Yeast phosphoglycerate kinase: investigation of catalytic function by site-directed mutagenesis

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A salt link buried in the domain interface of phosphoglycerate kinase has been implicated as being important in controlling the conformational transition from the open, or substrate-binding, to the closed, or catalytically competent, form of the enzyme. The residues contributing to the salt link are remote from the active site, but are connected to the substrate-binding sites through strands of β -sheet. It has been suggested that these residues may also mediate sulphate and anion activation. These assumptions have been tested by examining the properties of a site-directed mutant (histidine-388 $\rightarrow$ glutamine-388). The expression and overall structural integrity of the mutant, produced in yeast from a multicopy plasmid, remains essentially unaltered from the wild-type enzyme. However, the mutant enzyme has a k_{cat} reduced by 5-fold. The K_m for ATP is lowered by 3-fold, and the K_m for 3-phosphoglycerate is unaffected. The effects of sulphate on activity over a wide range of substrate concentrations appear to be the same for both the mutant and wild-type enzymes. These results lead to a reappraisal of the mechanistic role of the inter-domain histidine–glutamate interaction, as well as a refinement of the kinetic model of the enzyme.

INTRODUCTION

Phosphoglycerate kinase (PGK; EC 2.7.2.3) has been isolated from over 20 different species (Fifis & Scopes, 1978) and, in all cases, whether glycolytic, gluconeogenic or photosynthetic, has been shown to be active as a monomer of about 415 amino acid residues catalysing the reaction:

The crystallographic structure of the yeast enzyme is known at high resolution (Watson *et al.*, 1982), and the primary sequence of both the protein and its encoding gene have been determined (Hitzeman *et al.*, 1982; Perkins *et al.*, 1983). The kinetics of the enzyme, although complex, have received much attention and are of the rapid-equilibrium random-order type (Larsson-Raznikiewicz & Arvidsson, 1971). The accessibility of the yeast gene to genetic manipulation, and the availability of extensive structural and kinetic data, thus provide the means to test and extend our understanding of the mechanism of this kinase.

The crystal structures of both the horse (Banks *et al.*, 1979) and yeast enzymes revealed that the PGK molecule is composed of two distinct lobes joined by residues 200–205 (yeast numbering) connecting major secondary structure elements forming part of each domain. Residues 390–401 form an α -helix which lies between the two domains, with the final 13 residues forming an integral part of the *N*-terminal domain. The adenine ring of the nucleotide substrate binds in a deep slot in the *C*-terminal domain with the ribose ring

and phospho chain extending towards the domain interface as is shown in Fig. 1. The triose-binding site is situated in the domain interface at the amino end of the penultimate helix. Largely on the basis of the domain structure and the crystallographically determined substrate-binding sites, a hinge-bending model was proposed to explain the mechanism of the enzyme (Banks *et al.*, 1979). This model suggested that the enzyme exists in two states: in the absence of substrates the molecule adopts an 'open' conformation as seen by crystallography, but in the presence of substrates the two domains come together to produce the 'closed' form. The two lobes are presumed to form a more compact structure when substrates bind in order to exclude water from the active site, to reduce the dielectric environment and prevent abortive hydrolysis.

Evidence supporting the hinge-bending model came initially from three sources. Ultracentrifugation (Roustan *et al.*, 1980) and low-angle X-ray scattering (Pickover *et al.*, 1979) measurements both appeared to indicate that a more compact structure is formed when substrates bind. Modelling experiments based on these results indicated that the two lobes rotate by as much as 13° about a point in the domain interface. Distance mapping based on n.m.r. experiments (Tanswell *et al.*, 1976) has also revealed a conformational state where the ATP-binding site is much closer to the *N*-terminal domain than that seen in the crystal structure. Apart from this direct evidence for domain movement, the hinge-bending model also provides feasible explanations of chemical modification results (for example, Hjelmgren *et al.*, 1976; Markland *et al.*, 1975) and for kinetic experiments (Scopes, 1978a).

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Fig. 1. Schematic drawing of the yeast PGK molecule

The cylinders represent helices and the arrows are β -strands. The elements of the secondary structure are connected by lines drawn between each α carbon. The substrates ATP and 3-phosphoglycerate are drawn in space filling mode, as is the marked magnesium ion. The inset to the left depicts the position and nature of the glutamate-190/histidine-388 interaction, and the inset to the right shows the point mutation as built into the native enzyme. The position of the glutamine side chain relative to that of the carboxyl group of residue 190 was chosen by molecular graphics techniques. The drawing was produced with the help of a computer program written by Lesk & Hardman (1982).

Following the determination of the high-resolution structure of the yeast enzyme, the original hinge-bending model proposed by Blake and co-workers (Banks *et al.*, 1979) was extended by studies on the yeast enzyme (Watson *et al.*, 1982) to include a mechanism for the transfer of substrate-binding information to the hinge region. Examination of the domain interface at the hinge region revealed a hydrophobic pocket containing a buried glutamate. This residue is associated with the last α -helical segment of the *N*-terminal domain, and its carboxyl group forms a salt link with the imidazole ring of a histidine residue, as is shown in Fig. 1. The histidine extends from the *C*-terminal domain and is associated with the β -sheet structure which forms an integral part of both substrate-binding sites. Both glutamate-190 and histidine-388 (yeast numbering) are conserved in all known PGKs.

The conserved nature of the buried salt linkage, its unique environment surrounded by conserved aromatic residues, and its location relative to both substrate-binding sites suggest that this residue interaction may form part of the mechanism for controlling domain movement. In order to test this hypothesis, histidine-388

was changed to a glutamine residue by site-directed mutagenesis, and the properties of the resulting mutant enzyme have been compared with those of the wild-type molecule.

EXPERIMENTAL

Materials

Restriction endonuclease *Hind*III, DNA polymerase I large subunit (Klenow) and T4 DNA ligase (used for site-directed mutagenesis) were from Bethesda Research Laboratories (U.K.) Ltd. Restriction endonucleases *Bgl*II and *Bam*HI, T4 DNA ligase (used to form M13mp9-PGK), calf intestinal alkaline phosphatase, and polynucleotide kinase were from Boehringer Corporation (London) Ltd. [α - 32 P]dATP, [γ - 32 P]dATP and DNA sequencing materials were from Amersham (U.K.) Ltd. All restriction enzymes were used as recommended by the suppliers.

The following bacterial and yeast strains were used:

Escherichia coli JM101: $\Delta(lac-pro)$, *thi*, *supE*, *F'**traD36*, *proA*+*B*+, *lacI*^g, *lacZ* Δ M15
E. coli BMH 71-81 mutL: *mutL*, $\Delta(lac-pro)$, *supE*, *thi*/*F'**lacI*9, *lacZ* Δ M15, *proB*+*A*+
Saccharomyces cerevisiae MT302/28b: *leu2*-112, *leu2*-3

Oligonucleotide synthesis

The Omnifit manual DNA synthesizer was used to produce oligonucleotides by the phosphotriester method, as described by Sproat & Gait (1984). The deprotected oligonucleotide was purified by gel filtration on a Bio-Gel P2 column, and by polyacrylamide-gel electrophoresis. Protected mononucleotides, functionalized supports and mesitylene sulphononitrotriazole were from Cruachan Chemical Co. Ltd. 1-Methylimidazole was from Aldrich Chemical Co. Ltd., and was redistilled under vacuum with the temperature kept below 40 °C.

Site-directed mutagenesis

The gapped duplex prepared as shown in Fig. 2 was collected by ethanol precipitation. About 30 pmol (0.15 μ g) of the oligonucleotide specifying the single mismatch was added with 2 μ l of 200 mM-Tris-HCl/100 mM-MgCl₂, pH 7.8, and the volume was made to 17 μ l with sterile distilled water. The oligonucleotide was annealed by incubating at 65 °C for 1 min and at room temperature (20–23 °C) for 10 min, followed by chilling on ice. The extension/ligation reactions were done by adding 1 μ l of 10 mM-dATP, 1 μ l of a mixture containing 10 mM of all four dNTPs, 1 μ l (0.2 unit) of DNA polymerase Klenow fragment and 1 μ l (2 units) of T4 DNA ligase. The mixture was incubated at 15 °C for 1 h, and samples were taken for transfection of *E. coli* mutL cells, followed by plating out with *E. coli* JM101 cells.

Transformation of yeast

Recombinant plasmid pMA40b-*PGK (1 μ g) containing the mutant PGK gene was used to transform *S. cerevisiae* MT302/28b by the method of Hinnen *et al.* (1978). In a parallel experiment, plasmid pMA27 (1 μ g) containing the wild-type PGK gene was used for transformation of the same strain of *S. cerevisiae*.

Fig. 2. Strategy of site-directed mutagenesis

Plasmid pMA27 (top left) is derived from pBR322, the yeast 2μ plasmid, and a *Hind*III fragment from yeast chromosome III containing the PGK gene (Dobson *et al.*, 1982; Wilson *et al.*, 1984). The letters Bg, H, R and Ba denote sites of digestion by *Bgl*II, *Hind*III, *Eco*RI and *Bam*HI. (a) Plasmid pMA27 and phage M13mp9 were digested with *Hind*III (3 units/ μ g of DNA for 90 min at 37 °C), and the linear form of M13mp9 was treated with alkaline phosphatase. The 2.95 kbp fragment containing the PGK gene was purified by agarose gel electrophoresis. (b) The products of the ligation reaction (T4 DNA ligase, 4 units/ μ g of M13mp9 and 1.5 μ g of *Hind*III fragment, overnight at 4 °C) were used to transfect *E. coli* JM101. (c) Single-stranded DNA was prepared from white plaques and, as expected, was of two types, A or B, depending on the orientation of the *Hind*III fragment containing the PGK gene. Orientation A was chosen for the subsequent steps. (d) Phage M13mp9 was digested with *Hind*III and *Bam*HI (4 units of *Hind*III and 1 unit of *Bam*HI/ μ g of DNA for 90 min at 37 °C). The large linear fragment comprising most of the phage was separated from the small fragment by ethanol precipitation. (e) Circular, single-stranded M13mp9-PGK (0.5 μ g) and the linear *Hind*III/*Bam*HI fragment from M13mp9 (1 μ g) were mixed in 20 μ l of 0.16 M-NaCl, heated at 100 °C in a heated block for 10 min, cooled slowly (in the heated block) to 65 °C, and allowed to anneal at 65 °C overnight. Excess M13mp9 *Hind*III/*Bam*HI fragment was used to maximize the formation of gapped duplexes. (f) The gapped duplex thus formed was used as a template to direct mutagenesis. The oligonucleotide specifying a single T $\rightarrow$ A replacement

Enzyme assay

PGK activity was measured in the kinase direction by coupling with the glyceraldehyde-3-phosphate dehydrogenase reaction, and monitoring the decrease in absorbance at 340 nm (Scopes, 1975). Assays were done essentially as described by Scopes, initial velocity measurements being expressed as $V = (dA_{340}/dt)$ at $t = 0$ (in min^{-1}). All enzymes used were extensively dialysed to remove any traces of sulphate. Protein concentration of the PGK preparations was determined by amino acid analysis (Russell *et al.*, 1986) using the known amino acid composition of PGK.

ATP, NADH and 3-phosphoglycerate (calcium salt) were obtained from Sigma Chemical Co. Other chemicals used were of the AR grade. Experiments were done at a fixed free Mg^{2+} concentration of 1 mM. K_d values of 0.025 mM for MgATP^{2-} and 10 mM for 3-phosphoglycerate were used (see Scopes, 1978a), so that stock solutions of ATP and 3-phosphoglycerate were made up with 0.98 and 0.09 molar equivalents of Mg^{2+} , respectively.

RESULTS

Oligonucleotide-directed mutagenesis

Two aspects of the general strategy shown diagrammatically in Fig. 2 were found to be particularly important for the successful isolation of the mutant gene. Firstly, the use of the gapped duplex formed in step (e) for the extension/ligation reaction in step (f) ensured that the reaction time could be relatively short, as only a small portion of the circle needed to be synthesized. A short reaction time was important to minimize the problems encountered with slight contaminating nuclease activity. The recovery of complete double-stranded circles was very poor if the single-stranded form of M13mp9-PGK alone was used as a template. Secondly, the use of the repair deficient *mutL* strain of *E. coli* for transfection in step (f) was crucial. No mutant M13mp9-*PGK was obtained if strain JM101 alone was used, presumably

indicated by * was added, together with the Klenow fragment of DNA polymerase I, four dNTPs and T4 ligase. After extension and ligation, this mixture was used to transfect the *mutL* repair-deficient strain of *E. coli*, and then plated with *E. coli* JM101. Plaques producing single-stranded phage with the mutant PGK gene were detected by slot-blotting. The presence of the T $\rightarrow$ A base change was verified in one case by dideoxynucleotide sequencing with a synthetic primer complementary to a region near the mutation site. These colonies were used to transfect *E. coli* JM101, and the replicative form of the phage was purified. (g) The 2.95 kbp *Hind*III fragment with the mutant PGK gene was purified by agarose gel electrophoresis, and ligated to the large *Hind*III fragment comprising most of plasmid pMA40b. (This plasmid is very similar to pMA27, but has the *Eco*RI fragment in the opposite orientation so that only a relatively small piece of DNA is lost during the *Hind*III digestion/ligation experiment.) Only one of the two possible orientations of the PGK-containing fragment is shown. (h) The plasmid with the mutant PGK gene was used to transform yeast MT302/28b. The yeast cells were lysed with ammonia, and the mutant PGK isolated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration.

because of the normal mismatch recognition and repair system. However, the use of the *mutL* strain alone would run the risk of introducing extra unwanted mutations. Therefore transfection was initially with the *mutL* cells, which were then plated with JM101 cells. This minimized the number of times the M13mp9-*PGK molecules were synthesized in the mutation-prone *mutL* cells. It was clearly an advantage to propagate the double-stranded form of M13mp9-*PGK (which no longer had a mismatch) in the normal JM101 strain of *E. coli*.

Expression and isolation of mutant and recombinant wild-type PGK

Plasmids pMA40b-*PGK and pMA27, containing mutant and wild-type PGK genes respectively, were used to transform yeast (strain MT302/28b), which was then grown in liquid culture. Cells were harvested by centrifugation, freeze-dried, and lysed overnight in 0.5 M-ammonia/0.1% EDTA. SDS/polyacrylamide-gel electrophoresis of the crude extract showed a very high level of expression of both mutant and wild-type PGK, corresponding to about 80% of the proteins present. Material precipitated at 50% saturated $(\text{NH}_4)_2\text{SO}_4$ was discarded, and the remaining PGK protein collected at 80% saturated $(\text{NH}_4)_2\text{SO}_4$, pH 4.3 (adjusted with 0.5 M-lactic acid). The sample was then treated by gel filtration on Sephacryl S-200. The PGK so obtained was judged pure by SDS/polyacrylamide-gel electrophoresis, and by its absorption profile between 240 and 300 nm. About 100 mg of PGK was obtained from each gram of dried yeast (4 litres of culture).

Characterization of mutant and recombinant wild-type PGK

Both mutant and recombinant wild-type enzymes were cleaved with cyanogen bromide, and the fragment corresponding to residues 268-415 was isolated from each enzyme by gel filtration, following the procedure developed by Perkins *et al.* (1983). Tryptic peptides derived from these fragments were separated by high-pressure liquid chromatography (Fig. 3). Automated

Fig. 3. Isolation and sequence analysis of tryptic peptides corresponding to residues 386-403

Mutant and wild-type PGK were treated with CNBr, and the fragment corresponding to residues 268-415 was purified by gel filtration (Perkins *et al.*, 1983). Each fragment (200 nmol) was dissolved in 1 ml of 1% (w/v) NH_4HCO_3 and digested with trypsin (50 μg) for 22 h at 37 °C, and freeze-dried. The digestion mixture was dissolved in 3 ml of 0.1% trifluoroacetic acid, and peptides in a 0.3 ml sample were separated on a Waters Associates C_{18} $\mu\text{Bondapak}$ column eluted with a linear gradient of 0.1% trifluoroacetic acid and propan-2-ol as indicated in the two elution profiles: (a) peptides from wild-type PGK; (b) peptides from mutant PGK. The results of *N*-terminal sequence analysis of peak 7 material was Ile-Ser-His-Val-Ser-Thr-Gly-Gly-Gly-Ala- for the wild-type peptide, and Ile-Ser-Gln-Val-Ser-Thr-Gly-Gly-Gly-Ala- for the mutant peptide. The sequencing was done as described previously (Russell *et al.*, 1986) with a Beckman 890C liquid-phase sequencer and high pressure liquid chromatography separation of the phenylthiohydantoin amino acids. The initial yields were about 20 nmol, and the repetitive yields were 94% for both peptides.

Fig. 4. Activity of the native and mutant enzyme

(a) The kinetics of the native (▲—▲) and mutant (×—×) enzymes at various MgATP concentrations. The 3-phosphoglycerate concentration was 2.5 mM, NADH was 0.15 mM and sulphate was 40 mM. (b) The overall effects of sulphate on phosphoglycerate kinase activity. MgATP and 3-phosphoglycerate concentrations were 0.5 mM (▲—▲) and 5.0 mM (□—□) for the wild-type enzyme, and 0.5 mM (×—×) and 5.0 mM (■—■) for the mutant. NADH concentration was 0.15 mM.

sequence analysis of a peptide from the mutant PGK which eluted slightly later from the reverse-phase column than its wild-type counterpart (peak 7 in Fig. 3) showed that it had the following *N*-terminal sequence: Ile-Ser-Gln-Val-Ser-Thr-Gly-Gly-Gly-Ala-. Apart from the glutamine substitution in the third position (residue 388), this sequence corresponds to that of the wild-type enzyme between residues 386 and 403.

Parallel kinetic measurements have been made with both the mutant and wild-type enzymes. The relevant results suggest that the Michaelis constant for ATP but not for 3-phosphoglycerate is significantly altered by the mutation at position 388. The Lineweaver-Burk plot shown in Fig. 4(a) indicates that the K_m for ATP is lower for the mutant enzyme by a factor of about three (0.11 instead of 0.32 mM). Efforts to determine comparable Arrhenius plots were repeatedly thwarted by the loss of

activity of the mutant enzyme but not the wild-type enzyme, suggesting that the point mutation has reduced the overall stability of the enzyme.

Anions (especially sulphate) act as PGK effectors, being activators at low concentrations, but inhibitors at high concentrations (Khamis & Larsson-Raznikiewicz, 1981; Larsson-Raznikiewicz & Jansson, 1973). Scopes (1978b) has added to these observations by showing that such effects are dependent on the concentrations of the respective substrates. The results shown in Fig. 4(b) indicate that sulphate elicits the same complex activity response from both mutant and wild-type enzymes over a wide range of substrate concentrations.

DISCUSSION

A comparison of five different sequences of PGK from phylogenetically distant organisms [human, horse, yeast and trypanosome B and C isoenzymes (Osinga *et al.*, 1985)] shows that the molecular architecture around the domain interface is highly conserved. A naturally occurring mutant of the human enzyme (Fujii & Yoshida, 1980), and specific residue modification experiments (Hjelmgren *et al.*, 1976; Markland *et al.*, 1975), also indicate that this region of the molecule is critical for catalysis. The present results show that the mutation of histidine-388 has a measurable effect on the activity of yeast PGK. This single mutation reduces the catalytic constant by approximately 5-fold, and reduces the K_m for ATP approximately 3-fold. One consequence of the mutation is that the negative charge associated with the buried glutamate-190 cannot be neutralized by the uncharged glutamine-388. How has this change reduced the turnover and 'improved' the binding of ATP?

Initial rate measurements indicate that the enzyme has two binding sites for 3-phosphoglycerate, MgATP and sulphate ions (Khamis & Larsson-Raznikiewicz, 1981). These experiments also indicate that sulphate binding at the high affinity (or activating) site does not affect the K_m values for either 3-phosphoglycerate or MgATP. In contrast, binding at the low affinity site (implicated as being the active site) shows competitive inhibition with both substrates. Equilibrium gel filtration experiments (Scopes, 1978b) show, however, that sulphate, even at low concentrations, competes with 3-phosphoglycerate, 1,3-bisphosphoglycerate and MgATP, but not MgADP. Scatchard plots from the equilibrium gel filtration studies also show two binding sites for adenine nucleotide, but only one for phosphoglycerates (Scopes, 1978b).

It is tempting to speculate that the apparent anomalies in the kinetic data can be accommodated by a model invoking domain movement. Simply stated, the initial rate measurements on the ternary complex might be characteristic of a population predominantly in the closed form, whilst the equilibrium gel filtration experiments were done on the binary complex where the enzyme is likely to exist as the open conformer. This model might explain the differences in the number of binding sites, and the discrepancies about interactions between sulphate and substrates. Scopes has proposed a mechanism, however, which would account for the observed kinetic parameters by assuming that both sulphate and substrate bind only at the active site (Scopes, 1978b). In this model the release of 1,3-

bisphosphoglycerate is regarded as rate limiting, and composed of a two-step dissociation of each of the two phosphates. It is envisaged that this two-step dissociation is enhanced by substrates and by sulphate, and that secondary binding sites need not necessarily be relevant to the kinetic behaviour of the enzyme.

Treatment of yeast PGK with tetranitromethane leads to partial inactivation of the enzyme (Hjelmgren *et al.*, 1976; Markland *et al.*, 1975) concomitant with the nitration of tyrosine-193 (Perkins *et al.*, 1983). This specific modification does not appear to affect the K_m values for the substrates, although MgATP, but not 3-phosphoglycerate, can protect against nitration (Hjelmgren *et al.*, 1976). The crystal structure shows that residue 193 is remote from the active site, but located adjacent to the salt link bridging the two domains. It has been pointed out (Watson *et al.*, 1982) that nitration of this tyrosine would result in a relative displacement of the N- and C-terminal domains, thus preventing formation of the glutamate/histidine interaction observed in the open form of the structure.

It can be argued that the binding of MgATP at the active site may be responsible for perturbations at the domain interface which makes tyrosine-193 inaccessible to tetranitromethane. Alternatively, the location of a second nucleotide binding site (Khamis & Larsson-Raznikiewicz, 1981) located close to tyrosine-193 would help explain the protection against nitration afforded by MgATP. Kinetic studies with the mutant enzyme show that sulphate activation (see Fig. 4b) and substrate concentration effects are the same as with the wild-type enzyme. These findings demonstrate that the buried salt linkage associated with histidine-388 in the wild-type enzyme does not mediate the complex activation patterns of the enzyme.

Sequence conservation substantiates the suggestion that the domain interface is critical for the structural integrity of the enzyme. Even though it is spatially distinct from the active site, minor modifications to this region of the molecule affect catalysis. The lack of a sufficiently sensitive monitor of the conformational state of the enzyme in solution makes it difficult to assess the effect of the present mutation on domain orientation. The unaltered K_m for 3-phosphoglycerate suggests that any perturbation in enzyme structure/function cannot be simply accommodated in terms of a change in the equilibrium populations of open and closed states. The present work would suggest, however, that further mutagenesis and characterization experiments, including a residue change which will permit the introduction of a fluorescence label, will help to determine the function of the domain interface residues which play a significant role in the enzymic reaction.

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