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Crystallization and preliminary X-ray diffraction studies of 6-phosphogluconate dehydrogenase from *Lactococcus lactis*

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Abstract

6-Phosphogluconate dehydrogenase is one of the seven enzymes involved in the pentose phosphate pathway. Crystals of a mammalian and a protozoan enzyme have been obtained previously and structures determined. It is reported here that a bacterial 6-phosphogluconate dehydrogenase, from *Lactococcus lactis*, has been purified and used in crystallization trials. Large prisms suitable for a detailed structural analysis have been obtained and characterized as orthorhombic, space group *F*222, with a = 70.4, b = 105.7, c = 474.6 Å. Diffraction has been observed to 2.2 Å resolution using synchrotron radiation. Structural analysis, in combination with ongoing biochemical characterization, will assist the elucidation of the structure–activity relationships of this enzyme.

1. Introduction

The pentose phosphate or phosphogluconate metabolic pathway leads to the production of various carbohydrates which can be passed into the glycolytic pathway, NADPH for a wide range of biosynthetic processes and ribose 5-phosphate for use in DNA synthesis. 6-Phosphogluconate dehydrogenase (E.C. 1.1.1.44; 6PGDH) is the third enzyme of the pentose phosphate pathway and catalyses the oxidative decarboxylation of 6-phosphogluconate to D-ribulose 5-phosphate with the liberation of carbon dioxide and protonation of NADP. In concert with glucose-6-phosphate dehydrogenase, this provides most of the cellular NADPH required for reductive biosyntheses and maintenance of normal redox balance (Wood, 1986). The enzyme has been purified from a variety of sources and shown to be a homodimer with subunit mass of approximately 50 kDa.

A likely mechanism for the enzyme purified from *Candida albicans* has been proposed (Berdis & Cook, 1993). The structure of the enzyme from sheep liver (Adams *et al.*, 1991, 1994; Phillips *et al.*, 1995) has been determined at high resolution. 6PGDH from the parasitic protozoan *Trypanosoma brucei* has been cloned and overexpressed in *Escherichia coli* to facilitate production of large quantities of the enzyme (Barrett *et al.*, 1994) and a structure has been determined at 2.8 Å resolution (Phillips *et al.*, 1998). The most significant differences between the mammalian and trypanosome enzymes occur at the dimer interface. It is possible that such differences are critical, since the enzyme uses an alternating-site negative co-operativity, where an energetically favourable step of the overall catalytic process in one subunit is coupled to an energetically unfavourable step in the other (Rippa *et al.*, 1998). The gene encoding the enzyme from the Gram-positive bacterium *Lactococcus lactis* has recently been cloned and overexpressed in *E. coli* and the enzyme kinetics characterized (Tetaud *et al.*, 1998). The predicted translation product of the gene shows strong homology to 6PGDH from *E. coli* (68% identity), from sheep liver (55%) and from *T. brucei* (37%).

The recombinant *L. lactis* enzyme is much more stable when compared to the recombinant *T. brucei* variant and this may be attributed to the relative lack of cysteine residues within the bacterial enzyme, since the trypanosomal 6PGDH is prone to aggregation, which can be partially reversed by the addition of dithiothreitol (M. P. Barrett, unpublished results). Owing to this enhanced stability, the *L. lactis* enzyme offers advantages over the recombinant *T. brucei* protein for systematic studies of kinetic and chemical mechanisms. Previously, Pearse & Harris (1973) proposed a similar argument for structural studies on the 6PGDH from *Bacillus stearothermophilus*. Although crystals of that enzyme were obtained, no structure has yet been reported.

Loss of 6PGDH is lethal to eukaryotic cells, suggesting that it could be a target for antiparasite chemotherapy if significant differences between host and parasite versions of the enzyme can be exploited as sites of inhibition by chemical agents (Barrett, 1997). Structural information would be required to support such a program of research, which would improve understanding of 6PGDH and may provide details useful for the identification of novel antiparasitic compounds.

2. Results and discussion

2.1. *Preparation of recombinant 6-phosphogluconate dehydrogenase*

The *L. lactis* 6-phosphogluconate dehydrogenase gene was cloned into the T7-promoter based *E. coli* expression pET16b system (Studier *et al.*, 1990; Novagen) creating the plasmid pET16b-6PGDH, which adds a 10-histidine tag to the N-terminus of the protein. The tagged enzyme was produced in a soluble form, with kinetic parameters similar to those measured for the non-tagged variant of the enzyme (Tetaud *et al.*, 1998). *E. coli* strain BL21(DE3) was transformed with the plasmid pET16b-6PGDH and selected on Luria–Bertani broth (LB) plates containing 100 µg ml^{-1} of ampicillin. A positive colony was grown in 1 l of LB with ampicillin to an optical density of 0.6, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 m*M* and cell growth continued with vigorous aeration for 4 h. Cells were harvested by centrifugation (3000 r min^{-1} in a Sorvall centrifuge), then resuspended in 25 ml of 20 m*M* Tris–HCl, pH 7.2, and stored frozen in liquid nitrogen. The cells were thawed at 310 K and subjected to five rounds of sonication at 26 µm amplitude for 30 s interspersed with 30 s periods cooling on

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ice. Aliquots of the sample were transferred into 2 ml Eppendorf tubes and the soluble and insoluble fractions were separated by centrifugation at 12000 r min^{-1} in an Eppendorf bench-top centrifuge for 20 min. The specific activity of 6-phosphogluconate dehydrogenase in the supernatant was tested as described by Barrett *et al.* (1994). Extracts, with 50% glycerol added, were stored at 203 K.

The cell lysate was added to a nickel affinity column (Novagen) and washed according to the manufacturers' specifications, eluted with 1 *M* imidazole, 0.5 *M* NaCl and 20 m*M* Tris–HCl, pH 7.9, and the specific activity of eluted enzyme checked. The enzyme was dialysed overnight against 5 l of 50 m*M* Tris–HCl, pH 7.2, and loaded onto a 2′ 5′ ADP agarose column equilibrated with 50 m*M* Tris–HCl, pH 7.2. The column was washed with 50 m*M* Tris–HCl, pH 7.2, containing 120 m*M* NaCl, and the protein eluted in 50 m*M* Tris–HCl, pH 7.2, with 0.5 *M* NaCl. The specific activity of the nickel-chelate 2′ 5′ ADP affinity-purified enzyme was checked and overloaded samples run on SDS–PAGE gels stained by Coomassie blue to assess purity. Several contaminating bands were visible, even after this purification procedure. The sample was judged to be greater than 95% pure and was concentrated to 5 mg ml^{-1} in 20 m*M* Tris–HCl, pH 7.2, containing 50 m*M* potassium phosphate, for use in crystallization trials.

2.2. *Crystallization*

Conditions were tested based on consideration of previously successful crystal-growth experiments as documented in the Biological Macromolecule Crystallization Database (Gilliland *et al.*, 1994), and, in addition, screening according to the sparse-matrix approach (Jancarik & Kim, 1991) was carried out. Solutions purchased from Hampton Research (USA) were used in these experiments. Both hanging- and sitting-drop geometry were used at 277 and ∼293 K. Promising crystalline precipitates were obtained under a wide range of conditions. Further experiments produced blocks displaying well defined morphology. These crystals grow over a period of two weeks at room temperature in drops consisting of 5 µl of protein solution mixed with 1 µl of a solution of 0.2 *M* ammonium acetate, 0.1 *M* sodium citrate and 30%(*w*/*v*) PEG 4000. Under similar conditions needles up to 1 mm in length and 0.01 mm cross section were also obtained but were deemed unsuitable for diffraction studies.

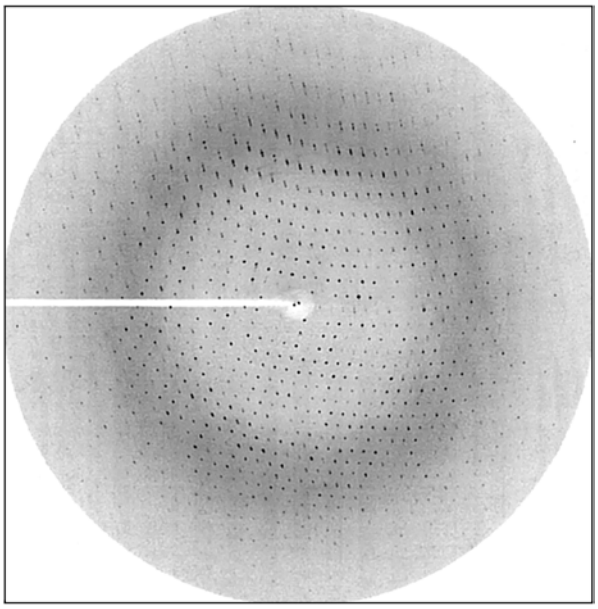

Fig. 1. An example of the diffraction pattern recorded on beamline BM14 at ESRF. The edge of the plate corresponds to 2.2 Å resolution.

2.3. *X-ray diffraction and unit-cell characterization*

A single specimen of maximum dimension 0.4 mm was mounted and sealed in a thin-walled glass capillary and exposed to X-rays on beamline BM14 at the European Synchrotron Radiation Facility, Grenoble. Data were measured using X-rays of wavelength 1.0 Å, 10 s exposures and a MAR 345 image-plate detector. Diffraction extended to 2.2 Å resolution. Owing to time limitations only 20° of data were recorded. The *HKL* suite of programs was used for autoindexing and data processing (Otwinowski, 1993). Initial calculations suggested monoclinic symmetry and space group *C*2 with a = 71.0, b = 106.2, c = 241.7 Å, $\alpha = \gamma = 90$, $\beta = 98.1°$. Data processed satisfactorily and gave an R_{sym} of 4.1% (17% of the theoretical data). However, autoindexing on alternative images and further processing suggested the crystal was in fact of higher orthorhombic symmetry, space group *F*222, with dimensions a = 70.4, b = 105.7, c = 474.6 Å. In this space group, 45152 measurements of 16386 unique reflections gave an R_{sym} of 3.2%. This represents 36.3% of the theoretical data between 30 and 2.2 Å resolution.

The enzyme is a homodimer with a subunit of 472 amino acids and molecular mass 52.4 kDa. The Matthews coefficient, V_m is 2.1 $Å^3$ Da^{-1} for a functional dimer in the asymmetric unit with about 41% solvent volume calculated according to Matthews (1968). These are reasonable values given that the range of typical V_m values observed for protein crystals is 1.68–3.53 $Å^3$ Da^{-1} and 27–65% solvent volume. That the crystals are well ordered and diffract to at least 2.2 Å resolution also hints at a low solvent content. Once a more complete data set is available, molecular-replacement methods using previously determined 6PGDH structures should facilitate structure solution and confirm the space group.

In summary, we have obtained crystals of *L. lactis* 6-phosphogluconate dehydrogenase. The crystals are well ordered, diffract to 2.2 Å resolution using synchrotron radiation and should facilitate a complete structure determination to compliment ongoing biochemical and kinetic analyses of this enzyme system.

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