6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHILUS*

B.M.F. PEARSE and J. Ieuan HARRIS
Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

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1. Introduction

The structural analysis of lactate dehydrogenase [1], malate dehydrogenase [2], liver alcohol dehydrogenase [3], and glyceraldehyde 3-phosphate dehydrogenase [4] show striking homologies within this group of proteins, particularly of the NAD-binding sites. However, no detailed structural information is available on a class of enzymes using NADP, of which 6-phosphogluconate dehydrogenase (6PGD) is a member.

6PGD has been purified and characterised from a number of sources including *Candida utilis* [5], human erythrocytes [6] and sheep liver. Crystals of the latter enzyme have been obtained [7]. We have sought to prepare large quantities of stable crystalline enzyme from the thermophillic bacterium, *Bacillus stearothermophilus*, from which stable crystals of GPDH were obtained [8]. We now report a procedure for preparing crystals, suitable for X-ray structural analysis of 6PGD from *B. stearothermophilus*. Some of the properties of the enzyme are also described.

2. Methods

2.2. *Purification*

6PGD was assayed at 25°C in 3 ml Tris–HCl buffer (pH 9.0, $I = 0.1$) containing 0.1 mM NADP and 1.0 mM 6-phosphogluconate. The increase in absorbance at 340 nm was followed using a thermostated Gilford 222A spectrophotometer. A unit of enzyme activity is defined as the number of μmoles of NADP reduced per min at 25°C.

B. stearothermophilus (strain 1503) was grown at the Microbiological Establishment, Porton, Wilts, U.K. The cells (20 kg wet weight) were disrupted and the soluble proteins extracted, giving a solution containing 80 000 units of 6PGD activity. The initial steps, for the large-scale purification of several enzymes, were carried out at Porton [9]. Fractionations were achieved using hydroxyapatite and DEAE-Sephadex. 16 000 units of 6PGD activity, representing a 20% yield of enzyme, with a specific activity of about 1 unit/mg. were present after separation from triose phosphate isomerase, aldolase and GPDH. Subsequent steps leading to the isolation of the pure enzyme were carried out in this laboratory. The enzyme solution was subjected to ion-exchange chromatography on CM-Sephadex at pH 5.6, resulting in a 10-fold purification. The 6PGD fraction was concentrated (in 0.1 I sodium acetate buffer pH 6.0, containing 1 mM EDTA, 0.1% β-ME and 10 μM NADP) to give a total protein concentration of about 20 mg/ml, with 200 units/ml of 6PGD activity. Microcrystals of the enzyme formed, on standing, at 0–4°C. These were redissolved in 0.1 I acetate buffer pH 6.0. The enzyme (with a specific activity of 20 units/mg) was stored in this solution at 0–4°C, with a crystal of thymol to prevent bacterial growth.

2.2. *Characterisation*

Sedimentation velocity experiments were done at

Abbreviations: β-ME, β-mercaptoethanol; 6PGD, 6-phosphogluconate dehydrogenase; GPDH, glyceraldehyde 3-phosphate dehydrogenase; NADP, nicotinamide adenine dinucleotide phosphate; EDTA, ethylene diamine tetra-acetic acid; SDS, sodium dodecyl sulphate.

Fig. 1. SDS-polyacrylamide gel electrophoresis of redissolved crystals of *B. stearothermophilus* 6PGD.

a speed of 60 000 rev./min on a Spinco Model E analytical ultracentrifuge at 20°C. Molecular weights were determined by sedimention equilibrium experiments [10] from plots of (1/r) / (dc/dr) versus ($c-c_m$) [11]. SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli, using 12.5% gels and a Tris–glycine buffer system at pH 8.5 [12].

Enzyme inhibition studies were performed by incubating the protein with 10 mM iodoacetamide at pH 8, in 0.1 *I* Tris–HCl buffer at room temperature. Samples were taken at intervals over 30 min and assayed in the usual way. In another experiment the incubation mixture also contained 1 mM 6-phosphogluconate. Carboxymethylation, tryptic digestion and peptide separation were carried out as described and referred to by Jörnvall and Harris [13].

2.3. *Crystallisation*

One ml of pure enzyme solution (4 mg/ml) was dialysed against 200 ml of 0.1 *I*.sodium acetate buffer pH 6.0, containing 1 mM EDTA, 0.1% (v/v) β-ME and 10 mM NADP, brought to 30% saturation with saturated ammonium sulphate solution. The protein solution was clarified by centrifuging at 10 000 rev./min. One hundred μl drops were placed carefully in shallow depressions in a glass tray, which was then placed in a dish containing 6 ml of acetate buffer and 9 ml of saturated ammonium sulphate. The container was sealed and left undisturbed at room temperature for several days.

3. Results

3.1. *Purity of the enzyme*

After the first fractionations at Porton, in which a proportion of the 6PGD was removed in other enzyme fractions, the yield was about 20%. The 6PGD was then purified a further 20-fold with a 35% yield. Thus 300 mg of the crystalline enzyme, with a specific activity of 20 units/mg, were obtained from the 20 kg of *B. stearothermophilus* cells.

The preparation gave a single band when examined by SDS-polyacrylamide gel electrophoresis, as shown in fig. 1. Electrophoresis of the protein on Cellogel at pH 8.6, with 10 μM NADP in the electrode buffer, gave a single band staining for protein and for 6PGD activity. The homogeneity of the protein was also demonstrated by sedimentation velocity experiments. At pH 8 and 0.1 *I*, a single symmetrical boundary with an $S_{20,w}$ value of 5.8 S was observed.

3.2. *Properties of the enzyme*

The enzyme, from *B. stearothermophilus*, is stable at relatively high temperatures. The percentage activity remaining after heating for 15 min at various temperatures (0.25 mg/ml enzyme in 0.1 *I* sodium acetate pH 6.0/1 mM EDTA/0.1% β-ME/10 μM NADP) is shown in fig. 2. The enzyme retains full activity at 60°C (the growth temperature of the organism) although it is denatured at higher temperatures. The

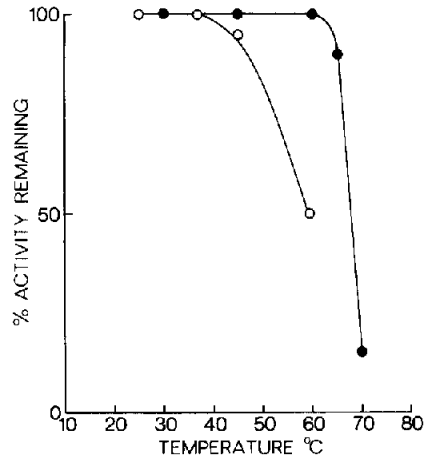

Fig. 2. Effect of temperature on the activity of *B. stearothermophilus* and human erythrocyte 6PGD. Solutions of the enzymes (0.25 mg/ml enzyme in 0.1 acetate pH 6.0/1 mM EDTA/0.1% β-ME/10 μM NADP) were incubated for 15 min at the indicated temperature and rapidly cooled in an ice bath before assay. (●-●-●) *B stearothermophilus* 6PGD, (o-o-o) Human erythrocyte 6PGD.

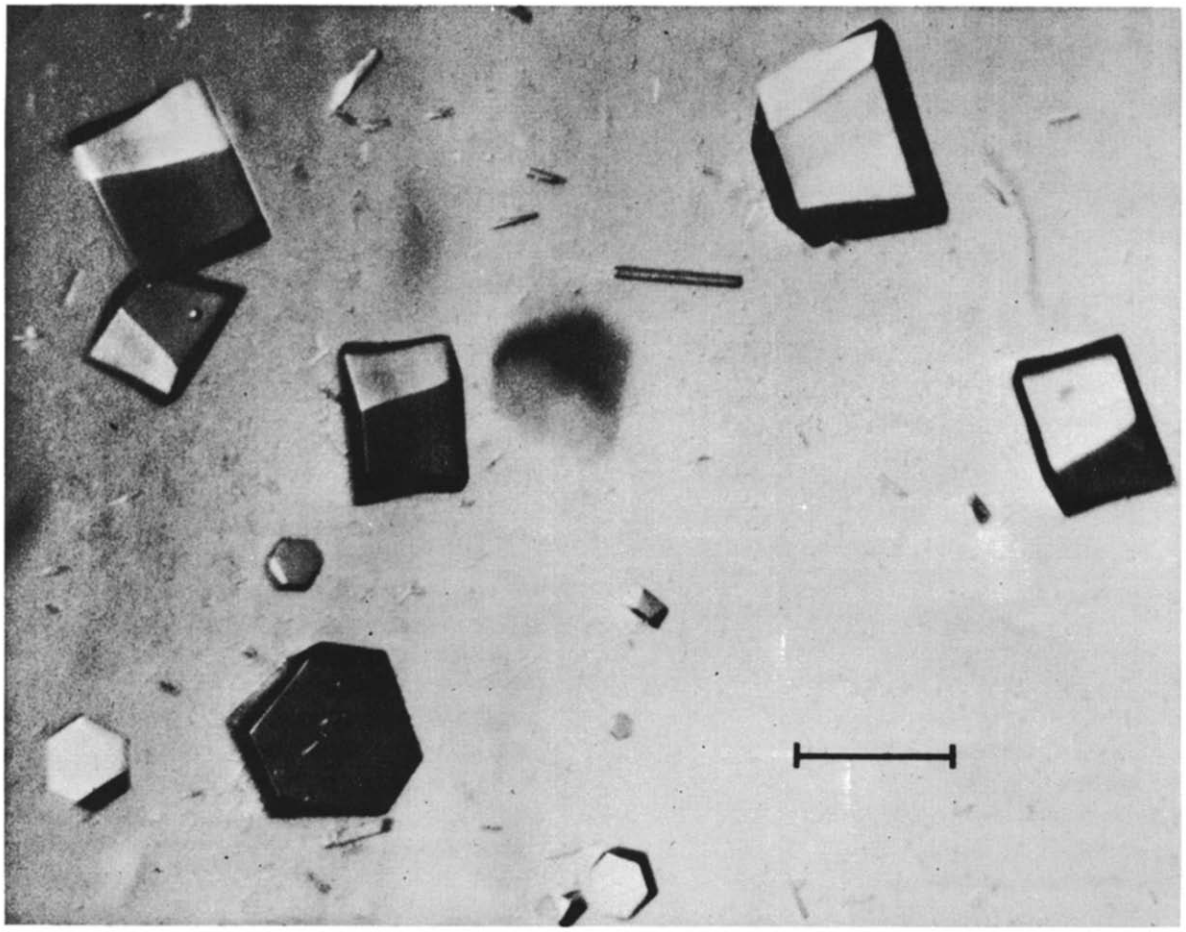

Fig. 3. Crystals of *B. stearothermophilus* 6PGD. The scale line represents 0.25 mm.

activity increases with temperature and an Arrhenius plot is approximately linear from 25°C to approx. 45°C. Above 45°C, the activity appears to rise more sharply with temperature, up to 60°C. This type of behaviour has also been observed for an ATPase from *B. stearothermophilus* [14].

The enzyme has a mol. wt. of 100 000 as determined by sedimentation equilibrium experiments at pH 6.0 and 1.1 *I*, pH 6.5 at 0.3 *I* and pH 8 at 0.1 *I*, with a protein concentration of 4 mg/ml. SDS-polyacrylamide gel electrophoresis (with marker proteins, bovine serum albumin, γ-globulin, GPDH and aldolase) showed that it is a dimeric enzyme, comprising two apparently identical subunits with a mol. wt. of 50 000.

When the enzyme was incubated with 10 mM iodacetamide, 80 % of the activity was lost after 30 min. The enzyme was, however, completely protected from this inactivation by the presence of its substrate, 6-phosphogluconate. Mapping of the tryptic peptides of the modified protein, after inactivation with [1-^{14}C]iodoacetamide, showed that the reaction resulted in the formation of a single radioactive peptide. The amount of radioactivity found in this peptide was about equivalent to that expected from the reaction of the inhibitor with two cysteines per mole (100 000 g) of enzyme. By contrast, if the protein was first denatured, and then treated with [2-^{14}C] iodoacetate, three equally radioactive peptides were detected. This indicated the presence of three unique cysteine residues in the primary structure.

3.3. *Crystal structure*

By the vapour diffusion technique, described in the Methods section, crystals measuring up to 0.2 mm in each dimension grew in a few days. Examples are shown in fig. 3. The crystals are distorted hexagonal prisms. Their symmetry is trigonal and the space group is $P3_121$ (or $P3_221$). The unit cell has dimensions a = b = 115 Å, c = 156 Å. There is probably one dimer with a mol. wt. of 100 000 per crystal asymmetric unit (unpublished results of D.M. Blow and M.J. Adams).

4. Discussion

Pure, crystalline 6PGD may be obtained from *B. stearothermophilus* in sufficient quantities for chemical structural studies. The specific activity (20 units/mg) at 25°C is of the same order of magnitude as that of the human erythrocyte enzyme (10 units/mg) at this temperature. The enzyme is much more stable to heat than that from red cells, which was previously purified [6]. Thus redissolved crystals retain complete activity after heating at 60°C for 15 min, whereas the human erythrocyte enzyme is 50% inactivated at this temperature (fig. 2). The enzyme preparation, in the storage conditions specified, retains full activity for at least six months.

In other respects, the thermophilic 6PGD resembles its counterparts from mesophilic sources. For instance, the *B. stearothermophilus* enzyme has the same dimeric sturcture (mol. wt. 100 000) as that established for 6PGD from other sources, including human erythrocytes [6] and *Candida utilis* [15]. Both the *B. stearothermophilus* and the human erythrocyte enzyme (unpublished work) are inhibited strongly by iodoacetamide. Both enzymes are protected from this inactivation by the presence of their substrate, 6-phosphogluconate. In the case of the *B. stearothermophilus* enzyme, the inhibition is due to reaction at a single cysteine residue. This group may therefore be near the binding site of the substrate and perhaps play a role in the functioning of the enzyme.

The thermal stability of the crystals of the *B. stearothermophilus* 6PGD are advantageous for future chemical and structural study. Knowledge of the spatial arrangement of the functional groups should provide an understanding of the role of NADP in the operation of this enzyme. A comparison can then be made with the role of NAD in the functioning of the glycolytic dehydrogenases.

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