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

Purification, crystallization and preliminary X-ray analysis of apo glyceraldehyde-3-phosphate dehydrogenase 1 (GAP1) from methicillin-resistant *Staphylococcus aureus* (MRSA252)

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

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

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

Received 2 January 2010
Accepted 2 March 2010

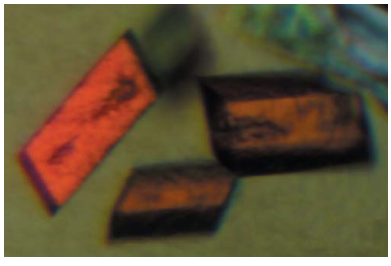
Glyceraldehyde-3-phosphate dehydrogenase 1 (GAP1) from methicillin-resistant *Staphylococcus aureus* (MRSA252) has been purified to homogeneity in the apo form. The protein was crystallized using the hanging-drop vapour-diffusion method. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 69.95$, $b = 93.68$, $c = 89.05$ Å, $\beta = 106.84^\circ$. X-ray diffraction data have been collected and processed to a maximum resolution of 2.2 Å. The presence of one tetramer in the asymmetric unit gives a Matthews coefficient (V_M) of 1.81 Å^3 Da^{-1} with a solvent content of 32%. The structure has been solved by molecular replacement and structure refinement is now in progress.

1. Introduction

Staphylococcus aureus is one of the most dreaded opportunistic nosocomial human pathogens and is responsible for many life-threatening diseases such as meningitis, pneumonia, osteomyelitis, endocarditis, septic arthritis, toxic shock syndrome and septicaemia (Archer, 1998). The resistance of this organism to antibiotics such as methicillin and vancomycin has further added to its already growing menace.

The glycolytic pathway, which is the primary ATP-synthesizing pathway of the cell, is one of the most important pathways for the survival of the microbe. Being predominant producers of ATP, the enzymes of the glycolytic pathway thus serve as potential targets for drug design against this pathway. The enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the sixth enzyme of the glycolytic pathway. It acts on glyceraldehyde 3-phosphate (G3P), converting it into 1,3-bisphosphoglycerate (1,3-BPG) and consuming inorganic phosphate, and harnesses 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. Firstly, the aldehyde group of G3P undergoes a nucleophilic attack by the catalytic cysteine to form a thiohemiacetal intermediate. The second step involves phosphorylation of the resulting thioester by nucleophilic attack of inorganic phosphate on the carbonyl group of the thioacylenzyme. The second step is preceded by the exchange of NADH for NAD, with the latter favouring the phosphorolysis step. NAD, being the cofactor, is not only crucial for the functionality of the enzyme but also plays a vital structural role. The role of coenzyme binding on cooperative interactions in tetrameric phosphorylating GAPDHs has been the subject of active research. Depending on the source of the enzyme, NAD binding to GAPDH has been shown to have either positive or negative cooperativity (Roitel *et al.*, 1999).

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).

The notorious methicillin-resistant strain of *Staphylococcus aureus* (MRSA252) contains two cytosolic GAPDHs: GAP1 (NCBI accession code YP_040254) and GAP2 (NCBI accession code YP_041153). We have previously reported the cloning, overexpression, purification, crystallization and preliminary X-ray crystallographic analyses of GAP1 bound to NAD (Mukherjee *et al.*, 2008). The structure was solved by molecular replacement and structural analysis showed that the cofactor binds in an extensive manner to the classical Rossmann fold and to part of the large S-shaped loop that is responsible for maintaining intersubunit contacts (S. Mukherjee, D. Dutta, B. Saha & A. K. Das, unpublished work). It is expected that there will be a significant structural difference between the apo (without NAD) and the holo (NAD-bound) forms of the enzyme. A comparison between these two forms will highlight interesting conformational changes induced by the cofactor that can be functionally correlated to its mechanism of catalysis and thus need to be investigated. Hence, the present study is targeted at the purification, crystallization and preliminary X-ray diffraction analyses of GAP1 from MRSA252 in the apo form.

2. Materials and methods

2.1. Purification of His_6-GAP1 in apo form

The cloning of the *gap*1 gene from MRSA252 in pQE30 expression vector and the overexpression of recombinant His_6-GAP1 in *Escherichia coli* M15 (pREP4) cells have been reported previously (Mukherjee *et al.*, 2008). During overexpression in *E. coli*, the heterologously expressed protein takes up cofactor from the host cells. Thus, the protein was first purified in its holo form and the dinucleotide was subsequently removed from the holoenzyme to obtain the purified apoenzyme.

The cells from 1 l of culture were resuspended in buffer *A* [10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 10 m*M* imidazole, 10%(*v*/*v*) glycerol] 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* and the protein was eluted with buffer *B* [10 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 300 m*M* imidazole, 10%(*v*/*v*) glycerol].

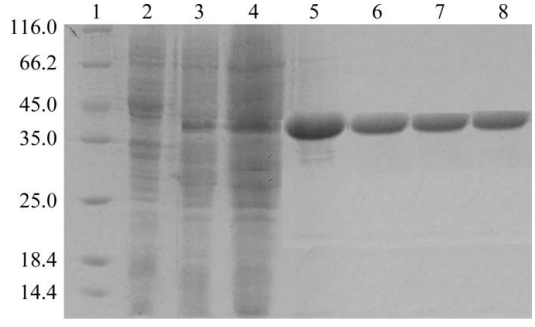


Figure 1
12% SDS–PAGE analysis of His_6-GAP1. Lane 1, premixed protein molecular-weight markers; lane 2, uninduced M15 (pREP4) cells; lane 3, induced M15 (pREP4) cells harbouring the recombinant plasmid; lane 4, supernatant; lane 5, Ni–NTA eluate; lane 6, purified holoenzyme after size-exclusion (Superdex 75) chromatography; lane 7, apoenzyme after activated charcoal treatment; lane 8, purified apoenzyme after size-exclusion (Superdex 75) chromatography.

The eluted protein was subjected to size-exclusion chromatography using Superdex 75 matrix in a C 16/70 column (GE Healthcare Biosciences) on an ÄKTAprime plus system (GE Healthcare Biosciences) equilibrated with buffer *C* [10 m*M* Tris–HCl pH 7.2, 50 m*M* NaCl, 2 m*M* DTT, 5.0 m*M* EDTA, 5%(*v*/*v*) glycerol]. 2 ml fractions were collected at a flow rate of 1 ml min^{-1}. Fractions containing the holoenzyme were pooled together. Activated charcoal was prepared following the protocol of Krimsky & Racker (1963). The charcoal was dried and suspended in buffer *C*, poured into a C 10/10 column and allowed to settle. A height of 1.5 cm of wet packed charcoal was used for each 100 mg of protein. The protein solution was passed through the column at a flow rate of 0.5 ml min^{-1}. The removal of NAD was confirmed by the absorbance ($OD_{280/260}$) ratio (Krimsky & Racker, 1963). To remove the EDTA that was present, the apoenzyme was concentrated and again subjected to gel-filtration chromatography using Superdex 75 on a C 16/70 column equilibrated with buffer *D* [50 m*M* Na_2HPO_4/NaH_2PO_4 buffer pH 8.0, 50 m*M* NaCl, 2 m*M* DTT, 5%(*v*/*v*) glycerol]. 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.

2.2. Crystallization

About 10 mg of purified protein was obtained per litre of culture. The purified protein was concentrated to 30 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 the commercially available Index screen from Hampton Research at 298 K. Small crystals were obtained using 0.1 *M* Tris–HCl pH 8.5, 30% PEG 3350. A fine screening around these conditions was performed using the hanging-drop vapour-diffusion method in 24-well Linbro plates by varying the pH, ionic strength, precipitant concentration and temperature of crystallization. Crystals appeared from 0.1 *M* Tris–HCl pH 8.2, 28%(*w*/*v*) PEG 3350 after a week at 298 K.

2.3. Data collection

Diffraction data were collected with Cu $K\alpha$ X-rays generated by our home source Rigaku Micromax HF007 Microfocus rotating-anode X-ray generator equipped with a Rigaku R-AXIS IV^{++} detector and a Varimax mirror system and operated at 40 kV and

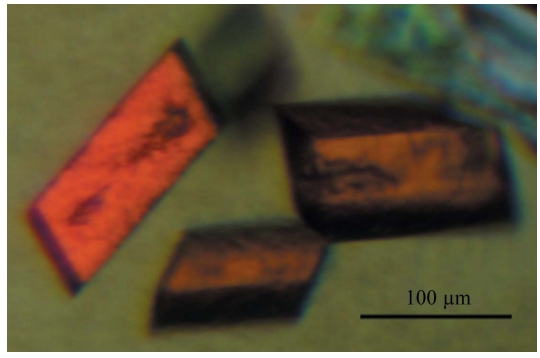


Figure 2
Crystals of apo GAP1. Crystals obtained from 0.1 *M* Tris–HCl pH 8.2, 28%(*w*/*v*) PEG 3350 at 298 K measured 0.12 × 0.04 × 0.012 mm.

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)	66.95
b (Å)	93.68
c (Å)	89.05
β (°)	106.84
Unit-cell volume (Å^3)	534635
Matthews coefficient (Å^3 Da^{-1})	1.81
Solvent content (%)	32
No. of monomers in asymmetric unit	4
Resolution range (Å)	33.34–2.20 (2.28–2.20)
Total reflections	192348
Unique reflections	53348
Redundancy	3.6 (3.3)
Completeness (%)	99.7 (97.3)
$R_{\rm merge}$† (%)	8.8 (42.8)
Average $I/\sigma(I)$	6.8 (2.3)

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

30 mA. The crystals were flash-cooled in liquid nitrogen at 100 K with a Rigaku X-stream 2000 cryosystem using the reservoir solution as cryoprotectant. The crystals diffracted to a maximum resolution of 2.2 Å. A total of 360 frames of data were collected with an oscillation angle of 0.5°, an exposure time of 2 min per frame and a crystal-to-detector distance of 160 mm. Diffraction data were processed with *d*TREK* v.9.8 software (Pflugrath, 1999).

3. Results and discussion

Recombinant His_6-tagged GAP1 from MRSA252 was successfully purified to homogeneity in the apo form (Fig. 1). The N-terminal hexahistidine tag was not removed from the protein. The purified holoenzyme was treated with specially treated activated charcoal to remove the bound cofactor. Being much smaller in size than the protein molecule, NAD preferentially enters the open pores of the activated charcoal, which adsorbs it effectively. As a result, the cofactor is abstracted from the holoenzyme. NAD, being a di-nucleotide, strongly absorbs at 260 nm. Thus, the absorbance ratio, which was initially 1.0 for the holoenzyme, increased to 1.9–2.0 in the case of the apoenzyme.

The crystals obtained from 0.1 *M* Tris–HCl pH 8.2, 28%(*w*/*v*) PEG 3350 at 298 K measured 0.12 × 0.04 × 0.012 mm (Fig. 2). Diffraction data were collected using a single crystal cryoprotected with 0.1 *M* Tris–HCl pH 8.2, 28%(*w*/*v*) PEG 3350. The crystals diffracted to a maximum resolution of 2.2 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 = 69.95, b = 93.68, c = 89.05 Å, β = 106.84°. Determination of the Matthews coefficient suggests the presence of 32% solvent content in the unit cell ($V_{\rm M}$ = 1.81 Å^3 Da^{-1}) with one tetramer in the asymmetric unit (Matthews, 1968). The overall completeness of the data set was 99.7%, with an $R_{\rm merge}$ of 8.8%. 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 (Collaborative Computational Project, Number 4, 1994) using a monomer of the holoenzyme (PDB code 3lvh; S. Mukherjee, D. Dutta, B. Saha & A. K. Das, unpublished work) as the search model. A promising solution with a homotetrameric structure was obtained (correlation coefficient of 0.65). A similar result was obtained using *Auto-Rickshaw*, an automated crystal structure-determination pipeline (Panjikar *et al.*, 2005). 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 33.5%. Final model building and restrained refinement using *REFMAC*5 are currently in progress.

This work was carried out with financial assistance from the Department of Biotechnology, Government of India. SM thanks the Council of Scientific and Industrial Research, Government of India for an individual fellowship. The authors acknowledge the kind help of Mr Dipankar Manna during data collection.

References

Archer, G. L. (1998). *Clin. Infect. Dis.* **26**, 1179–1181.
Boschi-Muller, S. & Branlant, G. (1999). *Arch. Biochem. Biophys.* **363**, 259–266.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Delgado, M. L., O'Connor, J. E., Azorin, I., Renau-Piqueras, J., Gil, M. L. & Gozalbo, D. (2001). *Microbiology*, **147**, 411–417.
Gil-Navarro, I., Gil, M. L., Casanova, M., O'Connor, J. E., Martinez, J. P. & Gozalbo, D. (1997). *J. Bacteriol.* **179**, 4992–4999.
Gozalbo, D., Gil-Navarro, I., Azorin, I., Renau-Piqueras, J., Martinez, J. P. & Gil, M. L. (1998). *Infect. Immun.* **66**, 2052–2059.
Harris, J. I. & Waters, M. (1976). *The Enzymes*, 3rd ed., edited by P. D. Boyer, ch. 13. New York: Academic Press.
Krimsky, I. & Racker, E. (1963). *Biochemistry*, 512–518.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Michels, S., Rogalska, E. & Branlant, G. (1996). *FEBS J.* **235**, 641–647.
Modun, B. & Williams, P. (1999). *Infect. Immun.* **67**, 1086–1092.
Moras, D., Olsen, K. W., Sabesan, M. N., Buehner, M., Ford, G. C. & Rossmann, M. G. (1975). *J. Biol. Chem.* **250**, 9137–9162.
Mukherjee, S., Dutta, D., Saha, B. & Das, A. K. (2008). *Acta Cryst.* F**64**, 929–932.
Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). *Acta Cryst.* D**53**, 240–255.
Pancholi, V. & Fischetti, V. A. (1992). *J. Exp. Med.* **176**, 415–426.
Panjikar, S., Parthasarathy, V., Lamzin, V. S., Weiss, M. S. & Tucker, P. A. (2005). *Acta Cryst.* D**61**, 449–457.
Pflugrath, J. W. (1999). *Acta Cryst.* D**55**, 1718–1725.
Roitel, O., Sergeinko, E. & Branlant, G. (1999). *Biochemistry*, **38**, 16084–16091.
Segal, H. L. & Boyer, P. D. (1953). *J. Biol. Chem.* **204**, 265–281.
Sirover, M. A. (1999). *Biochim. Biophys. Acta*, **1432**, 159–184.
Soukri, A., Mougin, A., Corbier, C., Wonacott, A., Branlant, C. & Branlant, G. (1989). *Biochemistry*, **28**, 2586–2592.
Taylor, J. M. & Heinrichs, D. E. (2002). *Mol. Microbiol.* **43**, 1603–1614.
Trentham, D. R. (1971). *Biochem. J.* **122**, 59–69.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.
Zang, W.-Q., Fieno, A. M., Grant, R. A. & Yen, T. S. (1998). *Virology*, **248**, 46–52.