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The magnesium protein interaction in nucleotide complexes of phosphoglycerate kinase

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Abstract. The X-ray structure determination of yeast phosphoglycerate kinase and subsequent substrate binding studies have helped to define the binding sites for the triose and nucleoside phosphate substrates. This communication deals with one feature of the binding site—the location of an aspartic acid residue close to the phosphoryl binding site of the nucleotide substrate—and relates this and other structural features of the active site to the properties of this enzyme as deduced from nuclear magnetic resonance studies.

Keywords. Enzyme; mechanism; crystallography; resonance; phosphoryl.

Introduction

X-ray diffraction studies of the yeast (Bryant *et al*., 1974) and horse muscle (Blake and Evans, 1974) enzymes have shown that the principal feature of the cofactor free enzyme is the occurrence of two widely separated domains of almost equal size connected by a flexible neck region. This bilobal feature of the enzyme together with the considerable fidelity of phosphoryl transfer has led to the suggestion that the lobes move towards each other when substrates bind in order to develop the complete active site (Banks *et al.*, 1979). It follows therefore that the enzyme should exist in at least two stable conformational states as is shown diagramatically in figure 1.

Small angle X-ray scattering experiments (Pickover *et al.*, 1979) have shown that, under physiological conditions, both reaction substrates are required for domain movement to occur. Roustan and his colleagues (Roustan *et al.*, 1980) have reached the same conclusion using sedimentation velocity measurements and have shown that sulphate ions also effect domain movement to produce a more compact form of the enzyme. These results would suggest therefore that any detailed structural or mechanistic type data obtained for this enzyme is necessarily dependent on the exact conditions used in the relevant experiments.

The sedimentation velocity measurements suggest that in the absence of salt the enzyme is in its open or substrate binding form whereas in solutions containing 10 mM ammonium sulphate the enzyme is in its compact or catalytically active form. In the absence of substrates increasing the salt concentration beyond 50 mM appears to return the enzyme to its substrate binding form. We have extended the original sedimentation velocity measurements to cover an ammonium sulphate concentration

Abbreviations used: PGK, Phosphoglycerate kinase; Mn-AMP-P-N-P, Mn-adenylyl β,γ-imidodiphosphate.

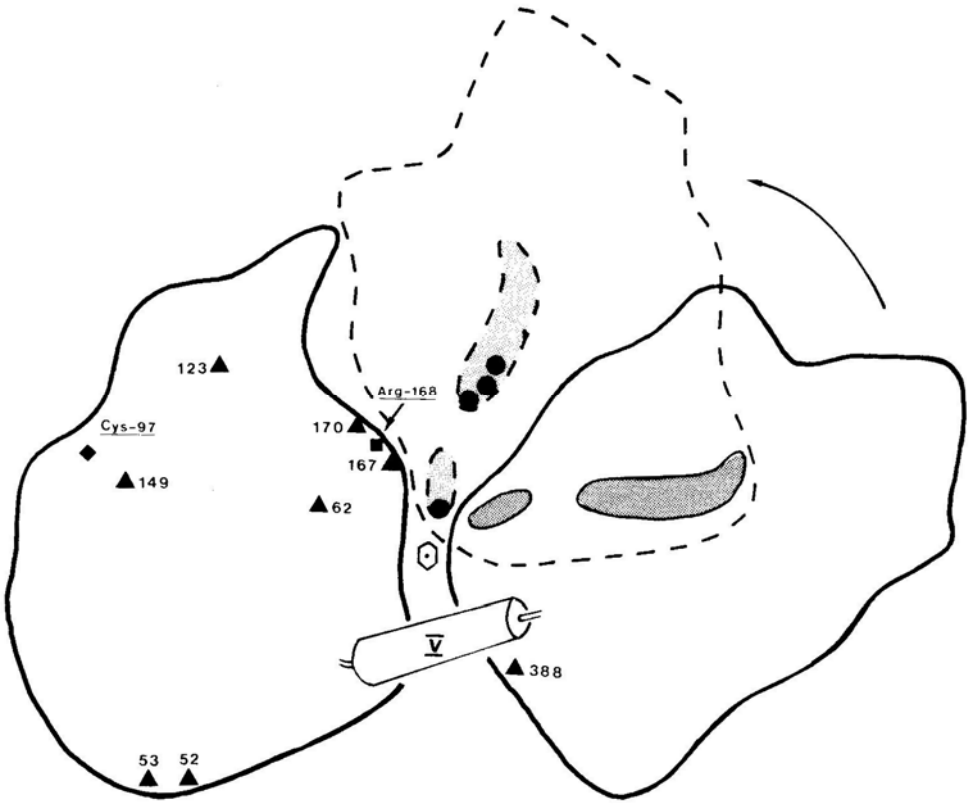

Figure 1. Outline drawing of yeast phosphoglycerate kinase showing the relative positions of the substrates and the eight histidine residues (▲) as observed in the crystal structure of the open form of the enzyme. The outline of the carboxyl domain, after rotation about a point above helix V (⊙), is drawn with a dashed line as also are the equivalent Mg · ATP and 3-PGA sites. For a discussion of the probable domain movement see Blake and Rice (1981). The positions of the phosphate groups (●) and of the arginine (■) whose guanidinium group could be involved in stabilising the transition state complex (see Watson *et al.,* 1982) are shown as they probably occur in the closed, and therefore active, form of the enzyme.

Seven of the eight histidine residues, six of the seven tyrosine residues, eleven of the thirteen arginine residues and the single cysteine residue are associated with the amino terminal domain. The surface tryptophan (308) is located near the adenine binding site and the buried tryptophan (333) is located near the phosphoryl binding site of the nucleotide substrates.

range from 1–200 mM. The new measurements confirm the high salt concentration observations of Roustan *et al.* (1980). The fact that sulphate binding at the inhibitor, as opposed to the activator site (Scopes, 1978), opens the enzyme is consistent with the results obtained from the X-ray structure studies refered to above which employed crystals prepared using ammonium sulphate as the precipitant.

Experiments using PGK solutions have shown that approximately 10 mM concentrations of ammonium sulphate helps to stabilise the enzyme during prolonged

experimental procedures (see Tanswell *et al.,* 1976). Nuclear magnetic resonance spectra are therefore typically recorded under conditions which select for the closed form of the enzyme. Since the crystallographic work has obviously been carried out on the open form of the molecule the important mechanistic observations made using these different, but complementary, techniques must be re-examined in terms of the domain movement properties of the enzyme.

The structure of the open form

The open or substrate binding form of the structures of the horse and yeast enzymes are very similar. As far as the active site region is concerned the structures can be considered to be virtually identical (Watson *et al.,* 1982). The nucleoside phosphates bind to the carboxyl domain with their phosphate chains extending towards the neck region of the molecule as is illustrated in figure 1. The adenine group binds in a deep depression on the enzyme's surface with its N6 nitrogen forming a hydrogen bond with a main chain carbonyl oxygen. The sugar is puckered in the C2′ *endo* form and is linked to the nucleotide in the *anti* conformation ($\chi \sim 100°$) with the 2′ and 3′ hydroxyls forming hydrogen bonds with the carboxyl groups of a glutamate and, in yeast, an aspartate (3′). The phosphate chain is linked to the sugar in the *trans-gauche* ($\chi \sim 180°$) conformation with the α and β phosphoryl groups in contact with a section of the main chain containing a glycine residue. Two lysine residues are located within bonding distance of the α and β phosphoryl groups. One of these positively charged residues interacts with the phosphate chain when Mg · ADP binds (Blake and Rice, 1981) to the enzyme and probably both with Mg · ATP (Watson *et al.,* 1982). Mg · ADP binding studies with crystals of the horse enzyme have indicated that the metal ion interacts with both the α and β phosphoryl groups and with the side chain of aspartate 374 (residue 372 in yeast). Mg · ATP binding experiments with both the horse and yeast enzymes are complicated by the presence of a sulphate ion which is located adjacent to the β-phosphoryl site. Experiments with crystals of the yeast enzyme using Mn-adenylyl β,γ-imidodiphosphate (Mn-AMP-P-N-P) have shown however that at pH 9·0 the γ-phosphoryl displaces the sulphate by forming hydrogen bonds with two main chain nitrogen atoms at the amino end of an α-helix as is shown in figure 2. Under these conditions the metal ion is judged to interact with the γ-phosphoryl and a carboxyl oxygen of aspartate 372 (but see discussion). Thus we see that crystallographic studies of phosphoglycerate kinase (PGK) from quite diverse species complement each other and indicate that a similarly positioned aspartate is located close to the metal ion in enzyme complexes with Mg · ADP and Mg · ATP.

Aspartate 372 is located at the amino end of an α-helix where movement of its carboxyl group, by rotation about the $C^{\alpha} - C^{\beta}$ bond, is restricted by neighbouring residues. Although the carboxyl group is necessarily, though somewhat surprisingly, located adjacent to the common phosphoryl binding site it appears to have just enough freedom of movement to interact with the metal ion in both its ADP and ATP associated positions as is shown in figure 2. This special structural situation suggests that, in the enzymic reaction, the role of the metal ion is to provide a linkage between the flexible phosphate chain and the protein.

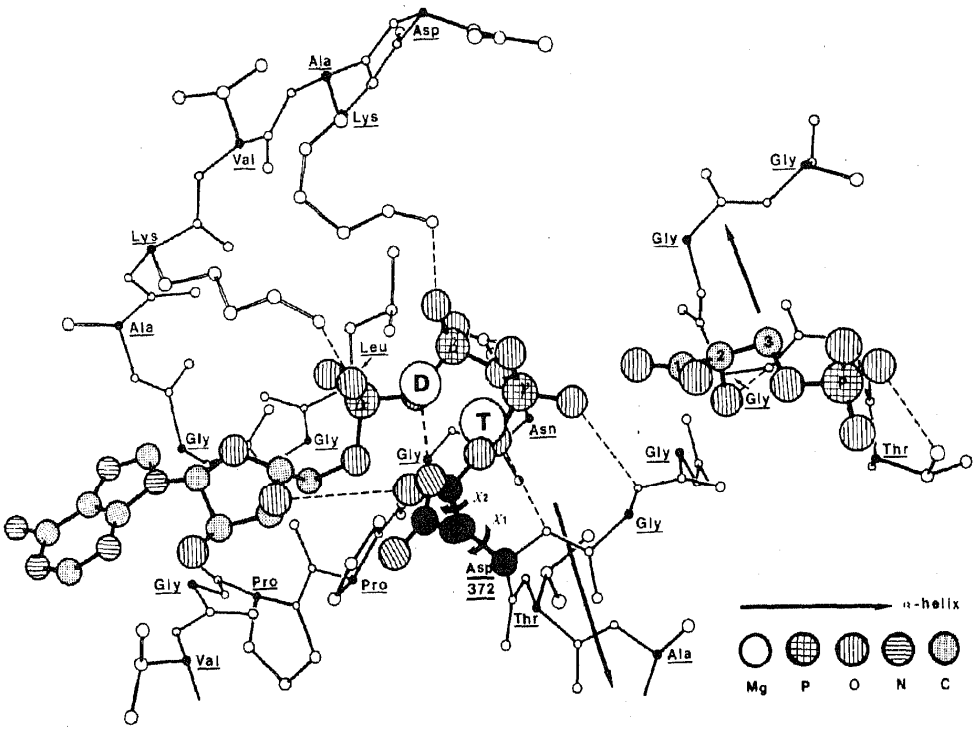

Figure 2. A drawing of the nucleoside phosphate binding site made using the co-ordinates of yeast PGK available from the Protein Data Bank. The sugar substrate, 3-PGA is also included to indicate its position relative to the protein bound Mg-ATP complex. The size of the substrate atoms and those of the side chain of Asp-372 have been increased relative to atoms of the protein. The approximate position adopted by Asp-372 in the Mn · ADP horse PGK complex (Blake and Rice, 1981) is also indicated. The sidechain rotation angles χ_1 and χ_2 describing the movement of Asp 372 from its position in ATP complex to that observed in the ADP complex are ~ 29° and ~ 24° respectively. The approximate positions of the magnesium ions, as determined from the ADP and ATP binding studies, are marked (D) and (T) respectively. Non covalently bonded atoms closer than 3·0A are joined by dashed lines.

Nuclear magnetic resonance studies

The PGK reaction, 3-PGA + Mg · ATP $\rightleftharpoons$ 1-3, DPG + Mg · ADP, is particularly suitable for study with ^{31}P NMR since both substrates and products contain phosphorous and are therefore visible in the ^{31}P resonance spectra. Rao *et al.* (1978) have utilised this feature of the reaction to study the enzyme under conditions where enzyme and substrates are present at approximately equal concentrations and report an equilibrium constant for the reaction (Keq = 0·8 ± 0·3) some three orders of magnitude greater than that observed using catalytic amounts of enzyme (Krietsch and Bucher, 1970). This finding can now be related to the effect of salt on the sedimentation velocity parameters. Since the resonance spectra were recorded using solutions containing

34 mM sulphate it seems reasonable to assume that the reported equilibrium result is for the closed enzyme; put another way these experiments have monitored the reaction equilibrium on the enzyme.

The extensive high resolution [270 MHz] proton magnetic resonance work of Tanswell *et al.* (1976) has led these workers to suggest that 'no group is close enough to bind directly to the first co-ordination sphere of the metal ion' in Mg · ATP complexes of PGK. Since the use of the X-ray crystallographic method appears to suggest that a protein metal ligand is formed when the enzyme binds nucleoside phosphate complexes it becomes necessary to consider the apparent anomaly which arises from the interpretation of the two sets of data.

First consider the histidine resonance studies of Tanswell *et al.* (1976). For comparative purposes the environment of each histidine residue, as found in the X-ray structure of the yeast enzyme, is given in table 1. Resonance 1 has an abnormal pK and could be related to His-388. Resonances 2a and 2b can be equated with the exposed residues 53 and 149 but resonances 3, 4 and 5 do not show a pH dependence even though the remaining five histidines (with the possible exception of His-62) are all exposed to solvent in the crystal structure. Two of the remaining histidine residues are in contact with tyrosine side chains suggesting that they (52 and 123) equate to resonance 6. Accumulating evidence suggests that histidines 62, 167 and 170 will be shielded from the solvent when the domains move together to form the fully developed active site (figure 1). A natural explanation of the NMR histidine titration studies is that they refer to the closed form of the enzyme. This conclusion is consistent with the fact that the experiments were carried out with solutions containing 15 mM ammonium sulphate. Additional confirmation for this deduction comes from the histidine metal ion distances derived in the same NMR study using paramagnetic difference spectro-

Table 1. A description of the general environment of each of the eight histidine residues as observed in the open form of yeast phosphoglycerate kinase. Residue numbers are listed in column N. The figures listed in column O (open) and C (closed) represent the distances (± 1A) from the metal ion to the centre of each imidazole ring. The data for the closed form of the structure were derived from the structure of the open form of the enzyme assuming domain movement commensurate with the metal histidine distances deduced from the paramagnetic resonance data of Tanswell *et al.* (1976). See also figure 1.

N	Imidazole environments	O	C
52	On surface but imidazole group in contact with Tyr 48.	40	34
53	Totally exposed.	45	39
62	Exposed but δ nitrogen probably bonded to Asn.	18	8
123	On surface but imidazole group in contact with Tyr 122.	30	18
149	Totally exposed.	38	28
167	Totally exposed but facing ATP binding site.	16	8
170	Totally exposed but facing ATP binding site.	19	8
388	Partly buried with δ nitrogen making a hydrogen bond with Glu 190.	18	20

scopy. The distances from the metal ion to three imidazole groups (~ 6A) are considerably less than the equivalent distances (~ 16A) measured for the three closest histidines (residues 62, 167 and 170) to the γ-phoshoryl site in the crystal structure.

The proton NMR studies show that a conformational change occurs on binding sulphate ions as indicated by the difference spectra produced on adding ammonium sulphate (15 mM) to salt free enzyme. Of direct relevance to the question of protein ligands for the metal ion is the observation that a sharp CH_2 resonance shifts significantly on adding sulphate and that this effect saturates at concentrations of about 0·5 mM (Tanswell *et al.,* 1976). This resonance (21) has been identified in experiments with enzyme nucleotide complexes as corresponding to a group which is close to the metal ion. The residues nearest to the metal ion in the open form structure are all glycines apart from aspartate 372 whose carboxyl group was thought to form an ionic bond with the magnesium (see structure section).

Discussion

This brief description of the ^{31}P and ^{1}HNMR experiments is perhaps sufficient to show that the crystallographic and NMR results cannot be compared directly since they relate to different forms of the enzyme. In one important sense—that of activity—the NMR experiments are clearly more relevant. In attempting to relate the open (and therefore inactive) form of the enzyme to that of the closed form we must assume that the equivalent domains have the same basic conformation in the two molecular states. The general correlation of the NMR data with the crystallographically determined structure, as has already been indicated for the aromatic resonances, would appear to support this assumption. The collective crystallographic and NMR evidence suggests therefore that the position of aspartate 372 relative to the ATP binding site is unlikely to change significantly during catalysis. Indeed the NMR observation that resonance 21 is characteristic of a methylene group of a polar amino acid (Tanswell *et al.,* 1976) provides support for this assumption.

The initial yeast PGK model building experiments appeared to indicate that the carboxyl group of aspartate 372 was positioned so as to form a hydrogen bond with the nucleotides sugar hydroxyl (3′) and a salt link to the magnesium as is shown in figure 2. A more detailed study of the active site region using the nucleotide derivative map and a computer graphics display system has shown that there is just sufficient flexibility in the positioning of the phosphate magnesium complex for the carboxyl magnesium interaction to be mediated by a water molecule. Such an arrangement would rationalise the X-ray and NMR observations and thus indicate that the interaction of the protein with the metal ion is second order. A similar type of ligand-water-metal complex has been proposed for pyruvate kinase to explain active site distances observed by NMR (Mildvan, 1981). In the pyruvate kinase system the position of the coordinating water molecule is thought to be between the metal and the substrate. The X-ray data for PGK indicates that the principal contacts of the magnesium ion are with the nucleotide substrate and the surrounding solvent rather than with the protein. This finding is consistent with the observation that the magnesium nucleotide complex is the true substrate for PGK (Larsson-Raznikiewicz, 1964).

It is generally assumed that the principal role of the divalent cation during catalysis is to enhance the effective polarisation of the transferable phosphoryl group to create a more electropositive site for oxygen attack. If this is the case then changes in the charge distribution must be sufficiently flexible to accommodate phosphoryl transfer either to or from the nucleotide substrate. Let us consider therefore the charge situation surrounding the transferable phosphoryl site in the open or substrate binding form of the enzyme. The PGK models of both the horse and yeast enzymes show that the only charged groups within potential bonding distance of the phosphate chain are those associated with the aspartate and the two lysine residues described above and illustrated in figure 2. The transferable phosphoryl group is located at the amino end of an α helix where the dipole effect (Hol *et al.,* 1978) will reduce the net negative charge of an ATP moiety by approximately one half unit. The environment surrounding the γ-phosphate is therefore essentially one of charge neutrality.

Water will be excluded from the active site on forming the closed enzyme thus preventing activated hydrolysis. The accompanying reduction in the ambient dielectric constant will increase the pK of aspartate 372 reducing the net negative charge at the active site. The active site will not be fully developed until the suitably positioned, and only non-ligated transferable phosphoryl oxygen (figure 2), interacts with one of the 'basic cluster' residues previously described by Blake and Rice (1981). In addition to effecting direct charge polarisation this interaction also satisfies the enzymological requirement that the transition state complex should be more stable than either of its precursors.

Model systems relating to enzyme catalysis must incorporate reversibility. The charge system described above for PGK is that which would relate to the formation of 1,3-DPG. Charge considerations show that when the nucleotide provides the attacking oxygen (as for the formation of 3-PGA) the movement of a charged ligand is required. It should be noted that the crystallographic studies already described indicate that two lysine residues interact with the phosphate chain of ATP but only one with ADP. The evidence presented would seem therefore to be compatible with the suggestion that catalysis is facilitated both by the introduction of positively charged ligand (shown as arginine 167 in figure 1) which completes the stabilisation of the transition state complex and by the change in the environment of a uniquely positioned carboxyl group (aspartate 372).

Since the combined crystallographic and NMR results suggest that aspartate 372 binds to Mg · ADP and Mg · ATP complexes of PGK by means of a water molecule it seems reasonable to postulate that this interaction may be necessary to position the somewhat flexible phosphate chain relative to other catalytically important residues. If this interpretation is correct, and solvated enzyme mediated interactions are a requirement for certain classes of ATP dependent reactions, then the bonding arrangement found in PGK might be expected at the active sites of other kinases. An examination of the relevant literature describing the structures of those enzymes for which sequence and structure information is available suggests that this may indeed be the case.

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