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Expression, purification, crystallization and preliminary X-ray diffraction studies of glyceraldehyde-3-phosphate dehydrogenase 1 from methicillin-resistant *Staphylococcus aureus* (MRSA252)

Somnath Mukherjee, Debajyoti Dutta, Baisakhee Saha and Amit Kumar Das*

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

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

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Glyceraldehyde-3-phosphate dehydrogenase 1 from methicillin-resistant *Staphylococcus aureus* (MRSA252) was cloned in pQE30 vector, overexpressed in *Escherichia coli* M15(pREP4) cells and purified to homogeneity. The protein was crystallized using the hanging-drop vapour-diffusion method. The crystals belonged to space group $P2_1$, with unit-cell parameters a = 65.23, b = 95.58, c = 87.91 Å, β = 106.5°. X-ray diffraction data were collected and processed to a maximum resolution of 2.0 Å. The presence of one tetramer in the asymmetric unit gave a Matthews coefficient (V_M) of 1.78 Å^3 Da^{-1} and a solvent content of 31%. The structure was solved by molecular replacement and structure refinement is now in progress.

1. Introduction

The glycolytic pathway is the primary ATP-synthesizing pathway of the cell. The enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the sixth enzyme of the glycolytic pathway. It acts on glyceraldehyde 3-phosphate (G3P) to convert it to 1,3-bisphosphoglycerate (1,3-BPG) and consumes inorganic phosphate to harness the energy into nicotinamide adenine dinucleotide (reduced) (NADH). The reaction mechanism has been intensively investigated, in particular for bacterial and eukaryotic GAPDHs (Segal & Boyer, 1953; Trentham, 1971; Harris & Waters, 1976; Soukri *et al.*, 1989; Michels *et al.*, 1996; Boschi-Muller & Branlant, 1999; Moras *et al.*, 1975), and consists of two steps: an oxidoreduction reaction followed by phosphorylation of the thioester. Initially, the aldehyde group of G3P undergoes a nucleophilic attack by the catalytic cysteine to form a thiohemiacetal intermediate. This is followed by a hydride transfer from the generated thiohemiacetal to C4 of the nicotinamide moiety of nicotinamide adenine dinucleotide (NAD), leading to the formation of a thioacylenzyme (Talfournier *et al.*, 1998). The transfer of the hydride ion is assisted by a nearby histidine, which plays the role of a general base catalyst. The second step involves the phosphorylation of the resulting thioester by the nucleophilic attack of inorganic phosphate on the carbonyl group of the thioacylenzyme. This second step is preceded by the exchange of NADH for NAD, with the latter favouring the phosphorolysis step. Although the role of GAPDH as a housekeeping enzyme has been well investigated, recent investigations have revealed new properties of this enzyme. These include localization on the cell surface, binding to cellular molecules (Pancholi & Fischetti, 1992; Gil-Navarro *et al.*, 1997; Delgado *et al.*, 2001; Gozalbo *et al.*, 1998; Zang *et al.*, 1998; Modun & Williams, 1999; Taylor & Heinrichs, 2002) and roles in apoptosis (Sirover, 1999). This glycolytic enzyme consists of two folding domains: the cofactor (NAD) binding domain and the catalytic domain. The NAD-binding domain forms a compact substructure and has a characteristic Rossmann fold. An important feature of the catalytic domain is an irregular S-shaped loop of polypeptide residues which is involved in intersubunit interaction (Biesecker *et al.*, 1977).

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Staphylococcus aureus, one of the most common causes of nosocomial infections, is responsible for a wide range of illness from minor skin infections to life-threatening diseases such as meningitis, pneumonia, toxic shock syndrome and septicaemia. The notorious methicillin-resistant strain of *S. aureus* (MRSA252) contains two cytosolic GAPDHs: GAP1 (NCBI accession code YP_040254) and GAP2 (NCBI accession code YP_041153). In some staphylococcal species (*S. aureus* BB, *S. epidermidis* 138 and *S. saprophyticus* 907), the transferritin binding protein (Tpn) shows GAPDH activity (Taylor & Heinrichs, 2002). The two cell surface-associated proteins GapB and GapC from *S. aureus* BM4-15 found in clinical isolates of bovine mastitis with differential plasmin and ferritin-binding activities also share considerable homology to several bacterial GAPDHs (Goji *et al.*, 2004). Such an important enzyme in *S. aureus* species thus needs to be studied from structural and mechanistic aspects. Hence, the present study targets the cloning, overexpression, purification, crystallization and preliminary X-ray diffraction studies of GAP1 from MRSA252.

2. Materials and methods

2.1. Cloning

The sequences corresponding to the open reading frame of GAP1 were amplified by PCR using MRSA252 genomic DNA as the template with the primer pair 5′-CGGGATCCATGGCAGTAAAAGTAGCAATTAATGG-3′ (forward primer with a *Bam*HI recognition site) and 5′-GCGCCCCAAGCTTTTATTTAGAAAGTTCAGCTAAG-3′ (reverse primer with a *Hin*dIII recognition site). The PCR product was purified, digested with *Bam*HI and *Hin*dIII and the amplicon was ligated into the *Bam*HI/*Hin*dIII-digested pQE30 expression vector using T4 DNA ligase. The recombinant DNA was then transformed in *Escherichia coli* M15(pREP4) cells and 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 GAP1 construct was grown in Luria broth 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 5 h at 310 K to maximize the overexpression of the recombinant protein in the cytosolic fraction. The cells from 1 l culture were 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 14 000 rev min^{-1} 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* and the protein was eluted with buffer *B* (10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 300 m*M* imidazole). The eluted protein was subjected to size-exclusion chromatography using Superdex 75 prep-grade matrix in a 16/70 C column (GE Healthcare Biosciences) equilibrated with buffer *C* (10 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 2 m*M* DTT) on an ÄKTAprime Plus system (GE Healthcare Biosciences). 2 ml fractions were collected at a flow rate of 1 ml min^{-1}. The fractions containing the desired protein were pooled together. The protein concentration was estimated by the method of Bradford (1976) and the purity was verified by 12% SDS–PAGE.

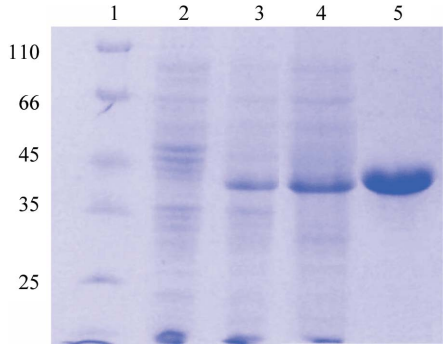


Figure 1
12% SDS–PAGE analysis. Lane 1, molecular-weight markers (kDa). Lane 2, uninduced M15 cells. Lane 3, induced M15 cells. Lane 4, supernatant. Lane 5, purified GAP1 after gel-exclusion chromatography.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.54
Space group	$P2_1$
Unit-cell parameters	
a (Å)	65.23
b (Å)	95.58
c (Å)	87.91
β (°)	106.5
Unit-cell volume (Å^3)	525520
Matthews coefficient (Å^3 Da^{-1})	1.78
Solvent content (%)	31
No. of monomers in ASU	4
Resolution range (Å)	23.54–2.0 (2.07–2.0)
Observed reflections	248982
Unique reflections	68281
Redundancy	3.65 (3.51)
Completeness (%)	97.6 (95.5)
$R_{\rm merge}$† (%)	7.5 (30.0)
Average $I/\sigma(I)$	9.4 (3.4)

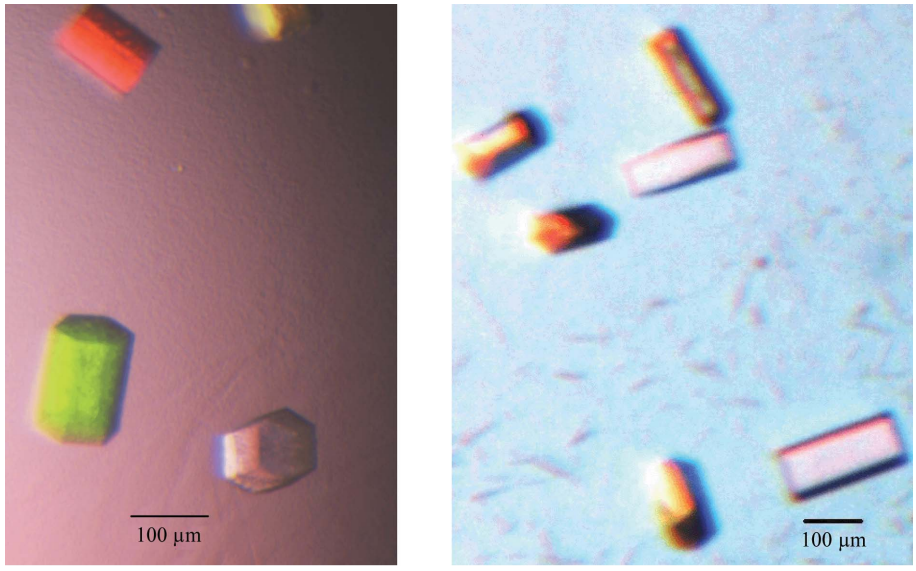
† $\sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of a reflection and $\langle I(hkl)\rangle$ is the mean intensity of reflection *hkl*.

2.3. Crystallization

The purified protein was concentrated to 35 mg ml^{-1} using Amicon Centriprep (10 kDa cutoff) and Amicon Centricon (10 kDa cutoff) concentrators. Initial crystallization trials were performed by the sitting-drop vapour-diffusion method in a 96-well Corning CrystalX microplate (Hampton Research). Droplets of 2 µl protein solution were mixed with an equal volume of mother liquor and equilibrated against 100 µl reservoir solution using commercially available sparse-matrix screens from Hampton Research (Crystal Screen and Crystal Screen II) at 298 K. Small crystals were obtained with (i) 0.2 *M* lithium sulfate, 0.1 *M* Tris–HCl pH 8.0, 30% PEG 4000 and (ii) 0.1 *M* HEPES pH 7.5, 70%(*v*/*v*) MPD. Varying the pH, ionic strength, precipitant concentration and temperature, fine screening around these conditions was performed using the hanging-drop vapour-diffusion method. Crystals appeared from 0.1 *M* HEPES pH 7.2, 42% MPD after 3 d at 298 K. Crystals were obtained from 0.1 *M* Tris–HCl pH 8.5, 20% PEG 4000 after one week at 277 K.

2.4. Data collection

The mother liquor for the crystals obtained from 0.1 *M* HEPES pH 7.2, 42% MPD could be used as a cryoprotectant owing to the high MPD content; the crystals from 0.1 *M* Tris–HCl pH 8.5, 20% PEG 4000 at 277 K were cryoprotected by dipping them into a solution of 0.1 *M* Tris–HCl pH 8.5, 20% PEG 4000, 15% glycerol. The diffraction



(*a*) (*b*)

Figure 2
Crystals of GAP1. (*a*) Crystals from 0.1 *M* HEPES pH 7.2, 42% MPD at 298 K measuring 0.15 × 0.11 × 0.04 mm. (*b*) The crystals from 0.1 *M* Tris–HCl pH 8.5, 20% PEG 4000 at 277 K grew to typical dimensions of 0.2 × 0.06 × 0.02 mm.

data were collected at our home source, which was equipped with a Rigaku R-AXIS IV^{++} detector, using Cu $K\alpha$ X-rays generated by a Rigaku Micromax HF007 Microfocus rotating-anode X-ray generator with a Varimax mirror system and operated at 40 kV and 30 mA. Crystals were flash-cooled in a liquid-nitrogen stream at 100 K using a Rigaku X-stream 2000. The crystals from 42% MPD diffracted to 15 Å. Therefore, data collection using these crystals did not proceed further. The crystals obtained from 20% PEG 4000 diffracted to a maximum resolution of 2 Å. A total of 360 frames of data were collected with an oscillation angle of 0.5°, an exposure time of 5 min per frame and a crystal-to-detector distance of 150 mm. Diffraction data were processed with *d*TREK* v.9.8 (Pflugrath, 1999).

3. Results and discussion

GAP1 from MRSA252 was successfully cloned and purified to homogeneity. The molecular weight of monomeric His$_6$-GAP1 predicted from the sequence (37.6 kDa) is also confirmed by 12% SDS–PAGE (Fig. 1). The crystals obtained from 0.1 *M* HEPES pH 7.2, 42% MPD at 298 K measured 0.15 × 0.11 × 0.04 mm (Fig. 2*a*), while those obtained from 0.1 *M* Tris–HCl pH 8.5, 20% PEG 4000 at 277 K grew to typical dimensions of 0.2 × 0.06 × 0.02 mm (Fig. 2*b*). Diffraction data were collected using a cryoprotected single crystal obtained using 0.1 *M* Tris–HCl pH 8.5, 20% PEG 4000. The crystals diffracted to a maximum resolution of 2 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 = 65.23, b = 95.58, c = 87.91 Å, β = 106.5°. Determination of the Matthews coefficient suggested a 31% solvent content in the unit cell (V_M = 1.78 Å^3 Da^{-1}) with one tetramer in the asymmetric unit (Matthews, 1968). A total of 248 982 observed reflections were merged to 68 281 unique reflections in the 23.54–2.0 Å resolution range. The overall completeness of the data set was 97.6%, with an R_{merge} of 7.5%. The data-collection and processing statistics are given in Table 1. The structure was solved using the molecular-replacement method with the program *MOLREP* (Vagin & Teplyakov, 1997) within the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Owing to a high sequence identity of 53%, a monomer of glyceraldehyde-3-phosphate from *Thermotoga maritima* (PDB code 1hdg; Korndörfer *et al.*, 1995) was used as the search model. Since the Matthews coefficient suggested the presence of a tetramer in the asymmetric unit, the number of monomers was set to four in the search parameters while running *MOLREP*. A promising solution with a homotetrameric structure was obtained (correlation coefficient of 0.53). The model was subsequently subjected to rigid-body refinement in *REFMAC*5 (Murshudov *et al.*, 1997) within the *CCP*4 package, giving an R factor of 46.6%. Final model building and restrained refinement using *REFMAC*5 are currently in progress. In parallel with refinement, we are preparing crystals of GAP1 complexed with substrate and coenzyme in order to study their mode of interaction with the enzyme.

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