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Crystallization and preliminary X-ray diffraction studies of β-phosphoglucomutase from *Lactococcus lactus*

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β-Phosphoglucomutase (β-PGM), a 28 kDa monomer, catalyzes the reversible conversion of β-D-glucose-1-phosphate to β-D-glucose-6-phosphate in maltose metabolism in a variety of organisms. Sequence analysis of β-PGM indicates that it is a member of the haloacid dehalogenase (HAD) enzyme superfamily, which evolved to cleave C—Cl, C—P and C—OP bonds in a variety of substrates. β-PGM has been crystallized using the hanging-drop method. Diffraction-quality crystals of the native protein have been obtained from two conditions, both belonging to space group $P2_12_12_1$, with unit-cell parameters a = 53.67, b = 92.78, c = 111.60 and a = 53.21, b = 57.01, c = 76.11 Å. To solve the phase problem, selenomethionine (SeMet) containing β-PGM crystals have been grown. The SeMet-containing crystals diffract to high resolution only when grown by microseeding with native crystals. A three-wavelength data set has been collected to 2.3 Å on crystals of the SeMet-substituted β-PGM. The structure solution is currently being attempted by the multi-wavelength anomalous diffraction (MAD) phasing method.

1. Introduction

β-Phosphoglucomutase (β-PGM) catalyzes the reversible conversion of β-D-glucose-1-phosphate (G1P) to β-D-glucose-6-phosphate (G6P) in the catabolic pathway of maltose metabolism (Qian *et al.*, 1994, 1997). The enzyme requires β-glucose-1,6-diphosphate (G-1,6-P_2) and Mg^{II} for activity (Marechal *et al.*, 1984). β-PGM belongs to the 2-L-halo-alkanoic dehalogenase (HAD) superfamily of hydrolases, which includes dehalogenases (C—Cl cleavage), phosphonatases (C—P cleavage), phosphotranferases and phosphatases (C—OP cleavage) (Koonin & Tatusov, 1994; Baker *et al.*, 1998; Collet *et al.*, 1998; Ridder *et al.*, 1999; Stokes & Green, 2000; Hisano *et al.*, 1996). Members of this family whose structures are known (haloacid dehalogenase, phosphonatase, Ca^{II} ATPase and phosphoserine phosphatase) contain an α/β core domain and a small α-helical cap domain. The active site is located at the interface of these two domains. The tertiary structure of the core domain is highly conserved to support a common active-site scaffold, while the tertiary structure of the cap domain has diverged to accommodate unique substrates and chemistries (Morais *et al.*, 2000). The core-domain active site is comprised of four loops, which corresponds to the four signature sequence motifs of the superfamily. The first Asp in motif 1 forms either an acylphosphate enzyme intermediate (phosphotransferases/phosphatases) or an alkyl ester intermediate (dehalogenases) during catalysis (Aravind *et al.*, 1998; Baker *et al.*, 1998; Li *et al.*, 1998).

In most HAD superfamily members the acylphosphate intermediate cannot be stabilized sufficiently for structure determination owing to the labile nature of this species. β-PGM is a unique member in this group since the form that binds substrate is the ground-state phosphorylated form, enabling the crystallization of this species. Besides yielding the first view of the phosphoenzyme in the superfamily, the structure should generate mechanistic insights. In addition to the invariant nucleophilic residue Asp8, motif I also contains a second conserved aspartate (Asp10). We propose that one or both of these residues participate in the transfer of a phosphoryl group between position C(1)OH and C(6)OH of the glucose phosphate substrate. In the ‘double Asp’ mechanism, enzyme phosphorylated at Asp10 binds the G6P isomer, while enzyme phosphorylated at Asp8 binds the G1P isomer. Phosphoryl transfer occurs to form the G-1,6-P_2 intermediate, which ultimately donates the phosphoryl group from the C(1) position to Asp8 or the C(6) position to Asp10, thus forming G6P or G1P, respectively. In the ‘single Asp’ mechanism, the enzyme phosphorylated at Asp8 binds G6P or G1P in an orientation that positions the free hydroxyl for in-line phosphoryl transfer. The G-1,6-P_2 intermediate formed must ‘flip’ in the active site or dissociate and then associate in an

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alternate orientation in order to allow the transfer of the substrate phosphoryl group to Asp8 and generation of the alternate glucose phosphate isomer.

The X-ray crystallographic structure of β-PGM would be the first structure of the HAD superfamily members with a stable phosphoenzyme intermediate. From this structure, we expect to determine how the superfamily active site has structurally diverged to stabilize this intermediate and how the mutase reaction is catalyzed.

2. Materials and methods

2.1. *L. lactus* β-phosphoglucomutase gene expression and protein purification

The *L. lactus* β-phosphoglucomutase gene (Qian *et al.*, 1994, 1997) was cloned for overexpression in *Escherichia coli* BL21 (DE3) (Novagen) using the pET3a plasmid vector. Cells were grown at 310 K in 2 l Luria–Bertani medium containing 100 µ*M* ampicillin to an OD_{600} of 1.0. The cells were induced with 0.4 m*M* IPTG and continuously grown at 303 K to an OD_{600} of 2.0. Harvested cells were resuspended in buffer *A* (50 m*M* HEPES, 5 m*M* $MgCl_2$ and 1 m*M* DTT pH 7.2) and lysed at 277 K with a French press at 69 MPa. The cell-free solution obtained by centrifugation was loaded onto a DEAE-Sepharose column and eluted at 277 K using a linear gradient of NaCl (0–0.5 *M*). Fractions containing β-PGM were pooled together and concentrated at 277 K with a Amicon protein concentrator (PM-10) before chromatographing on a Sephedex G-75 column in buffer *A* at 277 K. The active fractions were combined and concentrated to 20 mg ml^{-1}. The homogeneity of the β-PGM was confirmed by SDS–PAGE.

For the preparation of the selenomethionyl protein, the β-PGM-pET3a clone was used to transform the methionine-requiring auxotroph *E. coli* B384 (DE3). The protein-purification procedure was modified slightly to minimize oxidation of SeMet. The concentration of DTT in the chromatography buffers was increased to 10 m*M* and the concentration of $MgCl_2$ in buffer *A* was reduced to 1 m*M*. The chromatographic properties and activity of the SeMet β-PGM are consistent with the retention of the native fold. The K_m and V_{max} for the native and SeMet β-PGM were determined from the initial velocity data measured using the previously described coupled assay (Qian *et al.*, 1994). The initial velocity data were analyzed using the fortran *HYPERL* program of Cleland (1979).

2.2. Optimization of crystallization conditions

The wild-type protein was stored in 50 m*M* HEPES pH 7.5 containing 10 m*M* $MgCl_2$ and 0.1 m*M* DTT at 277 K. Prior to crystallization, the protein was transferred to 1 m*M* HEPES pH 7.5 containing 10 m*M* $MgCl_2$ and 0.1 m*M* DTT by repeated dilution and concentration in a Millipore Ultrafree concentrator. The final protein concentration of 15 mg ml^{-1} was determined *via* Bradford assay (BioRad). The preliminary crystallization conditions were obtained by the sparse-matrix sampling technique (Jancarik & Kim, 1991) with hanging-drop geometry using Crystal Screen I, Crystal Screen II and PEG Ion Screen (Hampton Research). Equal volumes (2.5 µl) of the protein solution and well solution were used. Crystals were obtained in two different conditions: from 0.2 *M* ammonium acetate, 0.1 *M* trisodium citrate dihydrate pH 5.6, 30%(*w*/*v*) PEG 4000 and from 0.2 *M* ammonium fluoride, 20%(*w*/*v*) PEG 3350 (unbuffered; measured pH 6.5). Crystals obtained from the initial screen were thin needles unsuitable for X-ray diffraction. Further optimization of these conditions to 0.15 *M* ammonium acetate, 0.1 *M* trisodium citrate dihydrate pH 4.5, 25%(*w*/*v*) PEG 4000 for the former and 0.1 *M* ammonium fluoride, 16%(*w*/*v*) PEG 3350 for the latter resulted in good-quality plate-like crystals suitable for diffraction. Crystals from both conditions usually grew in 4–5 d.

To grow SeMet-substituted β-PGM crystals, native β-PGM crystals were used as a source for microseeding experiments. A single native β-PGM crystal was removed into 5 µl well solution and crushed into small pieces using a blunt needle. The solution was then diluted to 100 µl and 1 µl of this mixture was used to seed a hanging drop containing 2.5 µl of selenomethionine β-PGM protein solution and 2.5 µl of well solution. Crystals grown using this seeding procedure were used as seeds in a second round of microseeding. One of the resulting crystals was used to collect the SeMet data set.

2.3. X-ray diffraction experiments

Crystals were flash-frozen for data collection in 100% Paratone-N (Hampton Research). The three-wavelength MAD data set for selenomethionine β-PGM was collected on beamline BM 14D at Argonne National Laboratories using a CCD detector. The *DENZO* and *SCALEPACK* programs were used for data indexing, reduction and scaling and determination of the unit-cell parameters (Otwinowski, 1990).

3. Results and discussion

High-level expression of the 28 kDa β-PGM from *L. lactus* was achieved in *E. coli* using the pET3a vector. The recombinant protein was purified to homogeneity in a yield of 7 mg per gram of cells (specific activity = 40 U mg^{-1}, compared with 18 U mg^{-1} reported for the native enzyme; Qian *et al.*, 1994). The steady-state kinetic constants measured at 298 K and pH 7.2 in the presence of β-G-1,6-P_2 and Mg^{II} cofactor are k_{cat} = 17 s^{-1} and K_m = 4.48 µ*M*. The values are comparable to those reported for *Bacillus subtilis* β-PGM (Mesak & Dahl, 2000).

A systematic search for crystallization conditions produced two crystal forms. Crystals from the condition with 0.15 *M* ammonium acetate, 0.1 *M* trisodium citrate dihydrate pH 4.5 and 25%(*w*/*v*) PEG 4000 were orthorhombic, with unit-cell parameters a = 53.21, b = 57.01, c = 76.11 Å, $\alpha = \beta = \gamma = 90°$, consistent with one monomer per asymmetric unit (Matthews, 1985). The best crystals from this condition were long thin rectangular plates with

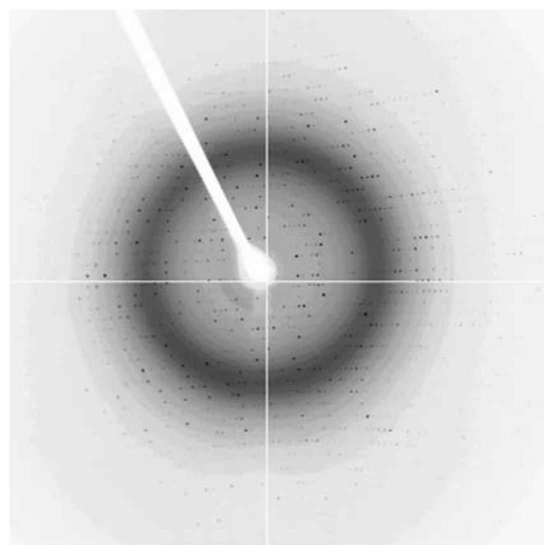

Figure 1
Diffraction of SeMet-substituted β-PGM crystals using synchroton radiation on beamline BM14-D at the Advanced Photon Source (see text for details). The resolution at the edge of the plate is 2.2 Å.

Table 1
Summary of crystallographic statistics.

Values for the outermost shell are given in parentheses.

	SeMet λ_1	SeMet λ_2	SeMet λ_3
Wavelength (Å)	0.9792369	0.9790073	0.96112713
Resolution (Å)	2.3	2.3	2.3
Total reflections	420727	419349	421192
Independent reflections	25469	25416	25922
Multiplicity	16.5	16.5	16.3
$R_{\rm merge}$ (%)	0.081 (0.191)	0.081 (0.192)	0.072 (0.190)
Completeness (%)	91.0 (60.8)	95.7 (81.8)	92.9 (61.4)
$I/\sigma(I)$	12.3 (3.5)	18.0 (4.9)	17.6 (4.2)
$I > 3\sigma$ (%)	85 (70)	87 (71)	86 (70)

dimensions of 1 × 0.3 × 0.1 mm. The crystals grown from the second condition with 0.2 *M* ammonium fluoride and 20%(*w*/*v*) polyethylene glycol 3350 had unit-cell parameters a = 53.67, b = 92.78, c = 111.60 Å, $\alpha = \beta = \gamma = 90^\circ$, with two molecules per asymmetric unit. The best crystals from this condition were thick rectangular plates with dimensions of 1 × 0.7 × 0.3 mm. From systematic absences in axial reflections in the data sets, the space group was determined to be $P2_12_12_1$ (Otwinowski, 1990) for both forms. The condition closer to the optimum physiological pH (6.5) was pursued, producing crystals that diffracted to better than 2.3 Å resolution.

Even though the HAD superfamily members share limited sequence identity (10–15%), attempts to use member proteins of known structure as search models in molecular replacement for member proteins of unknown structure have met with no success. Despite the common core superfamily fold, there are slight rearrangements in the secondary-structural elements with respect to one another, making molecular replacement impossible. Therefore, an attempt has been made to solve the phase problem for β-PGM by multiwavelength anomalous diffraction (MAD) methods using SeMet protein (Guss *et al.*, 1988; Hendrickson *et al.*, 1990). To crystallize SeMet-substituted β-PGM, the protein was grown under the same crystallization conditions as those optimized for native β-PGM. Although the crystal-growth period and the morphology were identical to those of the native protein, to our surprise the quality of diffraction was very poor (11 Å) compared with crystals of the native protein.

To obtain better quality SeMet crystals, native β-PGM crystals were used as a source for microseeding experiments. The microseeding procedure is commonly used for growing crystals of superior and more uniform crystallographic quality (Eichele *et al.*, 1979; Thaller *et al.*, 1981, 1985). The crystals resulting from the first round of microseeding were used for two successive rounds of microseeding to ensure better quality crystals and to exclude non-SeMet protein from the crystal. SeMet β-PGM crystals obtained in this manner were morphologically identical to their native counterparts and diffracted to 2.3 Å resolution (see Fig. 1). A three-wavelength MAD data set from these crystals has been collected. Table 1 shows the relevant statistics of the SeMet data sets. The structure determination of β-PGM using this data set is currently in progress. In the future, a complete native data set (no SeMet substitution) will be collected and the native structure will be solved by molecular replacement to ensure that no changes have been introduced by the use of SeMet. The structure of β-PGM will shed light on the mechanism(s) of phosphate stabilization and catalysis of phosphate transfer.

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