washed with buffer *C* and the bound protein was finally eluted using a linear gradient of 0.05–1 *M* NaCl in buffer *C*. The obtained protein was concentrated using a Vivaspin 20 concentrator (10 kDa molecular-weight cutoff, GE Healthcare Biosciences) and subjected to size-exclusion chromatography using Superdex 75 prep-grade matrix in a C 16/70 column (GE Healthcare Biosciences) on an ÄKTAprime plus system (GE Healthcare Biosciences) equilibrated with buffer *D* (10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl and 2 m*M* DTT). 2 ml fractions were collected at a flow rate of 1 ml min^{-1}. The protein was only obtained in monomeric form and the fractions containing the desired protein were pooled together. The protein concentration was estimated by the method of Bradford (1976) and its purity was verified by 12% SDS–PAGE.

2.3. Crystallization

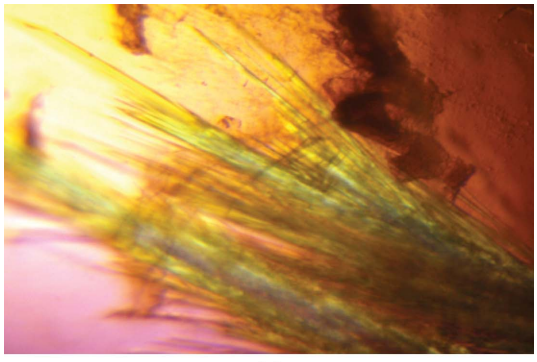
The purified protein was concentrated to 36 mg ml^{-1} using a Vivaspin 20 concentrator (10 kDa molecular-weight cutoff, GE Healthcare Biosciences). Droplets of 2 µl protein solution in buffer *D* were mixed with an equal volume of reservoir solution and equilibrated against 100 µl of the latter using commercially available sparse-matrix screens from Hampton Research (Crystal Screen and Crystal Screen 2) at 298 K. Clusters of crystals were obtained from 0.2 *M* $CaCl_2$, 0.1 *M* HEPES–NaOH pH 7.5, 28%(*v*/*v*) polyethylene glycol 400 (condition No. 14 of Crystal Screen) at 298 K. Varying the pH, ionic strength and precipitant concentration, a fine screening around these conditions was performed using the hanging-drop vapour-diffusion method in 24-well Linbro plates. Single crystals of larger dimensions and better morphology were obtained from 0.15 *M* $CaCl_2$, 0.1 *M* HEPES–NaOH pH 6.8, 20%(*w*/*v*) polyethylene glycol 2000 overnight at 298 K.

2.4. Data collection

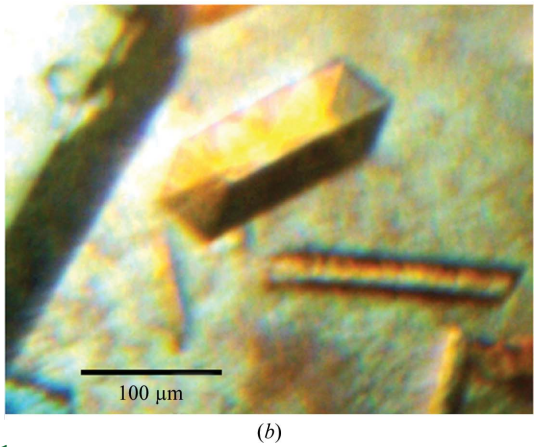
Diffraction data were collected at our home source equipped with a Rigaku R-AXIS IV^{++} detector using Cu *K*α (λ = 1.5418 Å) X-rays generated by a Rigaku Micromax-007 HF microfocus rotating-anode X-ray generator operated at 40 kV and 30 mA (Rigaku Americas Corporation). Single crystals were cryoprotected using 10%(*v*/*v*) glycerol in reservoir solution and were flash-cooled in a liquid-nitrogen stream at 100 K using a Rigaku X-stream 2000 cryosystem. The crystals diffracted to a maximum resolution of 2.3 Å. Diffraction data from a single cryoprotected crystal were collected over a range of 180° with an oscillation angle of 0.5°. A total of 360 frames were collected with an exposure time of 2 min per frame and a crystal-to-detector distance of 148 mm. Diffraction data were processed with *XDS* (Kabsch, 1993) and scaled in *SCALA* (Evans, 2006). The presence of the screw axis was also confirmed by *POINTLESS* (Evans, 2006) from the *CCP*4 program suite (Winn *et al.*, 2011).

3. Results and discussion

PGK from *S. aureus* MRSA252 was successfully cloned in pQE30 expression vector, overexpressed in *E. coli* SG13009 (pREP4) cells, purified to homogeneity using Ni Sepharose, ion-exchange and size-exclusion chromatography and crystallized. The initial crystals of His_6-PGK obtained from the Crystal Screen solution were clustered and of low quality (Fig. 1*a*) and thus were not suitable for diffraction. However, the single crystals obtained after optimization of the initial crystallization conditions were of much improved morphology and measured 0.15 × 0.045 × 0.045 mm (Fig. 1*b*), making them suitable for diffraction experiments. Diffraction data were collected using a cryoprotected single crystal that diffracted to a maximum resolution of 2.3 Å. Analysis of the symmetry and systematic absences in the recorded diffraction patterns indicated that the crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 45.14, b = 74.75, c = 58.67 Å, β = 95.72°. Determination of the Matthews coefficient (V_M = 2.26 Å^3 Da^{-1}) suggested the presence of 46% solvent content in the unit cell, with one molecule in the asymmetric unit (Matthews, 1968). A total of 62 963 observed reflections were merged to 16 871 unique reflections in the 19.58–2.30 Å resolution range. The overall completeness of the data set was 97.6%, with an R_{merge} of 9.4%. The data-collection and processing statistics are given in Table 1. The structure was solved using the molecular-replacement method with the *MOLREP* program (Vagin & Teplyakov, 1997) within the *CCP*4 package (Winn *et al.*, 2011) using PGK from *Bacillus stearothermophilus* (PDB entry 1php; sequence identity 52%; Davies *et al.*, 1994) as a model. A promising solution has been obtained.



(*a*)



(*b*)

Figure 1
The initial cluster of crystals obtained from 0.2 *M* $CaCl_2$, 0.1 *M* HEPES–NaOH pH 7.5, 28%(*v*/*v*) polyethylene glycol 400 (condition No. 14 of Crystal Screen, Hampton Research) using the sitting-drop vapour-diffusion method at 298 K. (*b*) Typical crystals of SaPGK grown from 0.15 *M* $CaCl_2$, 0.1 *M* HEPES–NaOH pH 6.8, 20%(*w*/*v*) polyethylene glycol 2000 at 298 K measured 0.15 × 0.045 × 0.045 mm.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Space group	$P2_1$
Unit-cell parameters	
a (Å)	45.14
b (Å)	74.75
c (Å)	58.67
β (°)	95.72
Unit-cell volume (Å^3)	196944
Matthews coefficient (Å^3 Da^{-1})	2.26
Solvent content (%)	45.6
No. of molecules in asymmetric unit	1
Resolution range (Å)	19.58–2.30 (2.42–2.30)
Observed reflections	62963
Unique reflections	16871
Multiplicity	3.7 (3.6)
Completeness (%)	97.6 (95.9)
R_{merge}† (%)	9.4 (23.5)
Average $I/\sigma(I)$	12.0 (5.2)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of the ith reflection and $\langle I(hkl)\rangle$ is the mean value for all equivalent measurement of reflection hkl.

Model building and refinement are currently in progress. Cocrystallization of SaPGK with nonhydrolysable substrate analogues is in progress to delineate the possible mode of interaction and the mechanism of the hinge-bend motion.

This work was carried out with financial assistance from the Department of Biotechnology, Government of India. ARC and SM thank the University Grants Commission and the Council of Scientific and Industrial Research, Government of India, respectively, for individual fellowships. Genomic DNA of MRSA252 was a kind gift from Professor H. G. Wiker, University of Bergen. The authors gratefully acknowledge the kind help of Mr Debajyoti Dutta.

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