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Phosphoglycerate kinase from the extreme thermophile *Thermus thermophilus*

Crystallization and preliminary X-ray data

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The glycolytic enzyme phosphoglycerate kinase has been prepared from the extreme thermophile *Thermus thermophilus*. In contrast to its eukaryote equivalents (yeast and horse muscle) this prokaryotic enzyme crystallizes from ammonium sulphate in the presence of nucleotide substrates. These crystals are of a form and size well suited to high resolution X-ray diffraction study using rotation camera techniques.

Glycolytic enzyme; Crystal growth; Nucleotide substrate; X-ray diffraction; (*Thermus thermophilus*)

1. INTRODUCTION

Phosphoglycerate kinase (PGK) is one of the two glycolytic enzymes responsible for the production of adenosine triphosphate (ATP) during glycolysis. The amino acid sequences of several different PGKs have been determined and in each case the enzyme has been found to be a monomer of approx. 45 kDa. High resolution X-ray structure studies of the horse [1] and yeast [2] enzymes have been reported. Not surprisingly both these structures are very similar and show that the molecule is made up of two quite discrete domains. The presence of a very basic region in the cleft between the two domains facing the binding site for the nucleotide moiety has led to the proposal that the enzyme exists in at least two forms [1]. The two predominant conformers can be thought of as an open or substrate binding form, for which the crystal structure is available, and a closed catalytically competent form.

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We have isolated and crystallized PGK from the extreme thermophile *Thermus thermophilus* with the intention of comparing this thermophilic enzyme with its mesophilic counterpart from yeast whose structure is already known. This comparison will then allow us to study, by genetically engineering into the yeast enzyme [3], those features of the thermophilic enzyme thought to be responsible for its stability at temperatures as high as 85°C.

We report here the procedure used to isolate and crystallize this enzyme. The crystals described are of a type ideally suited to X-ray diffraction study using rotation camera techniques [4]. They also represent the first crystals obtained of a prokaryotic PGK and are also unique in that, unlike their eukaryotic counterparts [1,2], they grow in the presence of nucleotide substrates.

2. MATERIALS AND METHODS

2.1. *Protein preparation*

Phosphoglycerate kinase was isolated from *T. thermophilus* HB-8 by a modification of the

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methods described by Nojima et al. [5]. 400 g of cells suspended in 2 l of buffer were homogenised by two passages through a Manton Gaulin press operated at 10000 psi. The buffer, buffer A, was made up using 0.05 M Tris-HCl adjusted to pH 7.5, 1 mM EDTA, 6 mM β-mercaptoethanol, 2×10^{-5} M phenylmethylsulphonyl fluoride (PMSF) and 2×10^{-5} M benzamidine (BAM). The cell debris was partially removed by centrifugation at 9000 rpm for 1.5 h. The resultant supernatant was applied directly to a pre-equilibrated DEAE Sephadex column, 2.5 × 30 cm, in buffer A. PGK bound to the column was eluted with buffer A containing 0.2 M NaCl. Solid ammonium sulphate (enzyme grade) was added, with stirring, to the column eluate until a concentration equivalent of 29 g/100 ml of solution was achieved. After standing overnight the precipitate was removed by centrifugation at 9000 rpm for 1 h. To each 100 ml portion of the supernatant a further 11 g of ammonium sulphate was added. After standing for a further hour the precipitate containing the PGK activity was collected by centrifugation as above. The precipitate was resuspended in buffer A and loaded onto a Sephacryl S200 column (5 cm × 100 cm) equilibrated in the same buffer. Fractions containing the relevant enzymic activity were pooled and dialysed against a buffer B containing 5 mM potassium phosphate, pH 6.5, 0.1 mM EDTA, 6 mM β-mercaptoethanol, 2×10^{-5} M PMSF and 2×10^{-5} M BAM. The dialysate was loaded onto a pre-equilibrated hydroxyapatite column (Biorad Bio-Gel HTP). Protein was eluted from the column with a linear gradient, 5 mM–40 mM NaCl, in buffer B. The active fraction, which still contained 10% contamination, was precipitated with ammonium sulphate and used directly for crystallization experiments and primary sequence analysis. In some cases the remaining contamination was removed by hydrophobic chromatography using phenyl-Sepharose (Pharmacia). The sample was applied to the column in buffer A containing 2 M NaCl and eluted with a reverse linear gradient, 2 M–0.2 M NaCl. Under these conditions the active fraction elutes from this column at a salt concentration of 1.7 M. All operations were carried out at 4°C.

The PGK enzyme was assayed at 22°C using a linked assay system with glyceraldehyde-3-phosphate dehydrogenase (yeast enzyme from Sigma or *Bacillums stearothermophilus* enzyme, a kind gift from Dr A. Wonacott). The change in absorbance of NADH was followed at 340 nm. The final assay solution in the cuvette contained 0.03 M triethanolamine, pH 7.5, 0.05 M KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM 3PGA, 4 mM ATP, 0.15 mM NADH and glyceraldehyde-3-phosphate dehydrogenase at from 1 to 30 mg/ml. The PGK protein concentration was determined spectrophotometrically using an $A^{1\%}_{1cm}$ of 5.0 at 280 nm. A final specific activity of 250 mol NADH converted/min per mg protein was obtained. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli and Favre [6] in order to check sample purity.

2.2. *Crystallization*

The protein was crystallized by the hanging-drop or sitting-drop vapour diffusion technique. The protein concentration was 4 mg/ml in the initial droplet in the presence of Pipes (piperazine-*N,N'*-bis[2-ethanesulphonic acid]) buffer, pH 6.8, 10 mM $MgCl_2$, 1 mM ATP, 10% $(NH_4)_2SO_4$. The precipitant was 37% $(NH_4)_2SO_4$ (enzyme grade) and crystallization was at 22°C.

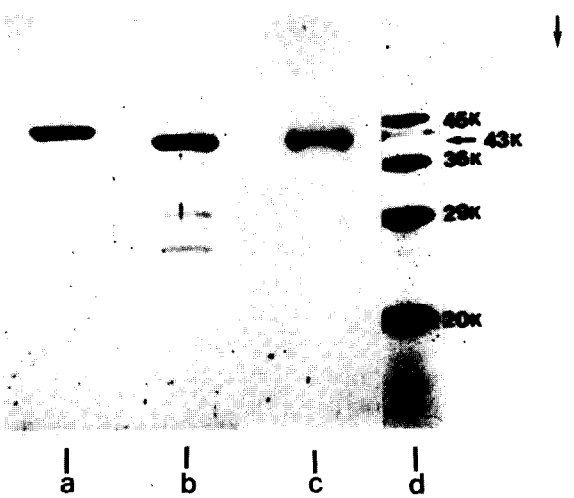

Fig.1. SDS-polyacrylamide gel showing: (a) yeast PGK; (b) *Thermus thermophilus* PGK; (c) redissolved *Thermus thermophilus* PGK crystal; (d) protein standards, yeast PGK (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and soya bean trypsin inhibitor (20 kDa).

2.3. *X-ray diffraction*

Diffraction patterns used for space group determination were recorded with a precession camera using a sealed X-ray tube producing Cukα radiation (Ni filter) with the generator operating at 40 kV and 30 mA.

3. RESULTS AND DISCUSSION

The yield of *T. thermophilus* PGK protein obtained was equivalent to 70 mg/kg of cells. The protein migrated on SDS-polyacrylamide gels [6] as a single band of molecular mass 43 kDa as is shown in fig.1. Gas-phase sequence analysis carried out on the complete peptide chain has helped determine the order of the first thirty or so amino acid residues. Using this information it has been possible to construct an oligonucleotide probe for use in the isolation of the *T. thermophilus* gene. This amino terminal sequence analysis also indicates that considerable homology must exist between this enzyme and its eukaryotic counterparts as is shown in fig.2.

Crystals of thermophilic PGK grow reproducibly in 7–14 days at room temperature from 37% $(NH_4)_2SO_4$ at 2–4 mg/ml protein. Large single crystals grow optimally at pH 6.8 and can be stored in a stabilising solution of 43% $(NH_4)_2SO_4$, Pipes buffer, pH 6.8, 10 mM $MgCl_2$, 1 mM ATP. The hexagonal shaped crystals such as

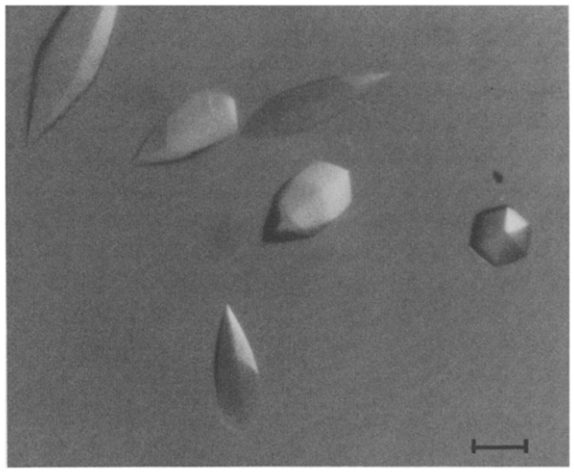

Fig.3. Crystals of *Thermus thermophilus* PGK. The bar represents a length of approx. 0.2 mm.

those shown in fig.3 are particularly stable to X-radiation allowing a new complete 3 Å resolution data set to be collected from a single specimen. Zero (see fig.4) and first level precession photographs show that the space group is either $P6_1$ or its enantiomorph $P6_5$ and that the unit cell dimensions are: $a = b = 144$ Å, $c = 77$ Å. Since the molecular masses of *T. thermophilus* and yeast PGKs are approximately the same (see fig.1), it can be shown from their respective crystal characteristics that the hexagonal unit cell must contain two molecules in each asymmetric unit.

PHOSPHOGLYCERATE KINASE N-TERMINAL SEQUENCE COMPARISON

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          1         5         10        15        20        25        30
          ¦         ¦         ¦         ¦         ¦         ¦         ¦
Yeast-  S L S S K L S V Q D L D L K D K R V F I R V D F N V P L D G K K
Horse-  S L S N K L T L D K L N V K G K R V V M R V D F N V P M K N - Q
Human-  S L S N K L T L D K L D V K G K R V V M R V D F N V P M K N N Q
Mouse-  S L S N K L T L D K L D V K G K R V V M R V D F N V P M K N N Q
T.br.B-S L K E R K S I N E C D L K G K K V L I R V D F N V P L D D G N
T.br.C-T L N E K K S I N E C D L K G K K V L I R V D F N V P V K N G K
Asp.nd-S L T S K L S I T D V D L K D K R V L I R V D F N V P L D K N D
T.th. -       M R T L L D L D P K G K R V L V R V D Y N V P V Q D G K
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Fig.2. The amino-terminal region of several eukaryotic PGKs compared with that obtained for the enzyme from *Thermus thermophilus*. The sequence obtained for the prokaryotic enzyme (*T.th*) was obtained using gas-phase sequence analysis. *T.br* and *Asp.nd* are abbreviations for *Trypanosoma brucei* and *Aspergillus nidulans*, respectively. The boxed regions enclose those residues which are totally conserved. The dashed line denotes the peptide region selected for construction of the oligonucleotide probe used to isolate the *Thermus thermophilus* gene. References to the sequence information are included in [7].

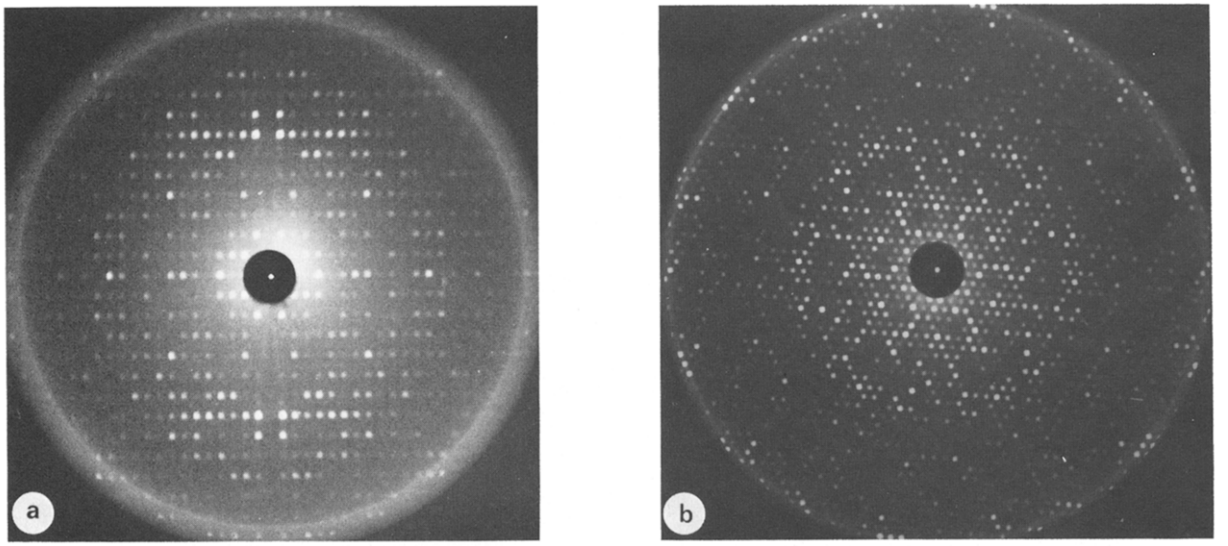

Fig.4. X-ray precession photographs of *Thermus thermophilus* PGK crystals with the 6-fold screw axis in (a) and at right angles (b) to the reciprocal lattice plane. Both diffraction photographs were recorded with precession angles of 7°.

The methods developed for growing crystals of yeast PGK fail to work if nucleotide substrates are added to the crystallizing solutions. Preformed crystals crack or shatter when relatively small quantities of nucleotide substrates are added to the surrounding mother liquor [1,2]. This phenomenon has been associated with a conformational change which is thought to take place during turnover and has so far thwarted all attempts to determine the structure of the closed or catalytically competent form of this enzyme. It is therefore of particular interest that crystals of *T. thermophilus* PGK grow in ammonium sulphate solutions only in the presence of added nucleotide substrates. These crystals can also be soaked in the second substrate, 3-phosphoglycerate, without losing crystallinity. We are fortunate therefore in that, in addition to providing us with a way of using evolution to investigate protein thermal stability, this study could also provide information about the catalytically competent form of a glycolytic kinase.

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