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Purification, crystallization and preliminary crystallographic analysis of phosphoglucose isomerase from the hyperthermophilic archaeon *Pyrococcus furiosus*

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The glycolytic enzyme phosphoglucose isomerase catalyses the reversible isomerization of glucose 6-phosphate to fructose 6-phosphate. The phosphoglucose isomerase from the hyperthermophilic archaeon *Pyrococcus furiosus*, which shows no sequence similarity to any known bacterial or eukaryotic phosphoglucose isomerase, has been cloned and overexpressed in *Escherichia coli*, purified and subsequently crystallized by the hanging-drop method of vapour diffusion using 1.6 *M* sodium citrate as the precipitant at pH 6.5. Multiple-wavelength anomalous dispersive X-ray data have been collected to a maximum resolution of 1.92 Å on a single selenomethionine-incorporated crystal. This crystal belongs to space group *C*2, with approximate unit-cell parameters $a = 84.7$, $b = 42.4$, $c = 57.3$ Å, $\beta = 120.6^\circ$ and a monomer in the asymmetric unit.

1. Introduction

Phosphoglucose isomerase (PGI; EC 5.3.1.9) catalyses the isomerization of glucose 6-phosphate to fructose 6-phosphate in the Embden–Meyerhof pathway and in the reverse direction in gluconeogenesis (Noltmann, 1972; Rose, 1975). The *Pyrococcus furiosus* PGI is a dimer of identical 21.5 kDa subunits (SWISS-PROT accession code P83194; Verhees *et al.*, 2001). Sequence comparisons have revealed that *P. furiosus* PGI is not related to its bacterial and eukaryal counterparts, for which a number of structures have been determined [*e.g. Bacillus stearothermophilus* (Sun *et al.*, 1999), rabbit (Davies & Muirhead, 2003) and human (Read *et al.*, 2001)]. However, sequence similarities do exist between the *P. furiosus* PGI enzyme and members of the cupin superfamily that comprises a variety of proteins generally involved in sugar binding (Dunwell *et al.*, 2000; Verhees *et al.*, 2001). This paper describes the cloning, purification, crystallization and preliminary X-ray analysis of *P. furiosus* PGI as a first step in the structure determination of this novel class of enzymes.

2. Overexpression and purification of selenomethionine protein

Wild-type PGI was initially purified from *P. furiosus* cell paste and the amino-acid sequence at the N-terminus was determined. The corresponding *pgi* gene was PCR amplified and cloned in pET24d (pLUW557), expressed in *Escherichia coli* BL21 (DE3) and purified (Verhees *et al.*, 2001). For production of the selenomethionine-incorporated protein, the pLUW557 construct was used to transform competent BL21 (DE3) cells. A single colony was cultured for 3 h at 310 K in 100 ml LB medium containing kanamycin (50 µg ml^{-1}) in a shaking incubator. The overnight culture was then used to inoculate two 2 l flasks each containing 500 ml of LB medium (50 mg l^{-1} kanamycin) and shaken at 310 K until the turbidity reached $OD_{600} = 1.0$. The contents of the two flasks were then transferred into a sterile centrifuge bottle and centrifuged at 4200 rev min^{-1} for 10 min at room temperature. The supernatant was subsequently discarded and the cell pellet resuspended in supplemented minimal media [1 g l^{-1} $(NH_4)_2SO_4$, 4.5 g l^{-1} KH_2PO_4, 10.5 g l^{-1} K_2HPO_4, 40 mg l^{-1} of all amino acids except methionine, 40 mg l^{-1} selenomethionine, 500 mg l^{-1} adenine, guanosine, thymine and uracil, 2 mg l^{-1} thiamine, 5 g l^{-1} glycerol, 500 mg l^{-1} sodium citrate.$2H_2O$ and 1 m*M* $MgSO_4.7H_2O$] containing 50 mg l^{-1} kanamycin at 310 K. The 50 ml of concentrated cell suspension was then used to inoculate a small bench-top fermenter containing 3.5 l of supplemented minimal media containing 50 mg l^{-1} kanamycin at 310 K. The turbidity of the culture was monitored closely to ensure growth remained in the logarithmic phase until an OD_{600} of 0.8 was reached (starting $OD_{600} = 0.35$). At this point. 2 m*M* IPTG was added to the culture. The culture was then fermented overnight at 310 K before harvesting the cells by centrifugation at 4200 rev min^{-1} and 277 K. The supernatant was discarded and the cell paste was frozen at 253 K for future use. Analysis of pre- and post-induction samples using SDS–PAGE showed a strong post-expression band at approximately 24 kDa, in good agreement with earlier results (Verhees *et al.*, 2001).

Purification was performed as previously described for the wild-type recombinant protein (Verhees *et al.*, 2001) with the following changes. Resuspension of overexpressing cells was performed in 40 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA (buffer *A*) and disrupted by ultrasonication for 3 × 10 s at 10 µm amplitude. The resulting cell-free extract was incubated at 338 K for 25 min and centrifuged at 70 000*g* for 20 min at 277 K. The lower temperature incubation step compared with that reported previously exposed the protein to less harsh conditions but nevertheless effectively removed the majority of the contaminating *E. coli* proteins. The soluble fraction was loaded onto a 20 ml Q-Sepharose Fast Flow column (Amersham Biosciences) equilibrated in buffer *A* and was eluted using a linear gradient of NaCl from 0 to 1 *M* in buffer *A*. The protein concentration of the fractions was measured using the method of Bradford (1976) with BioRad dye reagent (Bio-Rad Laboratories GmbH, Germany) and the purity was analysed by SDS–PAGE (NuPAGE 4–12% bis-tris gel; Novex). The purest fractions were combined and loaded onto a 16/60 HiLoad Superdex 200 column (Amerham Biosciences) equilibrated in buffer *B* (50 m*M* Tris–HCl pH 8.0, 0.5 *M* NaCl). PGI was eluted from the column with buffer *B*. Peak fractions were combined and concentrated on a Vivaspin concentrator to give a final concentration of 14 mg ml^{-1}. This equates to a yield of 18 mg of PGI per litre of cell culture.

3. Crystallization and preliminary X-ray analysis

The level of overexpression of selenomethionine-incorporated PGI was estimated to be approximately 15% of the total soluble cell protein. The purity of PGI, as determined by SDS–PAGE (4–12% bis-tris NuPAGE gel, Novex), was approximately 90%, with a yield of 21 mg of protein measured using the Bradford assay (Bradford, 1976). The level of selenomethionine incorporation in the PGI protein was determined by positive ionization nanoelectrospray mass spectrometry using the Q-Tof (Micromass UK, Ltd) mass spectrometer at the Mass Spectrometry Facility at the University of Leeds and was shown to be greater than 95%.

A preliminary crystallization screen was carried out by the hanging-drop method of vapour diffusion (290 K) using Hampton Research Crystal Screen kits and a protein concentration of approximately 14 mg ml^{-1}. Crystals of the methionine and selenomethionine protein grew optimally in 1.6 *M* sodium citrate dihydrate pH 6.5 (condition No. 28) after 2–3 weeks. These crystals had maximal dimensions of 0.5 × 0.5 × 0.2 mm and a plate-like morphology.

Table 1
X-ray data-collection statistics for the selenomethionine-incorporated PGI crystal collected on beamline BM14 at ESRF, Grenoble, France.

Values in parentheses refer to the highest resolution shell.

Data set	Peak (λ_1)	Inflection (λ_2)	Remote (λ_3)
Wavelength (Å)	0.97897	0.979389	0.89843
Resolution (Å)	50–2.09 (2.16–2.09)	50–2.09 (2.16–2.09)	50–1.92 (1.99–1.92)
Reflections measured	87131	85980	116064
Reflections rejected	437	474	299
Unique reflections	10188 (648)	10167 (666)	13134 (900)
Multiplicity	3.7 (3.2)	3.7 (3.0)	3.7 (3.1)
Completeness (%)	96.5 (66.0)	96.3 (64.2)	96.5 (66.7)
$\langle I/\sigma(I)\rangle$	38.9 (10.3)	37.7 (9.3)	32.5 (6.35)
$\langle I/\sigma(I)\rangle$, $I > 3\sigma$† (%)	91.6 (85.0)	90.5 (84.5)	85.4 (72.6)
$R_{\rm merge}$‡	0.064 (0.140)	0.055 (0.121)	0.057 (0.162)

† The percentage of reflections with $I/\sigma(I)$ greater than three. ‡ $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$, where I is the integrated intensity of a given reflection.

For data collection, a single selenomethionine-incorporated crystal was flash-frozen straight out of the drop at 100 K. Subsequently, multiple-wavelength anomalous diffraction (MAD) data were collected from this crystal to a maximum resolution of 1.92 Å using a MAR Research CCD detector on beamline BM14 at the ESRF, Grenoble, France. Three wavelengths were chosen near the selenium-absorption edge based on a fluorescence absorption spectrum obtained from the frozen crystal in order to maximize the f'' component (λ_1, peak), to minimize the f' component (λ_2, inflection) and to maximize $\Delta f'$ (λ_3, remote). To ensure all Bijvoet pairs were measured, a total of 120 images of 1.5° rotation per image were collected at each wavelength. The crystal had a mosaicity that varied between 0.5 and 0.9° depending on its orientation. The data for each wavelength were processed individually and scaled in such a way as to preserve the anomalous signal using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997). Details of the data-collection statistics are presented in Table 1.

Preliminary analysis of the X-ray diffraction data using the autoindexing routine in *DENZO* indicated that the crystal belonged to space group *C*2, with unit-cell parameters a = 84.7, b = 42.4, c = 57.3 Å, β = 120.6° and a corresponding unit-cell volume of 1.8 × 10^5 Å^3. Consideration of the values of $V_{\rm M}$ suggests that the asymmetric unit contains a monomer with a $V_{\rm M}$ value of 2.1 Å^3 Da^{-1}, which is within the range observed by Matthews for protein crystals (Matthews, 1977). A full structural determination is currently under way in order to provide insights into the key determinants of the function of this class of PGIs, for which no structures are currently available.

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