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Site-directed mutagenesis of yeast phosphoglycerate kinase

Arginines 65, 121 and 168

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In the absence of a structure of the closed form of phosphoglycerate kinase we have modified by site directed mutagenesis several of the residues which, on the basis of the open form structure, are likely to be involved in substrate binding and catalysis. Here we report on the kinetic and anion activation properties of the yeast enzyme modified at positions 65, 121 and 168. In each case an arginine, thought to be involved in the binding of the sugar substrate's non-transferable phosphate group, has been replaced by lysine (same charge) and by methionine (no charge). K_m values for 3-phosphoglycerate of all six mutant enzymes are only marginally higher than that of the wild-type enzyme. Removing the charge associated with two of the three arginine residues appears to influence (as judged by the measured K_m's) the binding of ATP. Although binding affinity is not necessarily coupled to turnover the substitutions which have the greatest effect on the K_m's do correlate with the reduction in enzymes maximum velocity. The one exception to this generalisation is the R65K mutant which, surprisingly, has a significantly higher k_{cat} than the wild-type enzyme. In the open form structure of the pig muscle enzyme each of the three substituted arginines residues are seen to make two hydrogen bonds to the sugar substrate's non-transferable phosphate. From this it might be expected that anion activation would be similarly affected by the substitution of any one of these three residues. Although the interpretation of such effects are complicated by the fact that one of the mutants (R65M) unfolds at low salt concentrations, this appears not to be the case. Replacing Arg^{121} and Arg^{168} with methionine reduces the anion activation whereas a lysine in either of these two positions practically destroys the effect. With the substitutions at residue 65 the opposite is observed in that the lysine mutant shows anion activation whereas the methionine mutant does not.

Glycolytic enzyme; Site-directed mutagenesis, Enzyme kinetics; Sugar substrate

1. INTRODUCTION

Phosphoglycerate kinase (EC 2.7.2.3) is the monomeric glycolytic enzyme responsible for catalysing the reaction

$$MgADP + 1,3\text{-DPG} \rightleftharpoons MgATP + 3\text{-PGA}.$$

Crystallographic structures are available for this protein from both eukaryote and prokaryote sources [1–4]. For both types of organism the structure of the protein is homologous and is seen to be composed of two distinct domains connected by a narrow waist region. It has been proposed that the enzyme undergoes a substrate-induced domain movement to generate the catalytically active form. The movement has been described as a mutual rotation of the two domains such that its ternary complex is more compact than the substrate-free or 'open' form of the enzyme [5]. The inference, that substrate movement generates the active or 'closed' conformer of the enzyme, lends itself readily to the ideas of Koshland [6] and later, Steitz and co-workers [7], who pointed out that kinases have a particular need to exclude water from their active sites. In other words these enzymes have mechanisms to prevent hydrolysis of the high energy phosphoester bond of the substrates by water.

The structural evidence suggests that the enzyme's active site is split into two separate parts which only come together when both substrates are bound. The domain movement necessary to form the catalytically competent active site presumably prevents water from interfering with catalysis and also facilitates nucleophilic attack of the phosphoryl acceptor by producing a low dielectric environment. There are several lines of evidence which appear to support this proposal. The domain structure and catalytic function of PGK is, in some respects, very similar to that of hexokinase [8] for which this kind of hinge-bending motion has been reasonably well documented. Blake and his colleagues first suggested that PGK underwent such a conformational change on the basis of the substrate binding sites deduced from their crystallographic studies. While the nucleotide binding site was determined by crystal soaking experiments, they inferred the binding site for the triose substrate on the basis of a cluster of positively charged

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residues on the N-terminal domain of the enzyme facing the bound nucleotide. These two sites were such that the two domains would have to move together in order to bring the substrates close enough to react. Following on from this proposal two pieces of experimental evidence became available which supported the general tenet of substrate induced domain movement in PGK.

Firstly, using low angle X-ray scattering from solutions of PGK, Pickover et al. [9] were able to show that the radius of gyration of the enzyme decreased by about 1 Å on adding substrates. Subsequently Roustan and his colleagues used ultracentrifugation techniques to measure the sedimentation coefficient of the enzyme in the presence and absence of substrates and anions [10]. Those workers found a change in the $S_{20,W}$ of 0.7 (3.2–3.9) on going from the apo to the ternary complex of the enzyme, and also on adding 10 mM ammonium sulphate to the apo form. Both sets of experimental results were similarly interpreted in terms of a substrate/anion-induced conformational change in the protein.

In an attempt to determine the conditions for the crystallisation of the closed form of the yeast enzyme we have carried out a wide range of ultracentrifugation experiments on yeast PGK. No significant change in the sedimentation value of the enzyme on the formation of the ternary complex or the addition of anions [11] has been observed even though the experimental conditions have been varied exhaustively. In the light of those results, and our inability over several years to crystallise any form of the yeast enzyme other than the open form, we have embarked on a series of site-directed mutagenic studies. These studies have concentrated on changing those residues facing the nucleotide binding site which could be responsible for sugar binding, the rationale behind these experiments being that if such residues were involved in substrate binding then their replacement by structurally similar but different amino acid residues would affect the catalytic properties of the enzyme and thus prove that domain movement, no matter how transient, occurs during turnover.

Since this work began Blake and his colleagues have succeeded in crystallising the pig enzyme in the presence of 3-PGA [3]. We have also succeeded in crystallising the enzyme from *Bacillus stearothermophilus* in the presence of the nucleotide substrate [4]. Both the binary complexes are of the open form of the structure but they are significant in that they help define a set of interactions for the relevant substrate. In this paper we describe the kinetic and ion-binding results obtained using mutant enzyme which differs from the wild-type enzyme for those residues determined by Blake and his co-workers to be involved in binding the non-transferable phosphate in the binary complex.

2. MATERIALS AND METHODS

ATP disodium salt and NADH disodium salt were purchased from Boehringer-Mannheim Biochemica, whilst D(-)3-phosphoglyceric acid trisodium salt was purchased from Sigma Chemical Co., Poole, UK. Enzyme grade ammonium sulphate was obtained from BDH Ltd., Poole, UK. All other chemicals were of AnalaR quality. *Bacillus stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was kindly supplied by Dr. Alan Wonacott (Imperial College, London).

2.1. *Site-directed mutagenesis*

Yeast PGK mutants were generated using oligonucleotide-directed, site-specific mutagenesis based on the double primer method of Zoller and Smith [12]. Utilising a vector previously described as pYE-PGK [13], mutants were overexpressed in *Saccharomyces cerevisiae* (Cir$^+$ Leu 2-3 112 Trp 1-1 his 3-15 Ura 3-52 PGK::Trp1) kindly supplied by Dr. Peter Piper (University College, London). Competent cells were prepared by a modified version (Piper, personal communication) of the lithium acetate transformation procedure described by Ito et al. [14]. Overexpressed protein was extracted and purified by alkaline lysis, ammonium sulphate fractionation and FPLC gel filtration as previously described [13].

2.2. *Kinetic analysis*

Michaelis–Menten kinetic parameters were determined in the direction of 1,3-diphospho-D-glycerate formation by a coupled assay with GAPDH. The measurement of initial rate was based on a decrease in absorbance of NADH at 340 nm (25°C). The reaction was performed in 30 mM triethanolamine-HCl, pH 7.5, containing 40 mM $(NH_4)_2SO_4$, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.15 mM NADH and 1 $\mu g \cdot ml^{-1}$ GAPDH. The concentration of free magnesium was maintained at 1 mM. Parallel kinetic experiments were performed so as to eliminate possible errors when comparing kinetic parameters obtained for the mutant with those of the wild-type enzyme.

The determination of K_d values for 3-PGA was made by ^{1}H NMR methods as previously described [15]. A relative molecular mass of 45,800, determined from amino acid sequence analysis of the yeast enzyme, was used to calculate k_{cat} values. The effect of sulphate on the enzyme activity of the mutant PGK's was compared with that of the wild-type enzyme. Both substrates were maintained at 0.5 mM, whilst the ammonium sulphate concentration was varied between 0–50 mM. The remaining assay conditions were as outlined above.

3. RESULTS

The crystallographic results of Harlos et al. [3] indicate that the phosphate of 3-PGA is bonded to the guanidinium groups of three arginines, at positions 65, 121 and 168 (yeast numbering), as is shown in diagrammatic form in Fig. 1. Each of these three residues has been replaced in separate experiments by a lysine (same charge) and a methionine (no charge). All six mutant enzymes have been crystallised in the same space group and with essentially the same unit cell dimensions as the wild-type enzyme. All six mutant enzymes have been studied by ^{1}H NMR using methods similar to those described previously [15]. The detailed X-ray and NMR results will be published elsewhere. In this communication we refer to the results of the X-ray and ^{1}H NMR experiments only where they have a bearing on the interpretation of the kinetic results presented in Table I and Fig. 2. While this work was in progress a study involving the replacement of Arg^{65} in yeast PGK by glutamine, serine and alanine was reported [17]. The results of those studies, although different in detail (different substitutions) provide similar information to the

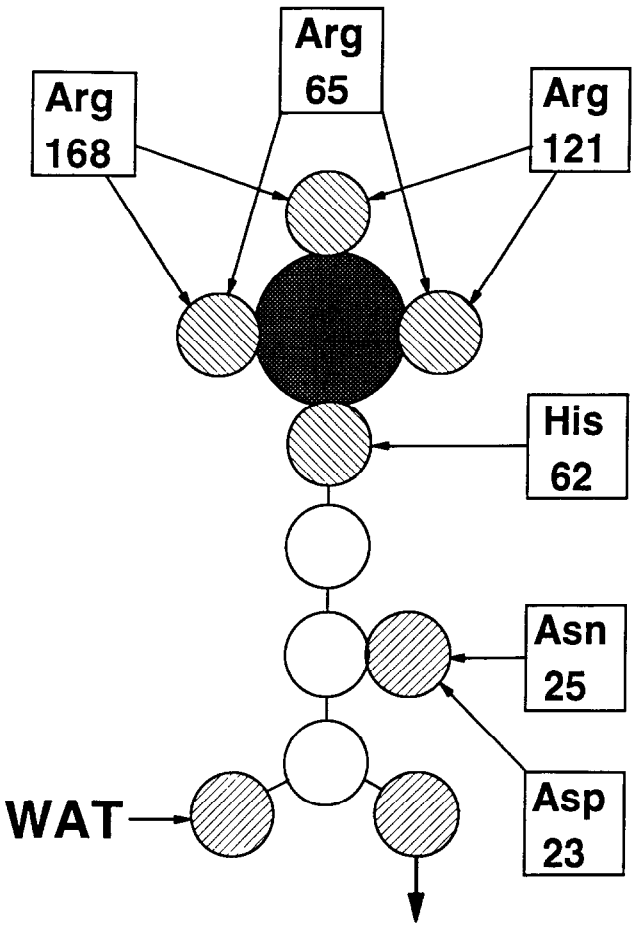

Fig. 1. A diagrammatic representation of 3-PGA binding as observed in a pig muscle binary complex [3]. Atoms are represented by circles. The phosphorous atom is shaded and the oxygen atoms are crossed by parallel lines. The arrows represent the proposed bonds between the sugar substrate and the enzyme side chains.

experiments reported here for the replacement of residue 65.

3.1. *Arg*^{65}

The R65K substitution has had no effect on the enzyme's K_m for MgATP, although a 2.2-fold increase in the K_m for 3-PGA is observed. This observation is consistent with the 3.6-fold increase in the K_d for this substrate. In addition to the increase in K_m and K_d seen for 3-PGA, a 1.5-fold increase in the k_{cat} is observed. As a result of the decrease in the enzyme's affinity for 3-PGA but increase in enzyme turnover, the catalytic efficiency of the enzyme is not significantly affected by the R65K substitution.

The R65M substitution results in a 4.9- and 20.6-fold increase in the K_m's for MgATP and 3-PGA, respectively. Both the X-ray and ^{1}H NMR data indicate that this substitution causes a significant structural perturbation close to the site of the mutation. These observations are consistent with the 7-fold decrease in k_{cat} and the concomitant 145-fold decrease in the enzyme's catalytic efficiency. The ^{1}H NMR results indicate that the K_d for 3-PGA exceeds 0.5 mM, a value which represents a 45-fold reduction in the enzyme's affinity for that substrate.

Fig. 2a shows that the R65K substitution reduces the salt activation effect observed with the wild-type. The same figure shows that the effect of the R65M mutation is to effectively abolish salt activation. This may be explained, in part, by the fact that the N-terminal domain of this mutant appears to unfold at low salt concentrations, as judged by the relevant NMR spectra. Perhaps the residual activity results from the re-folding of the domain, a feature which is supported by the fact that in high salt enzyme crystals can be obtained which are isomorphous with those of the wild-type enzyme.

3.2. *Arg*^{121}

The effect of both the R121K and R121M substitutions on the steady-state kinetics of PGK are almost identical. Both mutations result in an approximately 2-fold increase in the K_m for 3-PGA, whilst neither affect the K_m for MgATP. In the absence of salt, the solubility of both mutants is less than that of the wild-type enzyme. This complicates the NMR procedures used to measure K_d values, and as such neither value has been determined so far. An approximate 1.5-fold decrease in the k_{cat} results in an approximate 3.5-fold reduction in the catalytic efficiencies of both these enzymes.

The most surprising result obtained for the mutation at position 121 is that it is the change from arginine to lysine and not methionine which markedly reduces the anion activation effect. Taken at face value the results presented in Fig. 2b suggests that it is not the charge group associated with residue 121 which is responsible for the anion activation effect observed with the wild-type enzyme.

3.3. *Arg*^{168}

The substitution of the arginine at position 168 by either a lysine or methionine appears to alter the affinity of the enzyme for both sugar and nucleotide substrates, as is shown in Table I. The lysine substitution results in a 2.6-fold increase in the K_m for 3-PGA, consistent with the 2.3-fold increase in K_d for that substrate. The same substitution also increases the K_m for MgATP by a factor of 3. The methionine substitution results in a 8.3-fold increase in the K_m for 3-PGA, which, although lower than the increase in measured K_d (14-fold) for that substrate is within the expected error range of the two measurements. The same substitution also increases the K_m for MgATP 4-fold. The increase in the K_m's of both sugar and nucleotide substrates for both lysine and methionine mutants is reflected in the 5.5- (R168K) and 34.6- (R168M) fold decrease in their catalytic efficiencies.

Like the substitution at residue 121 it is not the loss

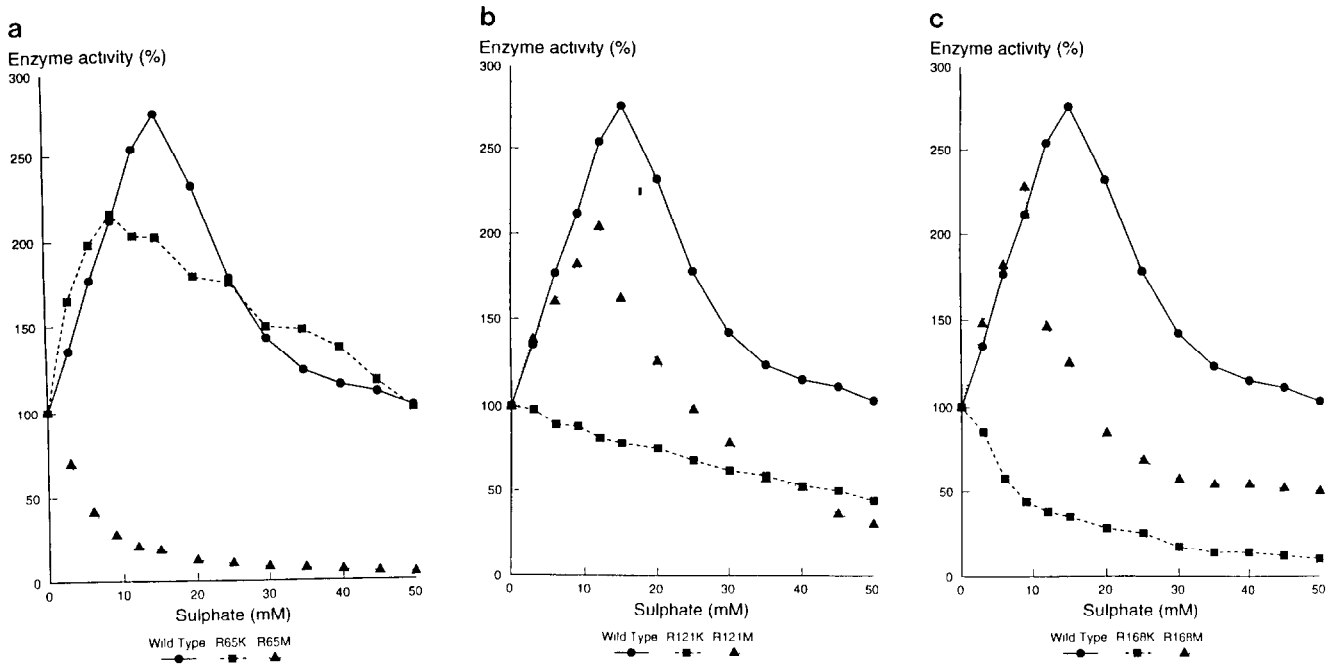

Fig. 2. Activity of yeast PGK modified by site-directed mutagenesis at positions 65 (a), 121 (b), and 168 (c). Each figure shows the activity as a function of the ammonium sulphate concentration present in the assay medium for the wild-type enzyme and the enzyme modified by replacing an arginine by lysine or by methionine.

of the charge which has the most marked effect on anion activation (see Fig. 2c). In contrast with the arginine replacement by lysine at position 121, which considerably reduces the salt activation effect (see Fig. 2b), the same substitution at residue 168 appears to act in such a way as to show only anion inhibition.

4. DISCUSSION

Two 3-PGA binding sites have been proposed for PGK based on crystallographic evidence. Neither site necessarily relates in detail to that found in the catalytically competent form of the enzyme. The putative site proposed by Blake and his colleagues [1] was located close to a 'patch' of basic residues on the domain surface facing, but separated by some 10 Å from, the phosphate end of the nucleotide binding site. The structure of the binary complex of the pig enzyme [3] shows that 3-PGA does indeed bind at that site. The second site, which was based on electron density observed in the Fourier map of the yeast enzyme [2], places the sugar substrates carboxyl group in a position for one of its oxygen's to make an in-line attack on the γ-phosphate of the bound ATP. That density was interpreted as representing substrate which had remained bound during the preparation of the enzyme. Sequence data has shown, however, that the residues implicated in the binding of the sugar phosphate are not conserved [20], although it is perhaps worthy of note that both replacements are of the most conservative type. The accumulated evidence, both from the sequence data and from the data obtained from the pig binary complex, would

Table I

The kinetic parameters for over-expressed wild-type PGK and PGK modified at positions 65, 121 and 168 by site-directed mutagenesis

PGK	K_m 3-PGA (mM)	K_m MgATP (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_d 3-PGA (mM)
Wild-type	0 77	0.33	343.5	$4\,5 \times 10^5$	0.011
R65K	1 70	0.33	503.8	$3\,0 \times 10^5$	0.040
R65M	15.89	1.62	48.9	3.1×10^3	> 0.500
R121K	1.92	0 36	232 1	1.2×10^5	–
R121M	2.00	0.37	255 7	$1\,3 \times 10^5$	–
R168K	2.01	0.99	164 1	8.2×10^4	0 025
R168M	6.40	1.34	85 5	$1\,3 \times 10^4$	0 160

The K_m values were determined in the presence of 40 mM ammonium sulphate, whereas the K_d values were measured in the absence of ammonium sulphate

suggest, therefore, that the binding site for 3-PGA is essentially that originally proposed by Blake and his colleagues [1]. The results obtained in the present study relating to the catalytically competent form of the enzyme have therefore been interpreted in terms of the substrate site determined for the pig binary complex. It must be emphasised, however, that the sugar binding site observed for the pig enzyme requires that the relative positions of the two domains move during catalysis, and that conformational changes associated with that movement, particularly those associated with the domain interface, could well alter the detailed bonding arrangement involving the substrate.

The structure of the binary complex indicates that Arg^{65}, Arg^{121} and Arg^{168} (yeast numbering) each form two bonds with chemically equivalent oxygen atoms associated with the non-transferable phosphate, as is shown in Fig. 1. The fourth, or linking oxygen is hydrogen bonded to the histidine at position 62. ^{1}H NMR studies with a yeast PGK, in which the histidine at position 62 was replaced by glutamine, have shown that 3-PGA binding induces the same conformational effects with the mutant as with the wild-type enzyme, although the affinity for that substrate is reduced some 2–3-fold [18]. The finding that the dissociation constant for glycerol-3-phosphate is close to that of 3-PGA [21] suggests that sugar substrate binding to PGK occurs without the involvement of the carboxyl group. That result implies that 3-PGA binding must depend on the interaction of the phosphate group, and to a lesser extent, the 2′-hydroxyl with the enzyme. What is surprising about the results presented here is that replacing the residues, which have been shown to interact with the phosphate group in the open form of the structure, have such a minimal affect on the K_m for the substrate.

The kinetic differences observed between wild-type enzyme and that substituted at positions 65, 121 and 168 by a residue of similar charge presumably reflects the small structural adjustments that are made at the non-transferable phosphate site to accommodate the change in side chain length on replacing arginine by lysine. The absence of a marked change on substituting any one of the three arginines by methionine can only be explained if it is assumed that the movement of a single ion, either into or out of the active site, compensates for the loss of charge. Such an explanation presumes that only two arginines of the three arginine side chains are required for phosphate binding, implying that PGK has evolved a mutationally insensitive substrate binding site. This is clearly a situation which could not have been anticipated in the absence of structural information.

What, if anything, do the salt activation effects tell us about the mechanism of the enzyme? Apparently little that we did not already know. The combined kinetic, crystallographic and ^{1}H NMR results obtained by studying a mutant PGK, in which Arg^{21} was replaced by lysine and by methionine, have shown that an anion binding site exists close to, but not necessarily coincident with, the sugar substrate's non-transferable phosphate [19]. That study showed that disturbing the structure associated with a side chain close to, but not interacting directly with, the sugar substrate was sufficient to abolish the anion activation effect. The anion binding results reported here, particularly for Arg^{121} and Arg^{168}, support those observations emphasising that it is not the loss of charge which affects salt activation but a change in the hydrogen bonding network close to the sugar binding site.

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