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Crystal Structure of the Binary Complex of Pig Muscle Phosphoglycerate Kinase and Its Substrate 3-Phospho-D-Glycerate

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ABSTRACT **Pig muscle phosphoglycerate kinase has been crystallized from polyethyleneglycol in the presence of its substrate 3-phospho-D-glycerate (3-PG) and the structure has been determined at 2.0 Å resolution. The structure was solved using the known structure of the substrate-free horse muscle enzyme and has been refined to a crystallographic *R*-factor of 21.5%. 3-Phospho-D-glycerate is bound to the N-domain of the enzyme through a network of hydrogen bonds to a cluster of basic amino acid residues and by electrostatic interactions between the negatively charged phosphate and these basic protein side chains. This binding site is in good agreement with earlier proposals [Banks et al., Nature (London) 279:773–777, 1979]. The phosphate oxygen atoms are hydrogen bonded to His-62, Arg-65, Arg-122, and Arg-170. The 2-hydroxyl group, which defines the D-isomer of 3PG, is hydrogen bonded to Asp-23 and Asn-25. The carboxyl group of 3-PG points away from the N-domain towards the C-domain and is hydrogen bonded via a water molecule to main chain nitrogen atoms of helix-14. The present structure of the 3-PG-bound pig muscle enzyme is compared with the structure of the substrate-free horse enzyme. Major changes include an ordering of helix-13 and a domain movement, which brings the N-domain closer to the ATP-binding C-domain. This domain movement consists of a 7.7° rotation, which is less than previously estimated for the ternary complex. Local changes close to the 3-PG binding site include an ordering of Arg-65 and a shift of helix-5.**

Key words: enzyme structure, substrate binding, X-ray crystallography, hinge bending, conformational changes

INTRODUCTION

Phosphoglycerate kinase (PGK, EC 2.7.2.3) catalyzes the high energy phosphoryl transfer reaction:

1,3-diphospho-D-glycerate + Mg-ADP ⇌
3-phospho-D-glycerate (3-PG) + Mg-ATP.

The enzyme has a molecular weight of around 46,000 and consists of a single polypeptide chain of 416 residues with a highly conserved amino acid sequence.[1] X-Ray crystallographic studies of the horse muscle[2,3] and the yeast[4] enzyme revealed that the molecule is composed of two domains of similar size, which correspond to the N- and C-terminal halves of the chain.

Substrate binding studies have shown that Mg-ATP and Mg-ADP are bound on the inner surface of the C-domain in the region above the cleft.[2,4] Whereas this binding sites is clear and consistant, equivalent conclusive evidence has not yet been reported for the location of the 3-phospho-D-glycerate substrate. In the absence of direct experimental data, a plausible binding site for the negatively charged 3-PG was identified on the N-terminal domain in a region opposite the ATP. This binding site consists of a cluster of basic residues (5 arginines and 3 histidines)[2,3] and has subsequently been referred to as the "basic patch." It has been shown recently by site-directed mutagenesis and NMR studies that two members of the basic patch, His-62 and Arg-170 (Arg-168 in yeast), are involved in the binding of 3-PG.[5,6]

When the substrates are bound to different domains they are too far apart to enable a direct phosphoryl transfer. This observation led to the suggestion that the enzyme has to undergo a large-scale hinge bending motion in order to bring both substrates together for catalysis.[2,3] On the basis of the domain structure and by analogy with hexokinase a closing of the cleft during catalysis has also been proposed by Anderson et al.[7] Small-angle X-ray scattering experiments have indicated a decrease in the

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TABLE I. Comparison of Horse and Pig Muscle PGK Crystals

Enzyme source	Horse muscle	Pig muscle
Crystallization conditions	50–60% saturated ammonium sulfate	18–25% (w/v) polyethylene glycol-8000
Substrate	—	10 mM 3-phospho-D-glycerate
Space group	$P2_1$	$P2_1$
Unit cell dimensions	$a = 50.8$Å $b = 106.9$Å $c = 36.3$Å $\beta = 98.6$Å	$a = 47.8$Å $b = 110.1$Å $c = 36.6$Å $\beta = 93.7$Å
Maximum resolution	2.5 Å	2.0 Å
Reference	Banks et al.,[2] Blake and Rice[3]	This paper

radius of gyration on the formation of the ternary complex which has been interpreted as a confirmation of a large-scale conformational change.[8,9] A detailed analysis of any substrate-induced conformational changes has, however, not yet been reported.

Experimental difficulties have up to now prevented a direct crystallographic determination of the 3-PG binding site. Crystals of both horse and yeast PGKs were grown from high concentrations of ammonium sulfate solutions and it is known that sulfate ions interfere with 3-PG binding by competitive inhibition.[10] Difference maps of the substrate-free horse enzyme revealed a sulfate group near Arg-170 and this was interpreted to indicate that the sulfate ion occupies the 3-PG binding site.[3] Soaking these crystals in 3-PG produced either no changes in the diffraction pattern[2] or shattered and dissolved the crystals at concentrations above 100 mM.[11,12] Intensity changes could be produced by exchanging the mother liquor for potassium tartrate and subsequently soaking the crystals in 3-PG. However, difference electron density maps calculated from these crystals produced a mass of features corresponding to extensive conformational changes throughout the molecule, which prevented the identification of the 3-PG binding site.[2,3] Soaking experiments with 1,3-diphospho-D-glycerate have not been carried out as this substrate is too unstable.

Based on indirect crystallographic evidence an alternative binding site for 3-PG, which does not require a large conformational change of the enzyme during catalysis, has been proposed for the yeast enzyme.[4] The inconsistency between these proposed 3-PG binding sites has given rise to some confusion in the literature on the catalytic mechanism of one of the basic housekeeping enzymes in metabolism. We therefore report here a high resolution X-ray study of the binary complex between 3-phospho-D-glycerate and PGK from pig muscle to identify the location of the binding site and to clarify the nature of the associated conformational change.

MATERIALS AND METHODS

Enzyme Preparation and Crystallization

3-Phosphoglycerate kinase was isolated from pig muscle by modification of the method described by Krietsch and Bücher.[13] After the ammonium sulfate fractionation similar to the one used by these authors a further chromatographic step on phosphocellulose was substituted for the previously used Sephadex G-75 gel filtration. The ammonium sulfate precipitate was dissolved in ice-cold 50 mM phosphate buffer pH 6.0, containing 1 mM EDTA and 5 mM 2-mercaptoethanol, and dialyzed against the same buffer to remove ammonium sulfate. The resulting solution was loaded onto a phosphocellulose column equilibrated with the above buffer. A linear gradient from 50 to 400 mM phosphate was applied to elute the protein. PGK-containing fractions were precipitated by the addition of solid ammonium sulfate.

From this stage the same protocol was followed as by Krietsch and Bücher,[13] including a DEAE Sephadex A-50 chromatography step. One difference which might be important to note is the presence of 2-mercaptoethanol during all purification steps. The preparation proved to be homogeneous as judged by SDS-PAGE.

PGK was stored in 3 M ammonium sulphate in the presence of 5 mM dithiothreitol at 5°C in the form of a microcrystalline precipitate. The specific activity of the preparation varied between 600 and 700 kat/mol measured as described previously.[14] For crystallization trials the enzyme was dialyzed against 30 mM Tris-HCl pH 7.0 to remove all the ammonium sulfate. The enzyme concentration was determined spectrophotometrically on the basis of $OD_{280}(1\%) = 0.69$.[13]

Single crystals of PGK were grown at 15°C within 1–3 months in hanging drops (20–30 μl in each) in the presence of 10 mM 3-phospho-D-glycerate, 5 mM dithiothreitol, 0.02% NaN_3, and 12–14% (w/v) polyethylene glycol-8000. The initial enzyme concentration was 10–15 mg/ml. The concentration of polyethylene glycol-8000 in the reservoirs varied between 18 and 25%. It was checked that 0.02% NaN_3 did not inhibit the enzyme. Under these crystallization conditions at least 80–90% of the enzyme activity was preserved after a few months. Crystals could also be grown in the presence of ATP as well as ATP plus 3-PG. In the absence of substrates, however, crystals could not be obtained from polyethyl-

TABLE II. Data Collection, Processing, and Refinement Statistics

Total number of reflections collected (resolution 100–1.92 Å)	58,316
Number of unique reflections	20,745
Number of unique reflections with $F > 5\sigma(F)$	19,014
R_{merge}* in intensities	6.9%
Completeness of data	
Resolution shell 100.00–3.49 Å	99.7%
3.49–2.77 Å	96.4%
2.77–2.47 Å	88.6%
2.47–2.20 Å	80.5%
2.20–2.04 Å	66.8%
Rigid body refinement (CORELS)	
One domain (residues 1–416), resolution 40–6 Å, 950 reflections	
R_{cryst}	41.3%
Two domains (residues 1–187 and 188–416), resolution 40–3 Å, 7,109 reflections	
R_{cryst}	42.1%
Molecular dynamics refinement (XPLOR)	
3,106 protein atoms	
40 water molecules	
1 phosphate group	
Resolution 8.0–2.0 Å, 19,057 reflections ($F > 0$)	
R_{cryst}	21.5%
rms deviation of bond length from ideality	0.025Å
rms deviation of bond angles from ideality	4.24°

$$*R_{merge} = \frac{\sum_h \sum_i | I_{i,h} - < I_h > |}{\sum_h \sum_i < I_h >}$$

where $I_{i,h}$ is the individual intensity measurement for each reflection h and $<I_h>$ is the mean intensity for this reflection,

$$R_{cryst} = \frac{\sum || F_{obs} | - | F_{calc} ||}{\sum | F_{obs} |}.$$

ene glycol. Crystals of the 3-PG binary complex are monoclinic, space group $P2_1$ with unit cell dimensions similar to those of the substrate-free horse muscle enzyme (Table I).

Data Collection and Refinement

X-ray intensity data were collected in 0.2°-oscillation frames using a Xentronics/Siemens area detector attached to a Rigaku rotating anode. All data were collected from three crystals, with most of the high resolution data originating from a crystal with dimensions 0.3 × 0.3 × 1.5 mm. The data were processed and analyzed using the XENGEN program package.[15] The data are nearly complete to 2 Å resolution (see Table II).

In the X-ray beam PGK crystals were very sensitive to the temperature and had to be kept at or above 22°C during data collections. At this temperature they were completely stable in the beam and diffracted strongly, but at 15°C or below they decayed rapidly and were completely ruined after a few frames. This is remarkable as the crystals were grown at 15°C and were also mounted and stored in capillary tubes at 15°C.

From the similarity in the unit cell dimensions it can be assumed that the binary crystals of the pig muscle enzyme are closely related to the known structure of the horse enzyme.[2,3] The isomorphous difference between the horse and pig structure factors calculated with the program DIFFER (D. Stuart, unpublished) was found to be 54.1%. This was considered too large to solve the structure by calculating a difference Fourier using the horse structure factors and phases.

The pig structure was determined by rigid body refinement using the program CORELS,[16] starting with the coordinates of the horse enzyme. In the first instance the whole molecule was treated as one rigid body. Several refinement cycles were calculated increasing the resolution from 8 to 6 Å. In order to

allow for a possible domain movement, the molecule was then divided into two parts (residues 1–187 and 188–416) and further cycles were calculated increasing the resolution stepwise from 6 to 3 Å. The crystallographic R-factor after the last rigid body refinement cycle was 42.1% using 7109 reflections in the resolution range 40–3.0 Å.

The resulting model was then further refined by molecular dynamics with the program XPLOR.[17] After each round of XPLOR $(2F_{obs}-F_{calc})\alpha_{calc}$ and $(F_{obs}-F_{calc})\alpha_{calc}$ electron density maps were calculated and these maps were compared with the model structure using the FRODO[18] graphics program. Calculated Fs were scaled to observed Fs using the program SHELLSCALE (D. Stuart, unpublished). In general clear density was found for main chain as well as side chain atoms. For any uncertain parts of the model OMIT maps were calculated leaving out the coordinates of the concerned residues in the structure factor calculation.

A part of the structure which did require a manual intervention was helix-13 (residues 371 to 386). In the original coordinate data set for the horse enzyme coordinates were present for helix-13 as a polyglycine sequence. Using these coordinates the refinement with XPLOR stopped at an R-factor of 25.4%. At this stage a $(2F_{obs}-F_{calc})\alpha_{calc}$ map was calculated for the horse enzyme and very little density was detected in the helix-13 region. Helix-13 is therefore disordered in the substrate-free horse structure. In all subsequent refinements horse coordinates for helix-13 were omitted and a new model was built for this helix. By the end of the refinement most residues of helix-13 could be fitted to electron density, but the positions of residues 385–387 still remained uncertain.

Other parts which did require some model building included some external loops. In these cases the overall density was rather weak and side chain density could not be detected for some residues, although the course of the main chain could still be traced. A number of external side chains, mainly lysine residues, must therefore be considered as disordered. At a later stage of the refinement individual temperature factors were refined and water molecules were placed in positions of extra density where a water molecule could form the appropriate hydrogen bonds.

As the sequence for pig PGK is not known, the horse PGK sequence[2] is used throughout the present paper. PGK is a highly conserved protein and an alignment of the three known mammalian PGK sequences mouse, human, and horse revealed at least 96% identity over the entire 416 amino acid residues.[1] In the present pig PGK structure the side chain density agrees as expected with the horse sequence in nearly all cases. The only exceptions are Thr-148, where we can only detect density of an Ala-148 side chain, and Thr-384, where we find density

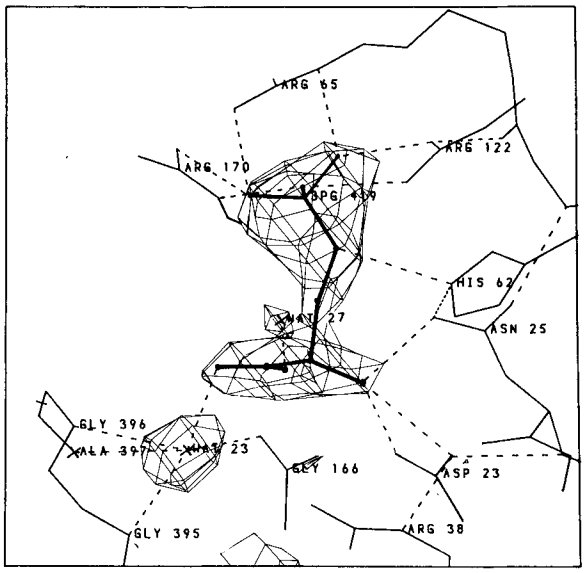

Fig. 1. Difference electron density map for 3-phospho-D-glycerate (3-PG) and two water molecules (WAT-23, WAT-27) in the basic patch region of pig muscle phosphoglycerate kinase (PGK). The map was calculated with coefficients $(F_{obs} - F_{calc})\alpha_{calc}$, where F_{obs} are the observed structure factors for the 3-PG-PGK complex and F_{calc}, α_{calc} are the structure factors and phases calculated from the final model, but without any contributions from 3-phospho-D-glycerate or the water molecules.

in accordance with Glu-384. In mouse and human Thr-148 is in fact replaced by Ala-148. However, we can not be certain about these possible substitutions, as residue 148 is an external residue and its side chain could also be disordered and residue 384 is close to the uncertain part 385–387 of helix-13.

By far the largest feature in all difference Fourier maps was found close to three arginine residues in the N-terminal domain. This density was identified as the phosphate group of 3-PG and as the refinement proceeded the other parts of 3-phospho-D-glycerate became apparent. The final cycle of XPLOR resulted in an R-factor of 21.5% for 19057 reflections in the 8.0–2.0 Å resolution range. This cycle used 3567 nonhydrogen atoms, which included 40 water molecules and the phosphate group of 3-phospho-D-glycerate (see Table II). Other atoms of 3-PG were not used in the refinement.

RESULTS

The 3-Phospho-D-Glycerate Binding Site

Difference electron density maps calculated after each round of XPLOR refinement consistantly revealed one major peak in the N-domain close to three arginine residues. Toward the end of the refinement the density maps were sufficiently detailed to fit the whole 3-PG molecule into electron density. The difference Fourier map of this region at the end of the refinement is shown in Figure 1. The map was calculated by omitting the coordinates of 3-PG and

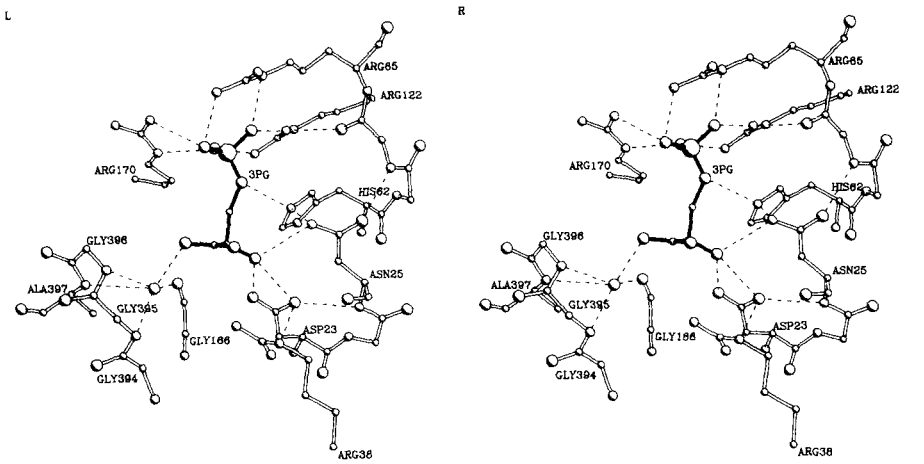

Fig. 2. Stereo plot of the 3-phospho-D-glycerate (3-PG) binding site of pig muscle phosphoglycerate kinase (PGK) showing the interactions of PGK with 3-PG and the ordered water molecule WAT-23.

surrounding water molecules from the structure factor calculation. The phosphate was placed at the position of highest electron density with the phosphate oxygens fitted into the protrusions. The 2-hydroxyl group is marked by a pronounced bulge and the 1-carboxyl group is also well defined. The L-isomer of 3-phosphoglycerate could not be fitted into the observed density.

An additional electron density feature was found within hydrogen-bonding distance to the carboxyl-oxygen O-12 of 3-PG. This roughly spherical feature is located close to the N-terminus of helix-14 on the helix axis and within hydrogen-bonding distance to the main chain NH groups of Gly-395, Gly-396, and Ala-397. From its shape, height, and environment we have interpreted this feature as an ordered water molecule (WAT-23). Another piece of density, which may represent a second water (WAT-27), was found within hydrogen bonding distance to the carboxyl-oxygen O-11 (Fig. 1).

3-Phospho-D-glycerate is bound to the N-domain of PGK through a network of hydrogen bonds and by electrostatic interactions between the negatively charged phosphate and the positively charged arginines. A stereo plot of the binding site is shown in Figure 2 and a schematic diagram of the interactions is given in Figure 3. The 3-phosphate group interacts with three arginine residues: Arg-65, Arg-122, and Arg-170. Arg-122 and Arg-170 each makes hydrogen bond interactions with two of the phosphate oxygens. Arg-65 is also close to the phosphate, but unexpectedly appears to have the plane of the guanidinium group placed almost parallel to the plane containing the three terminal oxygens of the phosphate, making hydrogen bond interactions geometrically difficult.

The bridge oxygen to the 3-phosphate group is in hydrogen bonding distance of the NE2-nitrogen of His-62. This histidine side chain is in turn hydrogen bonded to Arg-22 which is buried in the N-terminal domain. One consequence of this unusual hydrogen bond pairing may be to ensure that NE2 of His-62 is always protonated and thus able to donate a hydrogen bond to the bridge oxygen. The 2-hydroxyl group is within hydrogen bonding distance of two side chains: Asp-23 and Asn-25. Each of these residues is in turn hydrogen bonded to local main chain NH groups as shown in Figures 1 and 2. These interactions allow us to be certain that while Asp-23 accepts a hydrogen bond from the hydroxyl group, Asn-25 donates one. This pattern of hydrogen bond donation and acceptance is possible with a hydroxyl group, but would not be for an NH_2 or $(NH_3)^+$ group at the 2-position of the substrate, nor for a carbonyl at this position. This element of the binding site defines not only the stereospecificity of the enzyme but is also an important aspect of the chemical structure of its substrate.

Finally one oxygen of the 1-carboxyl group forms a hydrogen bond to the ordered water molecule Wat-23 located at the N-terminal end of helix-14. The main chain nitrogens of Gly-395, Gly-396, and Ala-397 orient Wat-23 so that the water–hydrogens are pointing away from the helix. These water–hydrogens can then act as donors in hydrogen bonds with the carboxyl-oxygen O-12 of 3-PG and the carbonyl group of Gly-166. A complete list of hydrogen bonds in the vicinity of the 3-PG binding site is given in Table III.

An alignment of all known PGK sequences was carried out with the program MULTALIGN.[19] Residues which form direct hydrogen bonds to 3-PG were found to be completely conserved, namely Asp-23, Asn-25, His-62, Arg-65, Arg-122, and Arg-170. Furthermore the three residues Gly-395, Gly-396, and Ala-397 which form the beginning of helix-14

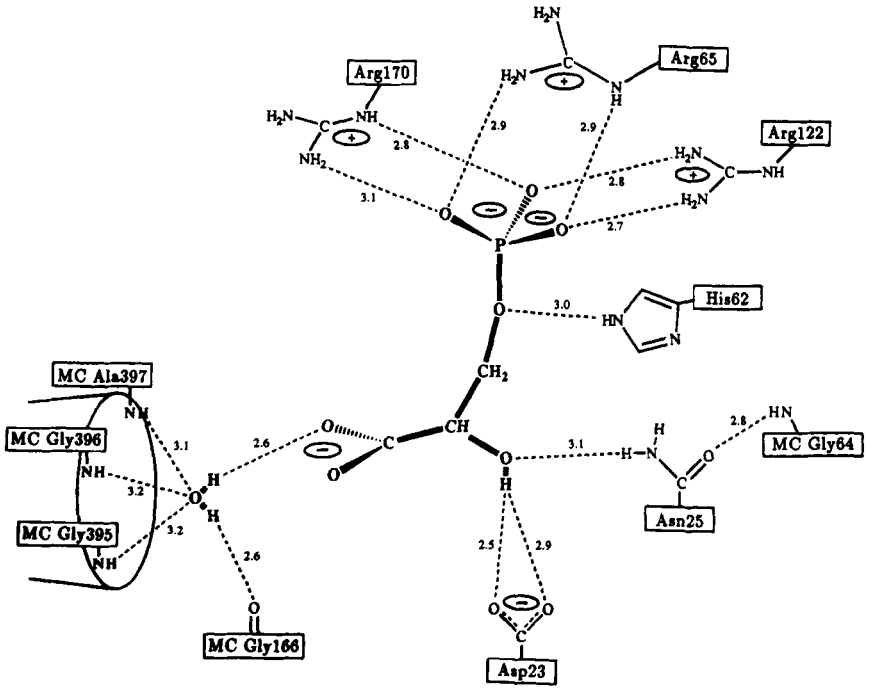

Fig. 3. Schematic diagram showing the hydrogen bond and electrostatic interactions of 3-phospho-D-glycerate with phosphoglycerate kinase and the water molecule WAT-23. Hydrogen bond distances are in Å.

and bind the ordered water are also completely conserved. Substitutions could be detected for Thr-167 and Gly-166, residues which do not interact directly with 3-PG. For example in *Thermus thermophilus*[20] Thr-167 is replaced by Ser-167 and in *Zymomonas mobilis*[21] Thr-167 is replaced by Ala-167 and Gly-166 by Ser-166.

It is interesting to note that some basic amino acids close to the 3-PG binding site namely Arg-38, His-169, and His-172 do not interact with 3-PG. However, Arg-38 seems to be in the correct position to take part in the phosphoryl transfer reaction.

Ordering of Helix-13

Possible conformational changes associated with the binding of 3-phospho-D-glycerate were analyzed by comparing the liganded structure of pig muscle with the substrate-free structure of horse muscle PGK. Preliminary experiments with crystals of pig muscle PGK grown from ammonium sulfate in the absence of substrates indicate that these crystals are closely isomorphous to the original crystals of the substrate-free horse muscle enzyme. It is therefore highly likely that the observed conformational changes are not a result of species variation but are due to structural changes induced by the binding of 3-PG. The most obvious change, which already had to be taken into account during the refinement, is an ordering of helix-13 (residues 371–386). This helix is located in the C-domain some distance away from the 3-PG binding site (Figure 4a and b) and is ordered in the present structure but disordered in the substrate-free horse muscle enzyme. Several studies underline the importance of this helix and support the assumption that the mobility of helix-13 can be changed by the binding of substrate. Any change of its structure or dynamics of this helix, possibly induced by 3-PG binding, could have important implications for the enzyme mechanism of action.

Substrate binding studies of the horse enzyme have shown that helix-13 is involved in the binding of ADP and ATP. Asp-374 of helix-13 interacts with the magnesium ion associated with the bound ADP. The β- and γ-phosphates of ATP are located close to the N-terminus of helix-13, but point away from the enzyme and do not interact with any of the side chains. It was suggested by Banks et al.[2] that the helix dipole acts as a phosphate binding helix as

TABLE III. Hydrogen Bonds at the 3-PG Binding Site

Donor	Acceptor	distance (Å)
NE Arg-65	OP2 3-PG	2.9
NH2 Arg-65	OP1 3-PG	2.9
NH1 Arg-122	OP3 3-PG	2.8
NH2 Arg-122	OP2 3-PG	2.7
NH2 Arg-170	OP1 3-PG	3.1
NE Arg-170	OP3 3-PG	2.8
NE2 His-62	OP4 3-PG	3.0
ND2 Asn-25	O21 3-PG	3.1
O21 3-PG	OD1 Asp-23	2.9
O21 3-PG	OD2 Asp-23	2.5
OH Water-23	O12 3-PG	2.6
OH Water-27	O11 3-PG	2.5
NH2 Arg-122	O Gly-64	2.8
NH1 Arg-21	ND1 His-62	3.2
N Gly-64	OD1 Asn-25	2.8
NE Arg-38	OD1 Asp-23	2.6
N Asn-25	OD1 Asp-23	2.4
OG1 Thr-167	OD2 Asp-23	2.8
N Gly-395	OH Water-23	3.2
N Gly-396	OH Water-23	3.2
N Ala-397	OH Water-23	3.1
OH Water-23	O Gly-166	2.6

described by Hol et al.[22] for other enzymes. The importance of this part of helix-13 is emphasized by the presence of a conserved glycine triplet, residues Gly-371, Gly-372, and Gly-373. One consequence of the ordering of helix-13 could be the binding of the β- or γ-phosphate through the helix-dipole, thereby bringing the phosphates into close contact with the protein. In yeast PGK helix-13 (labeled helix-XII in yeast) is ordered and the γ-phosphate of ATP is bound to the N-terminal end of this helix.[4]

Pig and horse PGK possess two fast reacting thiol groups and the reactivity of these groups can be reduced by the addition of substrates. The largest reduction in the reactivity was found with Mg-ADP, but a significant effect was also detected with 3-PG and Mg-ATP.[23,24] This effect has been interpreted as a ligand-induced structural change of the molecule, possibly related to the proposed hinge-bending mechanism. Cross-linking experiments revealed that in the pig enzyme the two fast reacting thiols are juxtaposed and are probably equivalent to the two cysteine residues Cys-378 and Cys-379 of the horse enzyme.[25] It was shown by Minard et al.[24] that in the horse enzyme the fast reacting cysteines are identical to Cys-378 and Cys-379. These residues are located in helix-13.[2] Human[26] and mouse[1] PGK also have cysteines at these positions. We can therefore assume that in the pig enzyme the two fast reacting thiols are at positions 378 and 379 as well. In the present structure the side chain of residue 378 is partially and that of residue 379 is completely buried within the molecule. Neither side chain is thus accessible from the solvent when the helix is ordered, which is in agreement with the reduced activity of the thiols in the presence of 3-PG.

A ligand-induced change in thiol reactivity could also be demonstrated in yeast PGK after the corresponding residues had been changed to cysteines by site-directed mutagenesis.[24] This effect is therefore not species dependent, but probably a general property of the molecule. How an ordering of helix-13 can be triggered by the binding of 3-PG, which takes place more than 10 Å units away in the N-domain, remains to be established.

Relative Movement of the Domains

Conformational changes involving the whole protein molecule were analyzed by fitting the substrate-free horse enzyme structure to the 3-PG liganded pig enzyme structure. Rotation matrices and translation vectors were calculated with the programme ASH (D. Stuart, unpublished) using α-carbon atoms only. Coordinates of helix-13 were not used in these calculations. In the first instance only the C-domains (residues 203–404) were superimposed (see Fig. 5). It can be seen that the N-domains have slightly different orientations and that in the pig structure the two domains are slightly closer together. In a second step the N-domains (residues 1–187) were superimposed and the domain movement was calculated as a 7.7° rotation plus a small translation. In the presence of 3-PG we can therefore observe a small hinge-bending motion, which brings the bound 3-PG closer to the ATP binding site. This allows the carboxyl group of 3-PG to interact with helix-14 via the water molecule. The observed domain movement for the 3-PG binary complex is, however, smaller than previously estimated for the ternary complex.[3,8,9,27]

Figure 5 shows the bound ATP molecule as determined for the horse enzyme. The β- and γ-phosphates project into the solvent and do not interact with any side chains of the enzyme. A movement of the β- and γ-phosphates toward the surface of the molecule, possibly induced by an ordering of helix-13 due to 3-PG binding as described above, would bring these phosphates closer to the 3-PG. Assuming that the ATP-phosphates are attached to the protein surface the distance between the γ-phosphate of ATP and the carboxyl oxygen group of 3-PG would reduce to approximately 7.2 Å. This would still be too large for any direct phosphoryl transfer, which requires a distance of 4.9 Å.[28]

The radius of gyration R_g of PGK, which has been determined experimentally by small angle X-ray scattering experiments,[8,9,29] can also be calculated from the coordinates. For these calculations, which were kindly carried out by D. Stuart, the horse coordinate set was used before and after the above described domain movement due to the binding of 3-PG. The R_g of the substrate-free horse enzyme was found to be 23.54 Å, which reduces to 23.36 Å after the domain movement. The decrease of 0.18 Å is consistent with X-ray scattering experiments of

Fig. 4. Ribbon diagram of the binary complex of phosphoglycerate kinase with its substrate 3-phospho-D-glycerate (3-PG): **(a)** the whole molecule and **(b)** the 3-PG binding site with some of the involved residues. ATP is shown as determined for the horse enzyme[2] and is included as a reference. α-helices 5, 7, 13, 14, and β-sheet L are highlighted. (b) also includes the two fast reacting cysteine residues Cys-378 and Cys-379 located in helix-13. Both figures were produced with the program RIBBONS.[39]

PGK in solution, although such a small change is probably close to the error margin of the scattering experiments. Pickover et al.[8] reported a decrease in R_g of 0.30 ± 0.50 Å, but Ptitsyn et al.[29] could not detect a change in R_g upon 3-PG binding. Larger changes in R_g (1.1–1.2 ± 0.3 Å) have been reported for the ternary complex.[8,9]

It is important to bear in mind that our present "substrate-free" structure is based on crystals which were grown in the presence of ammonium sulfate.

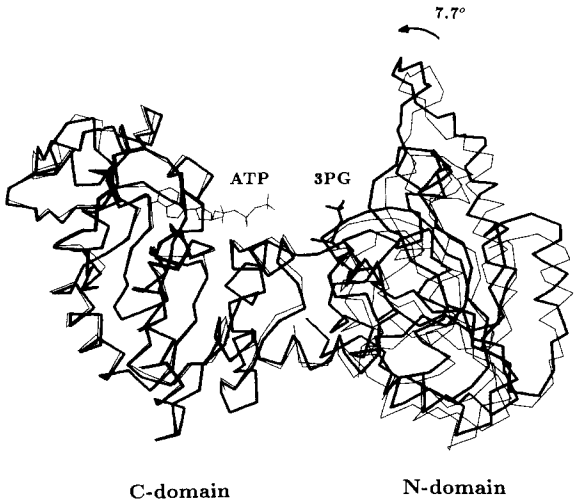

Fig. 5. Structural comparison of 3-PG bound pig phosphoglycerate kinase (PGK) (thick line) with substrate-free horse PGK (thin line). The C-domains (residues 203–404) of both structures are superimposed. ATP is shown as determined for the horse enzyme.[2] In the 3-PG bound pig enzyme the two domains are slightly closer together. This hinge-bending motion involves a 7.7° rotation of the N-domain (residues 1–187) and brings the 3-PG closer to the ATP.

This structure, which is used here as a reference, probably represents the sulfate-inhibited form of PGK. The crystal structure of the substrate-free enzyme in the absence of sulfate or phosphate is not available, as this form of PGK has not yet been crystallized.

Local Conformational Changes

Local changes in the structure were analyzed after the N- and C-domains had been superimposed separately. The difference Δd in α-carbon positions was calculated and is plotted in Figure 6 against residue number. The average main chain temperature factor (*B*-factor) for each residue of the pig enzyme is shown in Figure 6 for comparison. Residues in the N-domain generally have a much higher *B*-factor than residues of the C-domain. This indicates that the N-domain is slightly more mobile than the C-domain, which agrees well with studies on the domain flexibility.[30] The average rms deviation in α-carbon positions is 0.81 Å for the N-domain and 0.54 Å for the C-domain, also indicating that the N-domain is more flexible.

The difference curve usually parallels the *B*-factor curve reasonably well. High temperature factors indicate a less well-defined part of the structure and are found mostly in external loops (residues 29–31 and 133–138). Large differences found in these regions could be due to experimental errors in either the pig or the horse structure and may not represent a change in conformation.

One region where we can detect a conformational change is helix-5 (residues 165–170), as the differences Δd are large although the *B*-factors are comparatively small (Fig. 6a). Interestingly Gly-166, Thr-167, and Arg-170 of helix-5 are all involved in the binding of 3-PG (see Table III). Helix-5 is very short consisting only of a single turn. This helix has now moved closer to the 3-PG binding site. As a result the remaining residues of helix-5 Phe-165 and His-169 have also been shifted, although they do not themselves interact with 3-PG. Of particular interest is Phe-165 because it is close to an internal glutamic acid residue (Glu-192) and the movement of Phe-165 seems to shift Glu-192 as well.

Glu-192 is located in the hinge region of helix-7 at the domain interface between helix-7 and helix-14. It has been proposed that the hinge-bending mechanism involves a rotation of these two helices about their axis normal.[3,27] Glu-192 (Glu-190 in yeast) forms a salt bridge with His-390 (His-388 in yeast) and it has been suggested that this interaction may play a role in domain movement.[31] Mas et al.[32,33] have shown that Glu-192 is essential for the sulfate-induced activation of yeast PGK, but that His-390 is not essential for this activation. Other residues which could be involved in the domain movement are Ser-392 and Thr-393, located at the end of β-sheet L. However, at present it is difficult to establish the actual molecular details which lead to the hinge-bending.

The second place where we can detect a local conformational change is Arg-65. The C_α atom of Arg-65 has moved closer to the binding site and more importantly, the side chain of Arg-65 is now well ordered. The guanidine of Arg-65 projects over the phosphate of 3-PG, causing the phosphate group to be partly buried in the binding pocket. In the substrate-free horse structure the side chain of Arg-65 was disordered. The other residues which are involved in 3-PG binding do not change their main or side chain positions significantly.

DISCUSSION

Comparison With Kinetic Studies

The above details of the 3-PG binding site are in agreement with most of the conclusions derived from kinetic studies on substrate binding, for example:

1. The absolute requirement of the phosphate of 3-PG in the formation of its complex with the enzyme, as no binding of the phosphate-lacking analogue could be detected,[23];
2. The requirement of the dianionic form of the phosphate in respect to efficient substrate binding[34];
3. A much smaller binding contribution of the carboxylate of 3-PG as compared to its phosphate, as reflected by the relatively strong binding of the carboxyl-lacking analogue, D-glycerol-3-phosphate[35];
4. The importance of the 2-hydroxyl-group in the binding and stereospecificity of 3-PG, as deduced

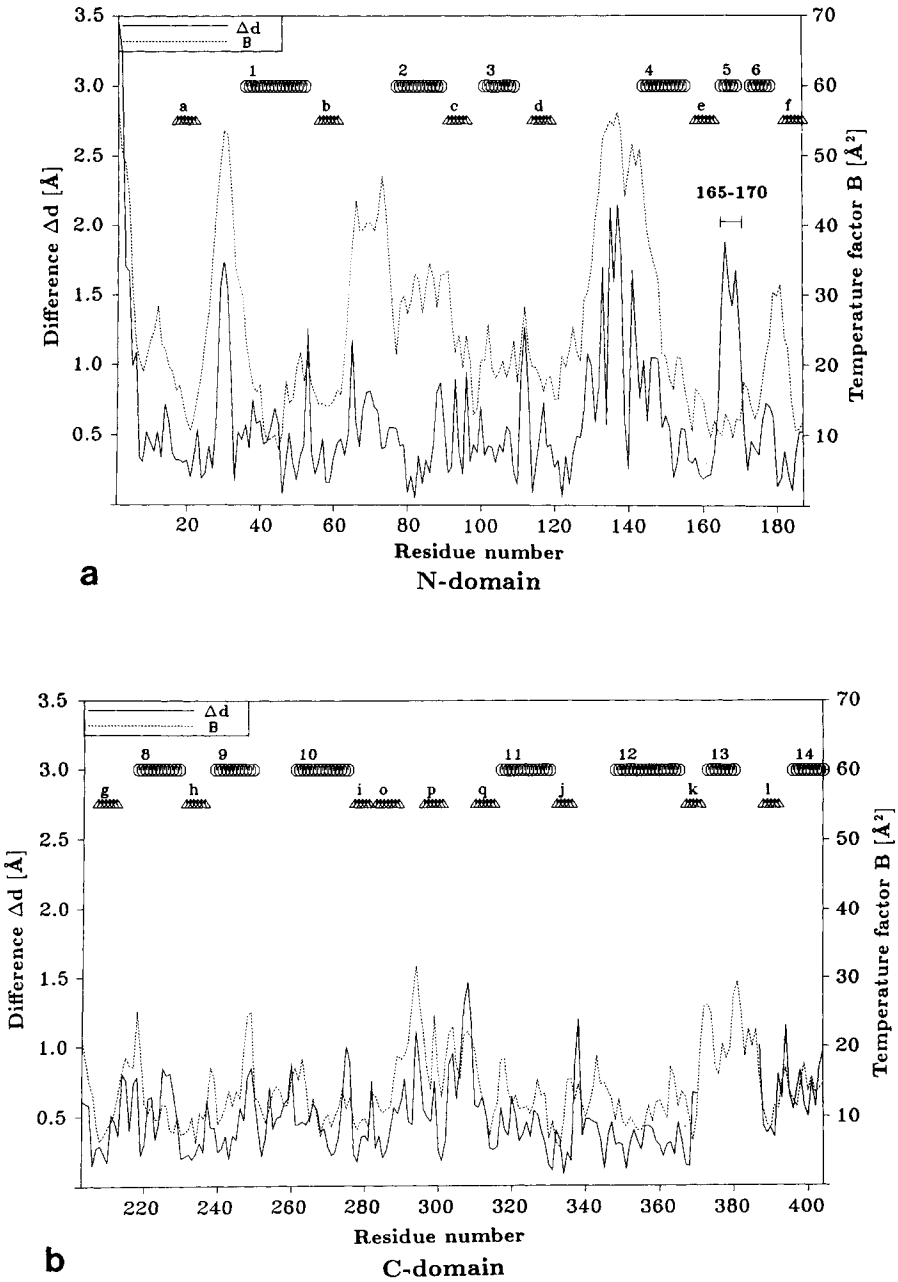

Fig. 6. Structural comparison of 3-PG bound pig phosphoglycerate kinase (PGK) with substrate-free horse PGK after superimposing N- and C-domain separately. The difference Δd in the α-carbon positions is plotted against residue number for the **(a)** N-domain, residues 1–187 and the **(b)** C-domain, residues 203–404. The same scale is used for both figures. The average main chain temperature factor B for each residue of the 3-PG bound pig enzyme is shown in dotted line for comparison. Secondary structural elements of α-helices and β-sheets are marked by circles and triangles and are labeled as in horse.[2]

from the lack of inhibition by L-glycerol-3-phosphate.[23]

Interestingly, there are also apparent inconsistencies with some kinetic studies. Although His-62 is involved in 3-PG binding, as shown here and deduced from site-directed mutagenesis experiments,[6] kinetic studies indicate that the His-62–bridge-oxygen interaction is not essential for the function of PGK. It was found that the methylene analogue of 3-PG (2-hydroxy-4-phosphonobutyrate), where the bridge-oxygen is replaced by a CH_2 group, is also a good substrate for PGK.[34,36]

Comparison With the Yeast Enzyme

The binding site described here is fully consistent with earlier predictions[2,3] and is supported by

site-directed mutagenesis experiments, which have been reported recently for the yeast enzyme. Mutants of yeast PGK were produced in which His-62 was replaced by Glu-62; Arg-170 (Arg-168 in yeast) was replaced by Lys-170 or Met-170; and His-172 (His-170 in yeast) was replaced by Asp-172. NMR-binding studies on these mutants have shown that His-62 and Arg-170 are involved in the binding of 3-PG, whereas His-172 is not of major importance.[5,6]

However, earlier crystallographic[4] and NMR[37] studies reported for yeast do not agree with the binding site described here. As with the horse enzyme, crystals of yeast PGK grown from ammonium sulfate failed to show any change in diffraction pattern when soaked in 3-PG. In this case however this was interpreted as indicating that 3-PG was already bound to the enzyme. A 3-PG molecule was therefore fitted to extra electron density, which was detected several Ångström units away from the basic patch region and closer to the ATP site. In this binding site the only basic residue involved in the binding of 3-PG is Arg-38, which has been proposed to hydrogen bond to the 3-phosphate group. The stereospecificity of PGK for the D-isomer of 3-PG was explained by a single hydrogen bond between the 2-hydroxyl group of 3-PG and the main chain nitrogen of Gly-395 (Gly-393 in yeast).[4,37]

We have checked our electron density map in the region corresponding to the proposed yeast binding site, but could not detect any extra density. An examination of the residues in pig muscle PGK which are directly involved in the binding of 3-PG revealed that all these residues are conserved in all known PGK sequences. If this information is coupled with the known high degree of conservation of structure and activity of PGKs it strongly suggests that the binding site we have described is similarly placed in other PGKs, including the yeast enzyme.

CONCLUSIONS

As suggested originally[2,3,38] the nucleotide and phosphoglycerate substrates are bound to different domains on PGK. In the substrate-free form of the enzyme these binding sites are located too far apart for the direct phosphoryl transfer to be possible. Some conformational change, possibly of a hinge bending type, is therefore needed to bring the two substrates into the appropriate geometrical relationship in the ternary complex. An indication that this type of conformational change may be in operation is given by the observation that the C- and N-terminal domains of the pig PGK-3-PG complex are rotated by 7.7° toward one another as compared with the native horse enzyme. The result of this rotation is to move the 3-PG toward the ATP, significantly closing but not completely eliminating the gap between them.

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