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# Phosphofructokinase: Structure and Control [and Discussion]

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# Phosphofructokinase: structure and control

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Phosphofructokinase from *Bacillus stearothermophilus* shows cooperative kinetics with respect to the substrate fructose-6-phosphate (F6P), allosteric activation by ADP, and inhibition by phosphoenolpyruvate. The crystal structure of the active conformation of the enzyme has been solved to 2.4 Å resolution, and three ligand-binding sites have been located. Two of these form the active site and bind the substrates F6P and ATP. The third site binds both allosteric activator and inhibitor. The complex of the enzyme with F6P and ADP has been partly refined at 2.4 Å resolution, and a model of ATP has been built into the active site by using the refined model of ADP and a 6 Å resolution map of bound 5′-adenylylimidodiphosphate (AMPPNP). The γ-phosphate of ATP is close to the 1-hydroxyl of F6P, in a suitable position for in-line phosphoryl transfer. The binding of the phosphate of F6P involves two arginines from a neighbouring subunit in the tetramer, which suggests that a rearrangement of the subunits could explain the cooperativity of substrate binding. The activator ADP is also bound by residues from two subunits.

## 1. Introduction

Phosphofructokinase (PFK, EC 2.7.1.11) catalyses the key control step of glycolysis, the phosphorylation of fructose-6-phosphate (F6P) by ATP to form fructose-1,6-bisphosphate ($F1,6P_2$):

$$F6P + ATP \,.\, Mg^{2+} \rightarrow F1,6P_2 + ADP \,.\, Mg^{2+}.$$

This paper describes the PFK from *Bacillus stearothermophilus*, but the enzyme has been studied from a wide variety of sources (for reviews see Bloxham & Lardy (1973) and Hofmann (1976)). The enzymes may be classified into three groups differing in their molecular mass: from mammals, from yeast, and from bacteria. The enzymes from various mammalian tissues have been most studied: these have a subunit molecular mass of 75000 to 95000, and form tetramers that aggregate into large oligomers. The enzyme from yeast is an $\alpha_4\beta_4$ octomer of subunit molecular masses 112000 and 118000. All the eukaryotic enzymes show complex allosteric behaviour, which may be summarized as inhibition by ATP and citrate, and activation by AMP, ADP and cyclic AMP. In contrast, the bacterial enzymes are much smaller, tetramers with subunit molecular masses of 32000–38000. Their allosteric behaviour is rather diverse, depending on individual requirements for metabolic control, but the enzymes from *Escherichia coli* (Blangy *et al.* 1968), *Thermus* X-1 (Cass & Stellwagen 1975), *Thermus aquaticus* and *B. stearothermophilus* (Hengartner & Harris 1975) all show similar kinetics, that is sigmoidal dependence of reaction rate on the concentration of the substrate F6P, allosteric activation by ADP, and inhibition by phosphoenolpyruvate (PEP). (See Sanwal (1970) for a discussion of control of glycolysis in bacteria.)

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In the absence of any amino-acid sequence or structural information on the mammalian or yeast PFKs, their relation to the bacterial enzymes is not clear. However, the general pattern of allosteric control shows some similarities between these various enzyme types, despite their different allosteric effectors.

1. The enzymes all show sigmoidal dependence of reaction rate on the concentration of the substrate F6P.

2. The activity of the enzymes responds to the energy level of the cell, increasing the rate of glycolysis when ATP is required. The eukaryotic enzymes achieve this by ATP inhibition and AMP activation, the bacterial enzymes by ADP activation.

3. The enzymes are inhibited by end-products of glycolysis. In the eukaryotic enzymes this inhibition is chiefly by citrate, in the bacterial enzymes by PEP.

These properties are summarized in figure 1.

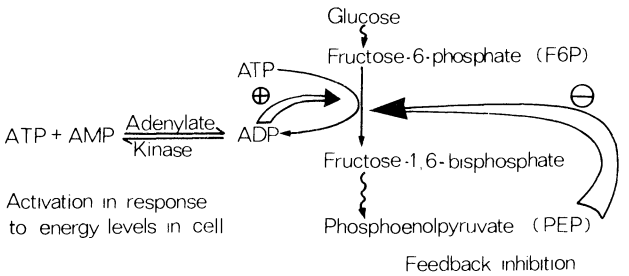


FIGURE 1. Phosphofructokinase in the control of glycolysis.

A thorough kinetic analysis of the PFK from *E. coli* was made by Blangy *et al.* (1968) the enzyme from *B. stearothermophilus* is very similar (H. Hengartner & R. Mulvey, unpubli The kinetics may be explained by a concerted two-state model (Monod *et al.* 1965) in which the enzyme is in equilibrium between an active R state and a less active T state. In the absence of ligands, the T state is favoured; binding of activators F6P or ADP favours the R state, and binding of the inhibitor PEP favours the T state. The enzyme is a *K* system, that is the R state has a higher binding affinity (lower $K_m$) for F6P than the T state, but both states have the same catalytic rate ($V_{max}$ and $k_{cat}$) once the substrate has been bound.

## 2. The determination of the crystal structure

### *Crystal forms*

We have solved the crystal structure of *B. stearothermophilus* PFK, crystallized from *ca.* 2 M potassium phosphate, pH 7.3, containing 2 mM F6P (Evans & Hudson 1979). These crystals belong to space group I222, cell dimensions 122.5 Å, 84.1 Å and 61.5 Å, with one subunit of the tetramer in the asymmetric unit. We identify this crystal form with the active R state of the enzyme, since it is crystallized in the presence of the cooperative substrate F6P, and binds other activating ligands without major structural changes (see §3). On the other hand, if the crystals are transferred to potassium tartrate solution in the absence of activating ligands, the crystals crack and change space group, presumably because removal of the ligands has allowed the transition to the T state. More recently, we have examined crystals grown in potassium tartrate in the presence of the allosteric inhibitors PEP or 2-phosphoglycollate. The latter compound is a weaker inhibitor than PEP, but is more stable to hydrolysis. These crystals

belong to space group $P2_12_12_1$, cell dimensions 131.5 Å, 114.7 Å and 96.2 Å, with a tetramer in the asymmetric unit. This crystal form probably contains the enzyme in the less active T state, since addition of F6P cracks the crystals rapidly. We are trying to solve the structure of the enzyme in this second crystal form.

### *The structure of the enzyme*

Native crystals for structure determination were prepared by washing out the F6P with 2.5 M phosphate solution. An electron density map was calculated from data to 2.4 Å resolution, by the usual method of isomorphous replacement with three heavy-atom derivatives (Evans & Hudson 1979). The model of the protein that was initially built into this map on a computer graphics system (Diamond 1980*a*, *b*) has now been refined by 31 cycles of restrained least-squares (Jack & Levitt 1978), although this refinement is not yet complete. The progress of the refinement was checked periodically by examining the difference map $(F_0-F_c)$ and the $2F_0-F_c$ map on the computer graphics, and rebuilding the model where necessary. During the refinement, three additional amino acid residues were inserted into the published amino acid sequence (Kolb *et al.* 1980), bringing the total to 319 residues: the insertion is Gly-Val-Tyr after residue 35.

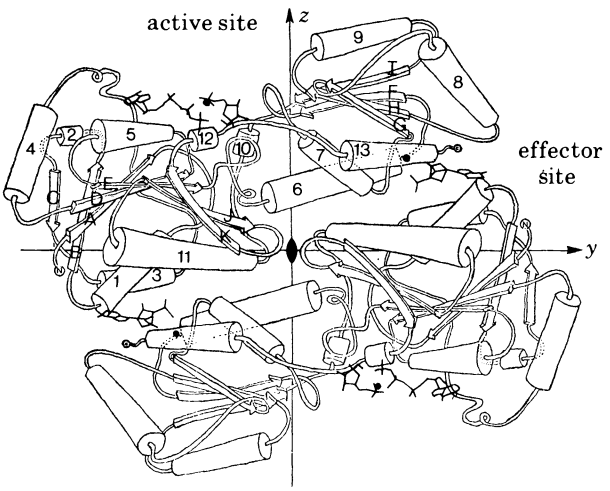


FIGURE 2

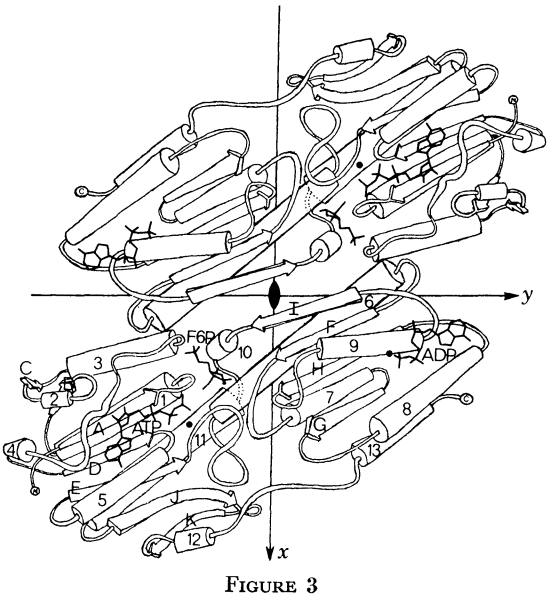


FIGURE 3

FIGURE 2. A schematic view of two subunits of the PFK tetramer, viewed along the *x* axis. The other two subunits lie behind the two shown. β-Sheet strands are represented by arrows (A–K) and α-helices by cylinders (1–13). Each subunit consists of two domains: domain 1 is on the left in the upper subunit. The substrates ATP and F6P are shown in the active site, and the activator ADP in the effector site.

FIGURE 3. Schematic view of two subunits viewed along the *z* axis, with the substrates and activator. The other two subunits lie behind the ones shown.

Figures 2 and 3 are schematic diagrams of the two subunits of PFK, with α-helices represented by cylinders and β-sheet strands by arrows. The subunit is clearly divided into two domains, each of which has a central β-sheet sandwiched between α-helices. Domain 1 has seven strands of β-sheet, the central five being parallel and the outer two anti-parallel to the others. Domain 2 has four parallel strands. The two sheets point towards a cleft between the domains which

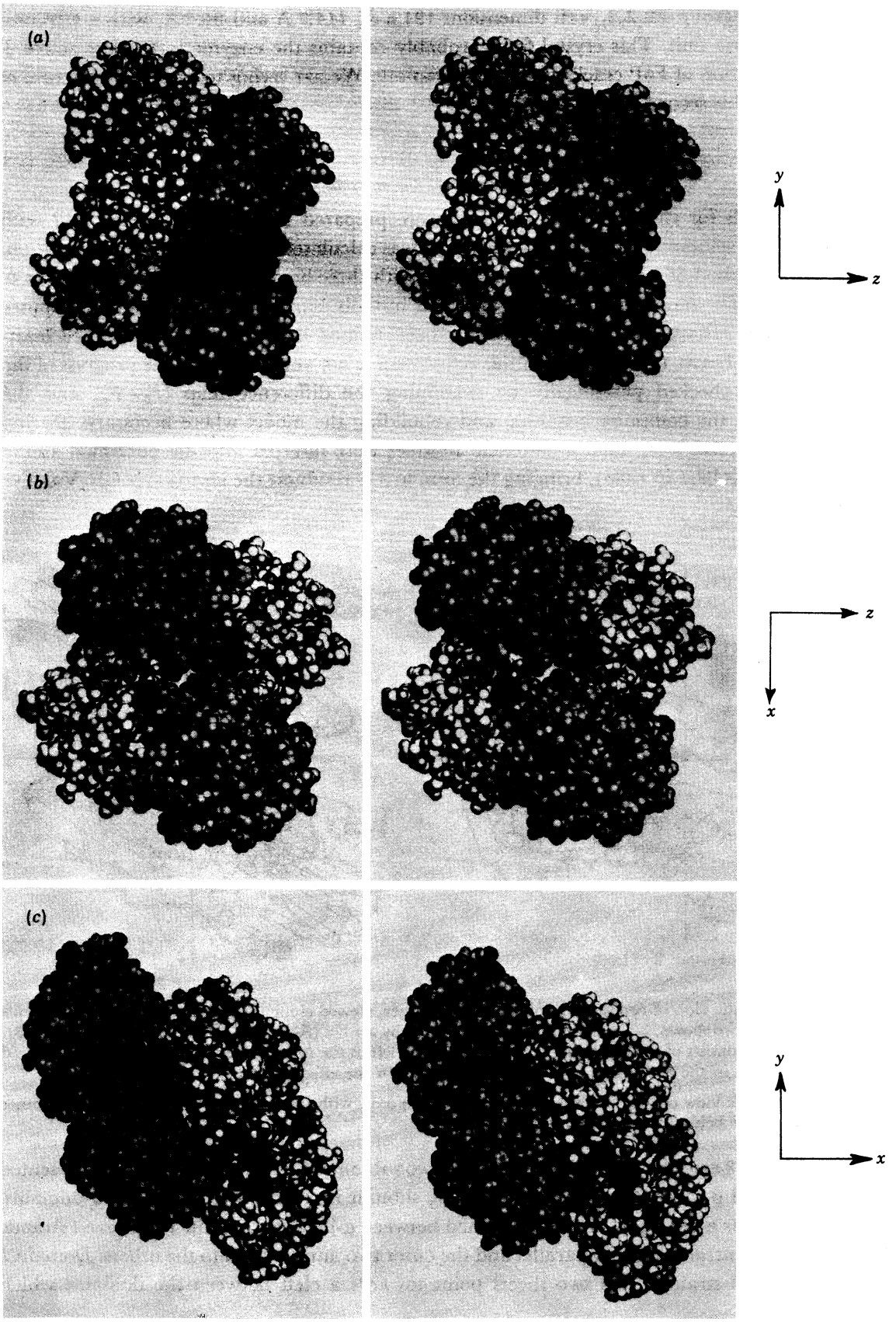


FIGURE 4. For description see opposite.



PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

forms the active site (see §3). Each subunit forms close contacts with only two of the other subunits in the tetramer: the third contact is very weak, only one arginine (residue 63) protrudes into the cavity along the *y* axis, and interacts with Asp 59 across this axis (see figure 4*b*). The subunit contacts are shown schematically in figures 2 and 3. The main interactions between the subunits related across the *x* axis (figure 2) are the packing of helices 6, 7 and 13 on to helices 3, 1 and 11, the β-bend between β-strands J and K interacting with its equivalent across the dyad axis, and a hydrophobic patch around the *x* dyad ('helix' 3 is not an α-helix, but is an irregular helical region). The main interactions across the *z* dyad (figure 3) are between the end of helix 6 and the loop above 'helix' 3: these are close to the F6P phosphate site and are partly shown in figures 5 and 6. Nearer to the *z* axis; the β-strands I do not form a close contact: their relative twist is in the wrong sense for a true antiparallel β-sheet, and the strands are a little too far apart (see figure 4*c*). This region of the subunit interface contains a number of ordered water molecules.

## 3. Ligand binding sites

### *Binding studies*

The binding of a number of substrates and effectors has been studied, mostly at 6 Å resolution, by soaking the crystals in solutions of the ligands. There are three binding sites per subunit, sites A and B, forming the active site, and site C, an effector site. The molecules that bind in these sites are summarized in table 1. Site A binds the substrate F6P or inorganic phosphate. Presumably it should also bind the product fructose-1,6-bisphosphate, but this compound

Table 1. Compounds binding to the three binding sites A, B and C

(Compounds in brackets have not been observed, but are assumed to bind in these sites.)

| active site | | effector site |
|---|---|---|
| A | B | C |
| F6P | $ADP/Mg^{2+}$ or $Mn^{2+}$ | $P_i$ |
| $P_i$ | (ATP) | $ADP/Mg^{2+}$ or $Mn^{2+}$ (activator) |
| $F1,6P_2$ | AMPPNP | PEP (inhibitor) |

binds rather weakly, and a crystal soaked in 50 mM $F1,6P_2$ showed no binding. The other substrate ATP should bind in site B, but crystals soaked in ATP solutions, with or without F6P, show only density for ADP, because of hydrolysis by the enzyme or turnover of the enzyme-catalysed reaction. However, the ATP analogue 5′-adenylylimidodiphosphate (AMPPNP) shows extra density for the γ-phosphate close to the F6P site. Site C binds both the activator ADP and the inhibitor PEP. These ligands can only bind when inorganic phosphate

### Description of figure 4

Figure 4. Stereo space-filling pictures of PFK with the subunits shaded differently, and the bound ligands in dark shading. (*a*) View of two subunits along the *x* axis from the centre of the tetramer (i.e. from behind figure 2). The activator ADP is between the subunits at the top and bottom of the picture, and the substrate F6P is shown at the sides. (*b*) View of the nearer half of the tetramer along the *y* axis, showing the cavity along this axis. The ADP molecules bound in the effector site are shown near the centre, and the phosphates of F6P can just be seen at the sides. (*c*) View of two subunits along the *z* axis from the outside of the tetramer, showing the open region around the centre of the subunits. The substrates F6P and ATP are drawn only on the right-hand subunit, to reveal the active site cleft on the other subunit.


PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

is displaced from the site by transferring the crystals from phosphate to solutions of potassium tartrate (2.5 M, pH 7.7) or sodium citrate (1.25 M, pH 7.3).

X-ray diffraction data to 2.4 Å resolution were measured from crystals soaked in F6P, ADP and $Mg^{2+}$ in 2.5 M potassium tartrate. The difference map from these data showed that F6P was bound in site A, and ADP . Mg in sites B and C. The F6P and the ADP in site B are only about half occupied. Models for the three ligands, and the conformational changes in the protein, were built on the computer graphics into the difference map and to a map with coefficients $2F(\text{complex}) - F(\text{native})$, taking the refined native protein coordinates as a starting point. These coordinates have now been refined for seven cycles by the same method as the native protein, with one check of the model on the computer graphics after five cycles. These partly refined coordinates are used in the following description, except that ATP has been model-built into site B by adding the third phosphate group to ADP and adjusting the positions of the β- and γ-phosphate groups to fit the 6 Å resolution map of AMPPNP: the β-phosphate group has not been moved far from its position in ADP. The metal position for ATP is taken from the 6 Å resolution difference map between $Mn^{2+}$ and $Mg^{2+}$ bound to AMPPNP.

## *Details of the binding sites*

### *Active site*

The active site lies in an extended cleft between the two domains of the subunit, with the 6-phosphate of F6P lying between the subunits related by the *z* dyad (figures 3 and 4*c*). The ATP molecule is bound almost entirely by domain 1, and the F6P by domain 2, except for two arginines, Arg 162 and Arg 243 from the other subunit, which bind to the F6P phosphate (figures 5 and 6). The catalytic site lies between the two domains.

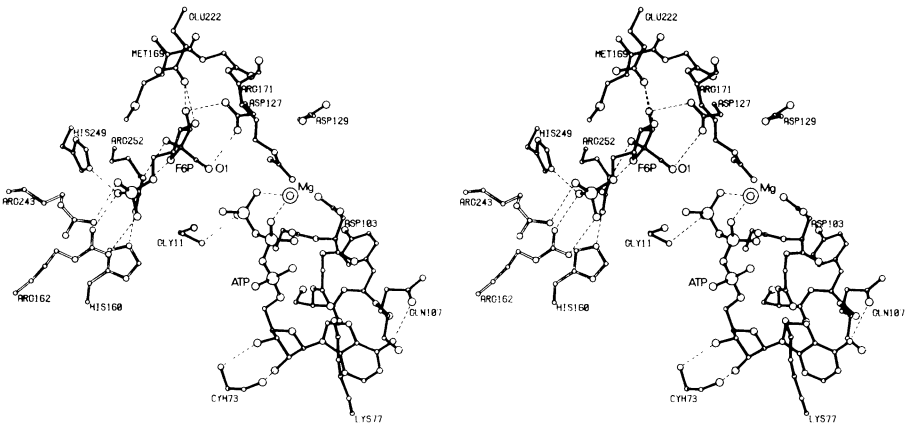


FIGURE 5. Stereo view of the active site region, viewed in the same direction as in figure 4*c*, showing F6P and ATP bound. Residues from the subunit related by the *z* dyad are drawn with open bonds. The enzyme-catalysed reaction involves the nucleophilic attack of the O1 of F6P on Pγ of ATP.

The ATP site lies between helix 5 (residues 102–108) and a surface loop between helices 3 and 4 (residues 72–77) (figure 5). This loop is not well ordered in either the native protein or the complex. The N6 amino group of the adenine is hydrogen-bonded to Gln 107, and the other side of the adenine makes a hydrophobic contact with the side chain of Lys 77. The two ribose hydroxyls are hydrogen-bonded to the main chain nitrogen and carbonyl of Cys 73. The ribose conformation is C3′-*endo*, with the C4′–C5′ bond *gauche* +, and the glycosyl bond

*anti* ($\chi = 36°$) (see, for example, Sundralingam 1975). The protein interactions with the phosphates and the $Mg^{2+}$ are not so clear, partly because of the uncertainty in the γ-phosphate position. The magnesium bridges the α- and β-phosphates in ADP, and moves about 2.8 Å to bridge the β- and γ-phosphates in AMPPNP. The metal chelate ring has been built as the Δ stereoisomer, which has been shown to be the active isomer for rabbit muscle PFK with chromium ATP (Dunaway-Mariano & Cleland 1980), but the 6 Å resolution map cannot reliably distinguish the isomers. The $Mg^{2+}$ ion seems to have no ligands from the protein either in ADP or in AMPPNP (Asp 103 is about 4 Å from both Mg positions). There may be some hydrogen bonding to the β-phosphate oxygens from the main chain of residues 102–104, and to a γ-phosphate oxygen from the main chain amide of Gly 11. Arg 171 may interact with the γ-phosphate in ATP and AMPPNP: figure 5 shows it in its position with ADP bound to the enzyme, and it is clearly too close to the Mg and to the γ-phosphate, so must move to a new position with the triphosphate. In the native enzyme this side chain is disordered.

In the catalytic site, the 1-hydroxyl of F6P is in a suitable place for nucleophilic attack on the γ-phosphate, although perhaps a little far away in the present model (O1 to Pγ 3.7 Å). On the other side of the 1-hydroxyl is the side chain of Asp 127, and although this group is again a little far away (O1 to Asp 127 $O_{\delta 2}$ 3.5 Å), it may act as a base catalyst for the reaction by increasing the nucleophilicity of the hydroxyl.

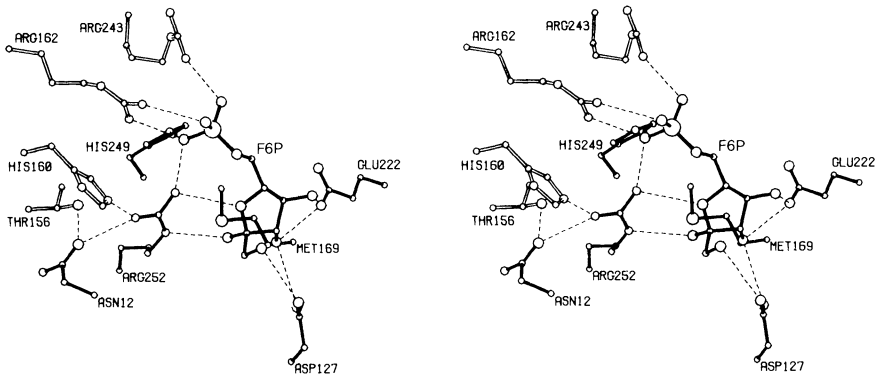


FIGURE 6. The binding site for F6P, with residues from the two subunits drawn with solid and open bonds. The view is approximately perpendicular to that in figure 5.

The binding of F6P is shown in figure 6. The most notable feature is the involvement of residues from the neighbouring subunit related by the dyad along *z*. The 6-phosphate group is bound by His 249 and Arg 252 from its own subunit, and by Arg 162 and Arg 243 from the neighbouring subunit. Arg 252 is central to a network of hydrogen bonds linking the F6P to the other subunit, to His 160, and through Asn 12 to Thr 156. These residues form a major part of the interaction between these two subunits. The sugar ring of F6P is bound by Met 169, Glu 222 and perhaps Asp 127. The binding by the two acid groups is not completely clear: the carboxyl of Glu 222 may need to be rotated by 90° so that both oxygens can hydrogen-bond to the sugar hydroxyls, and the 3-hydroxyl of the sugar presumably cannot be bound simultaneously to two acid groups. The presence of Asp 127 close to F6P O1 may explain the weaker binding of fructose-1,6-bisphosphate, by repulsion between the two negative charges.


PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

*Effector site*

The effector site lies in a deep cleft between the two subunits related by the dyad along *x* (figures 2 and 4*a*, *b*). ADP is bound with its diphosphate group and the $Mg^{2+}$ ion buried in the pocket, and the adenine group on the surface. This mode of binding explains the specificity of this site for nucleoside diphosphates, and its lower specificity for the base (Blangy *et al.* 1968). Groups from both subunits are involved in the binding of ADP, and groups from a third subunit are not far away: the side chain of Asp 59 shown in figure 7 is hydrogen-bonded to Arg 63 across the dyad along *y*. The $Mg^{2+}$ ion bridges the α- and β-phosphates of ADP, and is also bound by the main chain carbonyl of Gly 185, by Glu 187, and by a longer bond to His 215 (figure 7). Also shown in figure 7 is a possible water molecule bound to the magnesium ion, but this is rather close to the phosphates and may be wrong. The phosphates are tightly bound

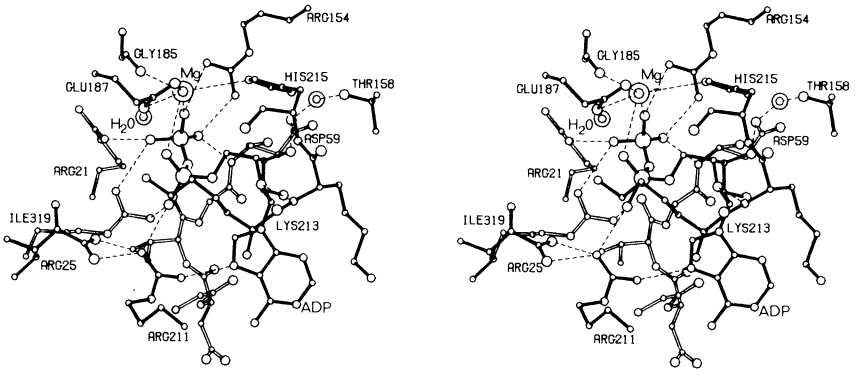


FIGURE 7. The binding site for the activator ADP, in the effector site, showing binding by two subunits (solid and open bonds). The putative water molecule near the magnesium ion is discussed in the text. The lower part of the adenine ring is exposed to solvent.

by groups from both subunits, four arginines, a lysine and a main chain amide: Arg 21, Arg 25, Arg 154 and the main chain of Asp 59 bind the β-phosphate, and Arg 25, Arg 211 and Lys 213 bind the α-phosphate. In the native crystals, inorganic phosphate binds in the place of the β-phosphate of ADP, and the phosphate group of the inhibitor PEP appears to bind in this position from the 6 Å resolution map. The ribose and adenine groups are less tightly bound: the ribose is hydrogen bonded through O3′ to Asp 59 and through O1′ to the main-chain amide of Lys 214. The adenine group is hydrogen-bonded by N7 to Arg 211, and the outer part of the adenine ring is exposed to solvent. The ribose is in the C2′-*endo* conformation, with C4′–C5′ bond *gauche* +, and the glycosyl bond *anti* ($\chi = 44°$).

The replacement of the $P_i$ ion in the native crystals by ADP.Mg causes some conformational changes around the binding site. The main chain of residues 212–215 moves down over the ADP, and the side chain of His 215 moves out of the way of the ribose to bind the magnesium ion. The side chain of Arg 211 swings round from a partly disordered position in the native enzyme to bridge between the α-phosphate and the adenine N7, bringing with it the carboxy-terminal residue Ile 319, which rotates about 180°.


PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

## 4. Discussion

With the current structural information on PFK, we can suggest some explanations for its allosteric behaviour and catalysis, but a more detailed explanation must await solution of the crystal structure of the T state of the enzyme, and the high-resolution study of complexes more similar to the true substrates, such as AMPPNP with F6P. The binding of the cooperative substrate F6P between two subunits suggests that the conformational transition between the R and T states of the enzyme involves the relative movement of the subunits in a quaternary structure change. The removal of Arg 162 and Arg 243 from the F6P binding site would clearly reduce the binding affinity for this ligand. The catalytic site lies between the two domains of the subunit, with the substrates ATP and F6P bound to different domains, so the lack of change of $k_{cat}$ between the two conformational states of the enzyme (Blangy *et al.* 1968) suggests that no substantial change of structure occurs in this region. This rules out the large relative movement of domains seen in hexokinase and phosphoglycerate kinase (Bennett & Steitz 1980; McDonald *et al.* 1978; Banks *et al.* 1979; Pickover *et al.* 1979). The most plausible model for the allosteric transition is thus the rearrangement of essentially rigid subunits into a new quarternary structure. The binding sites for the substrate F6P and the allosteric effectors ADP and PEP then cross-link the subunits, locking the structure into the preferred conformation.

Comparing the two main subunit interfaces, that around the *z* dyad is rather open, containing a number of water molecules, and many of the interactions are close to the F6P site (see figure 4*c*). The subunit interface across the *x* dyad, on the other hand, appears much tighter, involving the packing of three helices on three helices, and a large hydrophobic region around the *x* axis. However, both interfaces must change in the allosteric transition to explain the differential binding of the substrate and effectors.

Figure 4 was drawn by using a computer program written by Douglas Richardson and Arthur Lesk. P.J.H was a C.S.I.R.O. scholar. G.W.F. is supported by an M.R.C. research studentship.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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*Discussion*

H. G. Britton (*Department of Physiology, St Mary's Hospital Medical School, London, U.K.*). Is there any way in which the binding of one substrate to the active site of phosphofructokinase can influence the binding of the second substrate? Isotopic measurements with the rabbit muscle enzyme suggest that the binding of one substrate may increase the affinity of the enzyme for the other substrate by as much as a factor of 100.

P. R. Evans. For the phosphofructokinase from *E. coli*, Blangy *et al.* (*J. molec. Biol.* **31**, 13–35 (1968)) showed that the $K_m$ for ATP is independent of F6P concentration, and that the Hill coefficient and the concentration of F6P required to reach half-maximum velocity are not affected by ATP concentration (provided that ADP is excluded). This would imply no interaction between the substrate binding in the bacterial enzymes.


PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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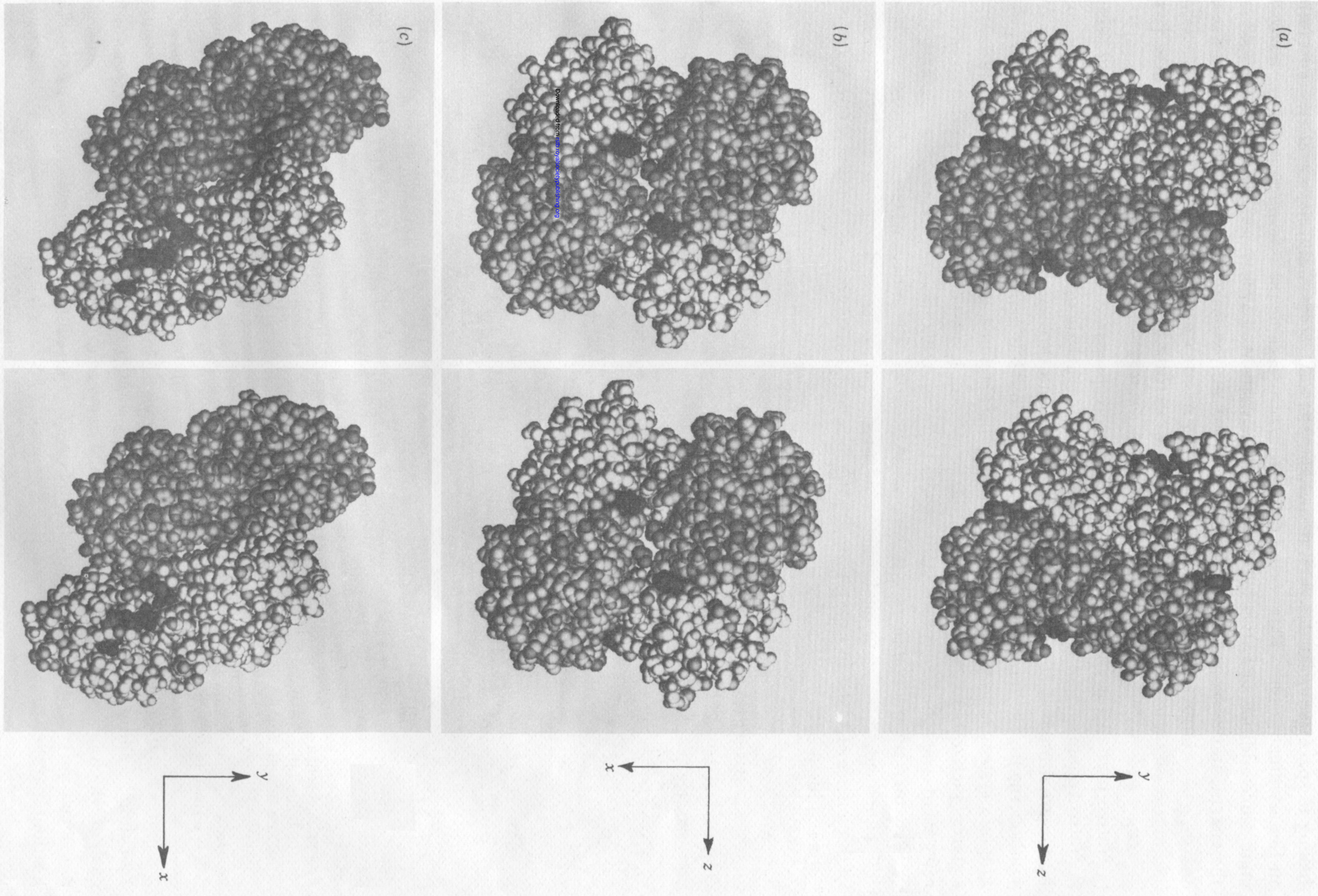




FIGURE 4. For description see opposite.