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Allosteric interactions of glycogen phosphorylase *b*

A crystallographic study of glucose 6-phosphate and inorganic phosphate binding to di-imidate-cross-linked phosphorylase *b*

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The binding to glycogen phosphorylase *b* of glucose 6-phosphate and inorganic phosphate (respectively allosteric inhibitor and substrate/activator of the enzyme) were studied in the crystal at 0.3 nm (3Å) resolution. Glucose 6-phosphate binds in the α-configuration at a site that is close to the AMP allosteric effector site at the subunit–subunit interface and promotes several conformational changes. The phosphate-binding site of the enzyme for glucose 6-phosphate involves contacts to two cationic residues, Arg-309 and Lys-247. This site is also occupied in the inorganic-phosphate-binding studies and is therefore identified as a high-affinity phosphate-binding site. It is distinct from the weaker phosphate-binding site of the enzyme for AMP, which is 0.27 nm (2.7Å) away. The glucose moiety of glucose 6-phosphate and the adenosine moiety of AMP do not overlap. The results provide a structural explanation for the kinetic observations that glucose 6-phosphate inhibition of AMP activation of phosphorylase *b* is partially competitive and highly co-operative. The results suggest that the transmission of allosteric conformational changes involves an increase in affinity at phosphate-binding sites and relative movements of α-helices. In order to study glucose 6-phosphate and phosphate binding it was necessary to cross-link the crystals. The use of dimethyl malondi-imidate as a new cross-linking reagent in protein crystallography is discussed.

Muscle glycogen phosphorylase can be activated either by covalent modification to form phosphorylase *a* or by non-covalent association with AMP (or IMP). In resting muscle phosphorylase *b* is inactive. It is generally assumed that the concentrations of the inhibitors of phosphorylase *b* (ATP, ADP and Glc-6-*P*) are such that they counterbalance the effects of the activators (AMP, IMP and P_i). In the present paper we report on the binding of the allosteric inhibitor, Glc-6-*P*, and the activator and substrate, P_i, to glycogen phosphorylase *b* in the crystal and attempt to provide a structural rationalization for the diverse effects of these metabolites.

Kinetic studies (Madsen & Scheckosky, 1967) have shown that Glc-6-*P* inhibits the AMP activation of phosphorylase *b* according to the model proposed by Monod *et al.* (1965). Later work on the glutaraldehyde-cross-linked enzyme showed that, despite their difference in structure, AMP and Glc-6-*P* are partially competitive (Wang *et al.*, 1970) and their effects are highly co-operative (Buc *et al.*, 1973; Buc-Caron & Buc, 1975; Battersby & Radda, 1979). Buc *et al.* (1973) and Morange *et al.*

Abbreviations used: DMM, dimethylmalondi-imidate; Glc-6-*P*, glucose 6-phosphate; Bes, 2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid. The nomenclature used to describe the atoms of amino acid residues follows that of the Protein Data Bank (Bernstein *et al.*, 1977), this nomenclature is similar to that of the IUPAC–IUB convention except that Roman letters replace the Greek letters.

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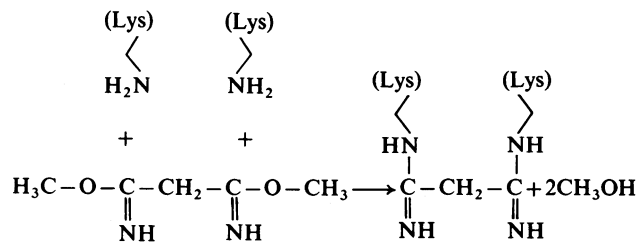
(1976) have shown that the binding of AMP to one subunit of phosphorylase *b* alters the conformation of both subunits so that the enzyme has a weak affinity for Glc-6-*P*. The binding of one molecule of AMP to the enzyme results in the release of two Glc-6-*P* molecules. From studies of a hybrid enzyme in which one of the subunits contains a covalent affinity label at the AMP-binding site Battersby & Radda (1979) have confirmed these heterotropic interactions. Thus they were able to show that binding of Glc-6-*P* to the unmodified subunit affects the adjacent modified subunit. Conversely the covalently modified subunit decreases the affinity of the unmodified subunit for Glc-6-*P*, increasing K_D from 0.03mM to 0.5mM.

The physiological significance of these effects observed *in vitro* depends on the intracellular concentrations of the metabolites *in vivo*, and precise values of these concentrations are not easy to obtain. Initially it was thought that the role of Glc-6-*P* was to keep phosphorylase *b* inactive in resting muscle in the presence of fairly significant concentrations of AMP. More recent results suggest that, although the total AMP concentration in resting muscle is about 50μmol/kg (Rahim *et al.*, 1976, 1980), the free AMP concentration is very much lower, about 0.1μmol/kg (Dawson *et al.*, 1980; Wilkie, 1981). At this concentration of AMP, activation of phosphorylase *b* is minimal. Nevertheless Glc-6-*P* may still be important in the prevention of non-useful degradation of glycogen and in the control of glycogenolysis under conditions in which formation of Glc-6-*P* exceeds disposal (Morgan & Parmeggiani, 1964). In resting muscle the total Glc-6-*P* concentration is about 0.18mmol/kg and may rise 10-fold on exercise (Rahim *et al.*, 1980). These concentrations are comparable with the affinity (K_D) of phosphorylase *b* for Glc-6-*P*. In the absence of other ligands, the K_D for α-Glc-6-*P* is 19μM (Battersby & Radda, 1976), whereas the efficacy of Glc-6-*P* as an inhibitor of AMP activation is represented by a rather higher constant $K_i \simeq 0.3$mM (Morgan & Parmeggiani, 1964) or $K_i \simeq 0.9$mM (Wang *et al.*, 1970). Early work suggested that Glc-6-*P* does not inhibit phosphorylase *a* (Morgan & Parmeggiani, 1964). However, binding studies have shown that Glc-6-*P* can bind to phosphorylase *a* with a dissociation constant of 6mM (Griffiths *et al.*, 1976), and Melpidou & Oikonomakos (1983) have shown that Glc-6-*P* inhibits phosphorylase *a* synergistically with glucose and can promote changes that make phosphorylase *a* more susceptible to dephosphorylation by phosphorylase phosphatase. Glc-6-*P* is formed as an intermediate at the branch point of several important metabolic pathways, namely glycogenolysis, glycolysis, gluconeogenesis and the pentose phosphate pathway. Its interactions with phosphorylase, as a representative of an enzyme–Glc-6-*P* complex, are therefore of interest.

Positive heterotropic interactions between AMP and P_i were first demonstrated by Helmreich & Cori (1964). The affinity of phosphorylase for AMP increases as P_i concentrations increase, and conversely the affinity for P_i increases as AMP concentrations increase. The affinity (K_D) of phosphorylase *b* for AMP in the absence of other ligands is about 80μM (Griffiths *et al.*, 1976), and in the presence of 10mM-P_i is about 43μM (Helmreich & Cori, 1964). Concentrations of P_i in muscle have been estimated to be between 3mM in heart (Morgan & Parmeggiani, 1964) to 6mM in frog muscle (Dawson *et al.*, 1980). In fatigued frog muscle concentrations of free AMP may rise to 22μmol/kg (Dawson *et al.*, 1980; Wilkie, 1981). Kinetic studies indicate that the K_m for P_i decreases from 23mM to 1.5mM as AMP concentrations increase from 15μM to 50μM (Helmreich & Cori, 1964).

At very high concentrations (approx. 150mM) P_i is able to activate phosphorylase in the absence of AMP (Buc, 1967). It was subsequently shown (Engers & Madsen, 1968) that other anions high in the Hofmeister series that are not substrates can also activate phosphorylase *b*. It was deduced that these anions produce activation by binding at the phosphate-recognition site of the enzyme for AMP.

Previous low-resolution crystallographic bind-



Scheme 1. *Proposed mechanism for the reaction of DMM with phosphorylase lysine-residue ε-amino groups to form a cross-linked amidine product (after Hunter & Ludwig, 1962)*

ing studies have shown that Glc-6-*P* binds close to the allosteric effector site with significant conformational changes (Johnson *et al.*, 1978). In a study at 0.4nm (4Å) resolution (Johnson *et al.*, 1979) on the binding of arsenate (as an analogue of phosphate; Helmreich & Cori, 1964), it was shown that at 100mM concentrations arsenate bound to the allosteric effector site and not to the catalytic site. In the presence of 5mM-AMP the nucleotide effectively displaced arsenate at the allosteric site but did not promote arsenate binding at the catalytic site. Attempts to extend these studies to high resolution were hampered by the fact that crystals soaked in Glc-6-*P* or P_i crack and become disordered. In order to obtain the present results crystals were cross-linked with DMM. Cross-linking enables us to study the binding in the crystal. It does not allow us to probe the extent of conformational changes, which can only be studied in co-crystallization experiments.

In our hands glutaraldehyde, the conventional cross-linking reagent of protein crystallographers, has not proved useful. After treatment crystals tended to be disordered or susceptible to radiation damage. Following the work of Hajdu *et al.* (1979), who have used a homologous series of alkyl di-imido esters to study the cross-linking patterns of different oligomeric states of phosphorylase, we explored the use of these reagents in the crystal. Alkyl di-imido esters react preferentially with ε-amino groups at high pH (pH10) with the formation of amidines (Hunter & Ludwig, 1962) (Scheme 1). At pH9 the initial product of the reaction is likely to be an *N*-alkyl imidate, which then partitions between reaction with ammonia to form amidine and hydrolysis to generate free amine (Browne & Kent, 1975). Hajdu *et al.* (1979) have shown that DMM stabilizes phosphorylase dimers under dissociating conditions and have suggested therefore that there are (at least) two lysine residues on different subunits whose ε-amino groups can come within 0.37nm (3.7Å) of one another across the subunit–subunit interface. DMM has proved an effective cross-linking reagent for phosphorylase *b* crystals, and has enabled us to study the binding of a number of 'difficult' metabolites, including Glc-6-*P*, that otherwise disrupt the crystals.

Methods

Rabbit muscle phosphorylase *b* was prepared by the method of Fischer & Krebs (1962) with minor modifications. Crystals were grown from a solution containing 25–30mg of phosphorylase/ml, 10mM-Bes, 10mM-magnesium acetate, 3mM-dithiothreitol, 2mM-IMP and 0.1mM-EDTA at pH6.7 as described previously (Johnson *et al.*, 1974). The crystals are tetragonal, space group $P4_32_12$. Unit-cell dimensions measured from precession photographs were found to be $a = b = 12.86 \pm 0.02$nm (128.6 ± 0.2Å), $c = 11.66 \pm 0.02$nm (116.6 ± 0.2Å). The crystallographic asymmetric unit contains one subunit of the enzyme (M_r 97412), and the two subunits of the physiologically active dimer are related by the crystallographic twofold axis at $z = \frac{1}{2}$.

Glc-6-*P* and other reagents were obtained from Sigma Chemical Co. DMM was a gift from Dr. Peter Friedrich (Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary).

For cross-linking, a 2mg/ml solution of DMM in 0.1M-triethanolamine/HCl/10mM-magnesium acetate, pH7.8, was prepared and crystals were transferred within 1min of dissolving the cross-linking reagent. After reaction for 2h at room temperature, the crystals were washed with 0.1M-triethanolamine/HCl, 10mM-magnesium acetate, pH7.1, to stop the reaction and to provide a convenient pH for crystallographic experiments. Cross-linking with imido esters is inefficient at pH8 (Browne & Kent, 1975), and it is likely that most of the reagent decomposed during the reaction time. Part of the success of this reagent in stabilizing the phosphorylase *b* crystals without introducing strain may be because the reaction with the protein is not complete. More complete cross-linking could probably have been achieved by multiple additions of freshly prepared di-imido ester, but in these experiments the crystals were sufficiently stabilized by only one treatment.

The cross-linked crystals were soaked in solutions containing 100mM-Glc-6-*P* in 0.1M-triethanolamine/HCl/10mM-magnesium acetate, pH7.1, for 24h.

Preliminary studies with 10mM- and 100mM-phosphate (or -arsenate) showed that the anions bound solely at the allosteric effector site and not at the catalytic site. In view of the weak affinity for P_i in the T state of the enzyme (results summarized in the introduction), it was decided to try even higher concentrations. Accordingly, cross-linked crystals were soaked in a solution of 500mM-K_2HPO_4 in 0.2M-triethanolamine/HCl/10mM-magnesium acetate, pH6.9, for 24h.

Three-dimensional data were collected by using the Arndt–Wonacott oscillation camera with Ni-filtered Cu K_α-X-radiation from an Elliott rotating anode GX6 run at 40kV and 40mA. The source-to-crystal and crystal-to-film distances were 150mm and 96mm respectively. A total rotation of 45° of the crystal about *c* with oscillation range $\Delta\phi = 2°$ and exposure times of 30×10^2s/deg allowed approx. 80% of the data available to 0.3nm (3Å) resolution to be measured. The choice of $\Delta\phi = 2°$ is a compromise. It allows a data set to be collected with the minimum of films for subsequent process-

ing but with some 20% of high-angle reflexions rejected because of overlapping spots. Some 25% of the data from the blind region [which was not measured and which represents some 3% of the data at 0.3nm (3Å) resolution] were obtained from an *h0l* precession photograph ($\mu = 15°$).

The data were processed as described by Wilson & Yeates (1979) and scaled to the native data.

The crystal structure of phosphorylase *b* was determined as 0.3nm (3Å) resolution by using conventional heavy-atom isomorphous-replacement techniques (Weber *et al.*, 1978). The resolution was extended to 0.2nm (2Å) with data recorded by using synchrotron radiation (Wilson *et al.*, 1983) and the structure refined by the method of restrained least-squares crystallographic refinement procedures (Konnert, 1976; Hendrickson & Konnert, 1980). At the present stage in the analysis the crystallographic *R* value for some 52329 reflexions in the 0.5–0.2nm (5–2Å) resolution range is 0.37. The overall root-mean-square deviation from ideal bond lengths for some 6640 atoms is 0.0018nm (0.018Å). The refinement has some way to go before completion, but analysis of difference Fourier syntheses shows that the bulk of the model is essentially correct. Difference Fourier syntheses were computed by using the phases obtained from a combination of those determined by isomorphous-replacement techniques and those obtained from the crystallographic refinement. The overall figure of merit at 0.3nm (3Å) resolution was 0.77. Two difference maps were computed with coefficients $F_{\text{Glc-6-}P} - F_{\text{P}}$ and $F_{\text{Glc-6-}P} - F_{\text{DMM}}$ where F_{P}, F_{DMM} and $F_{\text{Glc-6-}P}$ are the native, cross-linked and cross-linked Glc-6-*P* structure-factor amplitudes respectively. Similar difference Fouriers were computed for the cross-linked enzyme–P_i complex.

Binding sites were located on small-scale maps of the complete asymmetric unit. The difference maps for Glc-6-*P* and P_i were then examined in detail by using the program FRODO (Jones, 1978) implemented on an Evans and Sutherland Picture System II/PDP 11/70 computer. The single-crystal structure of Glc-6-*P* was not known at the time of these studies. The fit was obtained by using a regularized glucose molecule (Tanaka *et al.*, 1976) with a phosphate ester attached to the primary alcohol group. The phosphate ester P–O bond angle and bond length were 118° and 0.16nm (1.6Å) respectively. The phosphorus atom had tetrahedral geometry with the three P–O bond lengths of 0.155nm (1.55Å).

In the analysis of interactions, groups of atoms were considered to be in Van der Waals contact if their separations are less than 0.45nm (4.5Å). Potential hydrogen bonds are noted if the distance between donor and acceptor atoms is less than 0.35nm (3.5Å) and if the hydrogen bond is linear to within 40%. These rather generous limits reflect the level of precision of our current co-ordinates.

Solvent contact area calculations (Lee & Richards, 1971; Chothia, 1974; Richards, 1977) were performed by using a probe radius of 0.14nm (1.4Å) to simulate a water molecule. In general, these calculations assumed that there had been no changes in conformation of the protein. Several of the small conformational changes indicated in the difference Fourier synthesis for Glc-6-*P* are complex, involving movement of two related portions of the polypeptide chain. A proper analysis of these movements awaits the crystallographic refinement of the metabolite complexes. However, a clear indication for a simple movement of Arg-309 side chain was given in the difference map for Glc-6-*P* and P_i, and this change was included in the calculations.

Results

A general description of the phosphorylase *b* molecule has been given in previous publications (Weber *et al.*, 1978; Johnson *et al.*, 1979; Jenkins *et al.*, 1981; Stura *et al.*, 1983). The results, together with an explanation of the nomenclature, are summarized in Fig. 1. The allosteric effector site (site N) is located close to the subunit–subunit interface and makes contacts with residues solely from the *N*-terminal domain (residues 19–320). The catalytic site (site C) is situated at the centre of the subunit, where the three structured domains come together and which is close to the essential cofactor, pyridoxal 5'-phosphate. Site C is some 3.2nm (32Å) from site N. The amino acid sequence of phosphorylase has been determined by Titani *et al.* (1977).

Data processing

Comparison of precession photographs showed that there were no changes from the native crystals in unit-cell dimensions [to within 0.02nm (0.2Å)] for the cross-linked enzyme, the cross-linked-enzyme–Glc-6-*P* or the cross-linked-enzyme–P_I crystals. A summary of the three-dimensional-data-processing statistics is given in Table 1. The cross-linked-enzyme–Glc-6-*P* crystals had weak intensities at high angle and consequently the merging *R* values are poor.

Effects of cross-linking

The DMM difference Fourier synthesis indicated that no substantial changes had taken place in the protein structure. The largest peak above 'noise' level (height 300 arbitrary units for one grid point only) occurs in the region of the allosteric effector site but in a position distinct from that

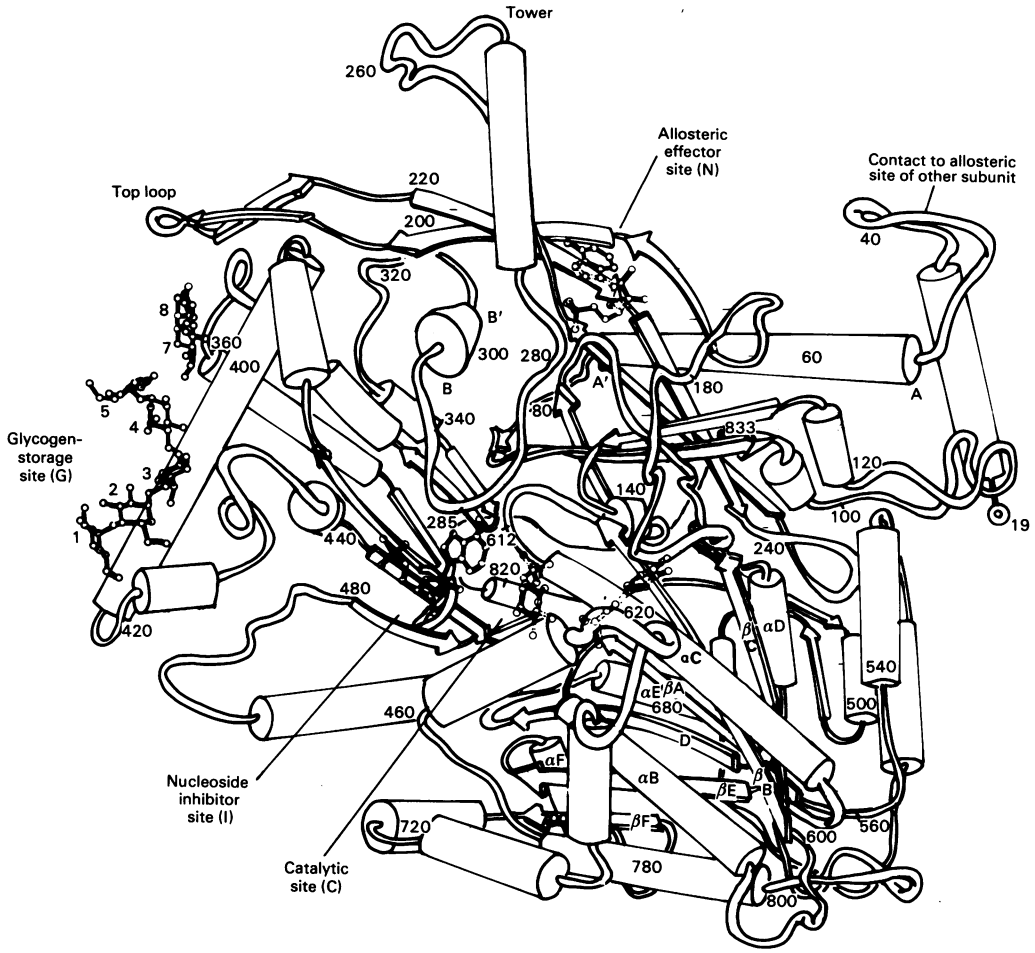


Fig. 1. *Schematic diagram of the phosphorylase b subunit viewed down the crystallographic y axis*
α-Helices and β-strands are represented by cylinders and arrows respectively. The first 18 residues from the *N*-terminus and the last eight residues from the *C*-terminus have not been located in the electron-density map. AMP is shown bound at the allosteric effector site N, maltoheptaose at the glycogen-storage site G, glucose 1-phosphate and pyridoxal 5′-phosphate at the catalytic site C and AMP at the inhibitor site I.

occupied in AMP-binding studies. The co-ordinates of this peak [$x = 2.43$ nm (24.3 Å), $y = 0.45$ nm (4.5 Å), $z = 5.16$ nm (51.6 Å)] place it so that it is about 0.47 nm (4.7 Å) from the carbonyl oxygen atom of Val-40′, from the symmetry related subunit, and rather more distant from three cationic residues, Lys-41′ [distance to NZ atom = 0.93 nm (9.3 Å)], Lys-191 [distance to NZ atom = 0.89 nm (8.9 Å)] and Arg-193 [distance to NH1 atom = 0.48 nm (4.8 Å)]. In our first interpretation of the structure, it appeared that Lys-41′, from the symmetry related subunit, was appropriately placed to make contact with this peak and to form a cross-link with Lys-191, after a conformational change of the latter residue. In our more recent interpretation Lys-41′ points out into solution and there would need to be a rearrangement of this residue in order to make contact with the observed peak in the difference Fourier synthesis.

None of the other smaller peaks in the difference Fourier synthesis appear to be associated with lysine residues. The α-amino group of phosphorylase is acetylated.

Hence it is not possible at the present time to give a simple explanation of the ability of diimidates to stabilize phosphorylase *b* crystals.

Glc-6-P binding

The difference Fourier synthesis for Glc-6-*P* showed a single peak close to the allosteric effector site in which the phosphate and sugar moieties were easily distinguished. The maximum peak height was 320 arbitrary units compared with a 'noise' level of approx. 80 arbitrary units. There

Table 1. *Data-processing statistics for oscillation-camera X-ray diffraction data*

Merging R is defined as:

$$R = \sum_h \sum_i |\bar{I}(h) - I_i(h)| / \sum_h \sum_i I_i(h)$$

where $\bar{I}(h)$ is the mean intensity of reflexion h for which there are i measurements.

Data set	Resolution (nm)	Total no. terms measured	No. of unique terms	Merging R	Fractional change in structure factor
Native	0.3 (3 Å)	64977	18173	0.088	–
DMM	0.3 (3 Å)*	43896	16220	0.108	0.133
DMM + Glc-6-*P*	0.3 (3 Å)*	51826	15399	0.141	0.173
DMM + K_2HPO_4	0.3 (3 Å)*	39905	14842	0.086	0.108

* Nominal resolution: see the text.

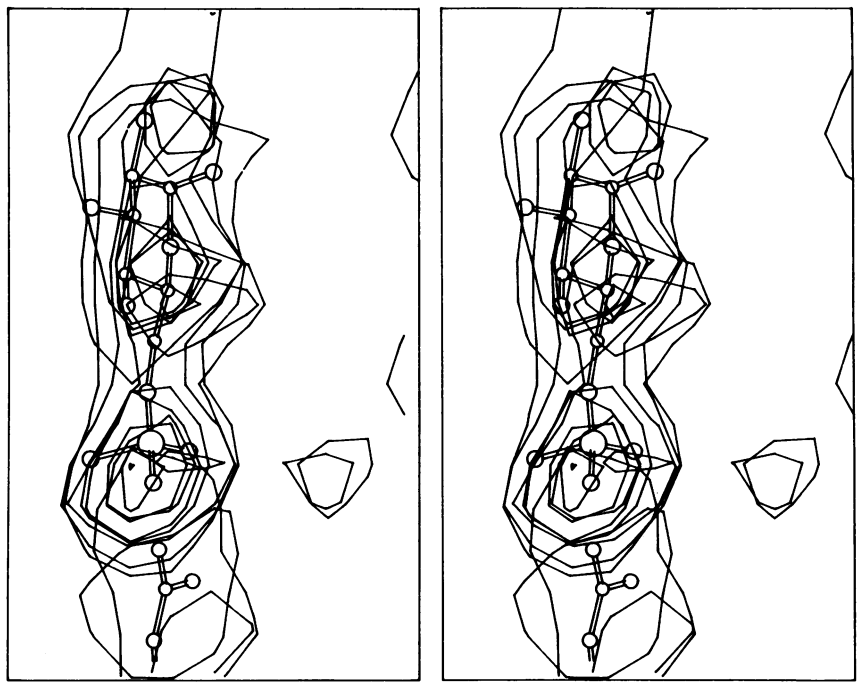


Fig. 2. *Stereo diagram of the difference electron density for Glc-6-P with the fit for α-Glc-6-P superimposed*
The new position of the guanidinium group of Arg-309 is also shown. Contour levels are 80, 160 and 240 arbitrary units.

was no indication of binding at the catalytic site or at any other site. The electron density of the sugar moiety of Glc-6-*P* partially overlapped the position of the peak seen in the DMM difference Fourier synthesis. Detailed analysis was therefore carried out by using the difference map with $F_{\text{Glc-6-P}} - F_{\text{DMM}}$ as coefficients so that density from the cross-link would not interfere with the interpretation. Cross-linking does not appear to alter Glc-6-*P* binding. Comparison of two-dimensional 0.3 nm (3 Å) difference Fourier syntheses from data recorded with the precession camera showed no differences in the binding of Glc-6-*P* between cross-linked and the native enzyme crystals.

The fit of Glc-6-*P* to its electron density is shown in Fig. 2. The orientation of the molecule could be defined unambiguously from the high contours representing the phosphate and the centre of the glucopyranose ring and the well-defined neck of density connecting the two. An equilibrium mix-

ture of Glc-6-*P* contains 38.5% α-anomer and 61.5% β-anomer (Bailey *et al.*, 1968). Both α- and β-configurations were explored, and it was concluded that the α-configuration gave a better fit to the electron density. The conformation of the phosphate ester is *trans,trans* (torsion angle O-5–C-5–C-6–O-6 = −166°; torsion angle C-2–C-6–O-6–P = −168°).

After the work had been completed, the single-crystal structure of the β-anomer of Glc-6-*P* was determined (Katti *et al.*, 1982), in which the glucopyranose ring has the standard $^4C_1$ chair conformation (as used in the present work), but the torsion angles O-5–C-5–C-6–O-6 and C-5–C-6–O-6–P (−70.3° and −118.3° respectively) are significantly different. In the single-crystal structure, the conformation about C-6–O-6 departs from the usual *trans* geometry and the smaller angle appears to be stabilized by an internal hydrogen bond between one of the phosphate oxygen atoms and O-4 of the glucopyranose ring. The 'folded' structure seen in the single-crystal work does not fit the electron density observed for Glc-6-*P* binding to phosphorylase *b*. We conclude the structures for the phosphate moiety are different. The differences probably arise from the differences in chelation of the phosphate. In the single-crystal work, the phosphate interacts with a Ba^{2+} ion and several water molecules. In the enzyme-bound structure the phosphate interacts with two cationic groups (discussed below). Both structures for Glc-6-*P* appear reasonable from the point of view of stereochemistry.

The binding site for Glc-6-*P* is close to but only partially overlaps the allosteric AMP-binding site. The general disposition of the effector site in the molecule is shown in Figs. 1 and 3(*a*). It is situated at the subunit–subunit interface, where the *C*-terminal ends of the two long α-helices, the A–A′ (residues 51–75) and the B–B′ (residues 291–312) helices, come together. The site is lined below by the central core of the β-sheet of the *N*-terminal domain (parallel strands of residues numbers 82–87, 153–159 and 241–248 and the antiparallel strand 227–233) and is bounded on one side by the start of the top loop comprising residues 193 and 194. The site is covered by the cap region of the symmetry-related subunit, residues 40′–47′. (Residues from the symmetry-related subunit are denoted by the superscript prime).

There are clear indications in the difference map for conformational changes at the region of the Glc-6-*P*-binding site. The first of these involves the side chain from Arg-309 on the B–B′ helix, which moves so as to make contact with the phosphate group of Glc-6-*P*. In the native-enzyme electron-density map this residue is reasonably well located, although the density is not as strong as that for well-localized side chains. The possible mobility of this arginine residue is reflected in the rather large temperature factors for side-chain atoms [average $B = 0.65\,nm^2$ ($65\,Å^2$)] compared with those for the main-chain atoms of this residue [average $B = 0.46\,nm^2$ ($46\,Å^2$)]. [*B*, the temperature factor, is related to the mean square amplitude of harmonic displacement ($\bar{U}^2$) by the expression $B = 8\pi^2 \cdot \bar{U}^2$.] In the Glc-6-*P* difference map positive contours indicate that the side chain shifts approx. 0.45 nm (4.5 Å) and becomes more localized (Figs. 2 and 4). The other major local conformational change involves residues from the symmetry-related cap region of the molecule. The carbonyl oxygen of Val-40′ makes a strong hydrogen bond to the hydroxy O-2 atom of Glc-6-*P*. There are positive contours in this region that, with associated negative features, indicate shifts in the side-chain atoms of Val-40′ and the main-chain atoms of Lys-41′ and the adjacent segment of chain around His-36′. It appears that Val-40′ and the main-chain atoms of Lys-41′ move to accommodate the Glc-6-*P* molecule and in so doing disturb other regions of the protein. In addition, there are also indications of movements of the A–A′ helix. These concerted movements are difficult to interpret from examination of the difference Fourier synthesis, and their detailed analysis awaits refinement of the metabolite–enzyme complex. They represent the largest conformational changes seen in our difference X-ray-crystallographic maps to date.

The intermolecular contacts between Glc-6-*P* and phosphorylase *b* are shown in Fig. 3. The phosphate group is stabilized by its interaction with two basic groups; the O-9 atom interacts with the NH2 group of Arg-309 from the B–B′ helix after the conformational change described above, and the O-8 atom interacts with the NZ group of Lys-247 from the underlying β-sheet. Arg-193 is in the vicinity of the site but is over 0.5 nm (5 Å) away. For the glucose moiety, the hydroxy O-2 atom acts as a donor in a hydrogen bond with the main-chain carbonyl oxygen atom of Val-40′. The hydroxy O-3 atom also hydrogen-bonds to this main chain atom. The hydroxy O-4 atom and the O-6 atom make no polar contacts. The ring O-5 atom can accept a hydrogen bond from the NE2 group of Gln-71 from the A–A′ helix. The α-hydroxy O-1 atom makes weak contacts with the NE2 group of Gln-71, the NH1 group of Arg-193 and the carbonyl oxygen atom of Val-40′. The major Van der Waals interactions involve contacts between the C-1 and C-5 (and to some extent the C-2 and C-6) atoms of the sugar and the side-chain carbon atoms of Gln-71 and Trp-67. The plane of the indole ring of Trp-67 is approximately perpendicular to the plane of the glucopyranose ring, and so this particular glucose–

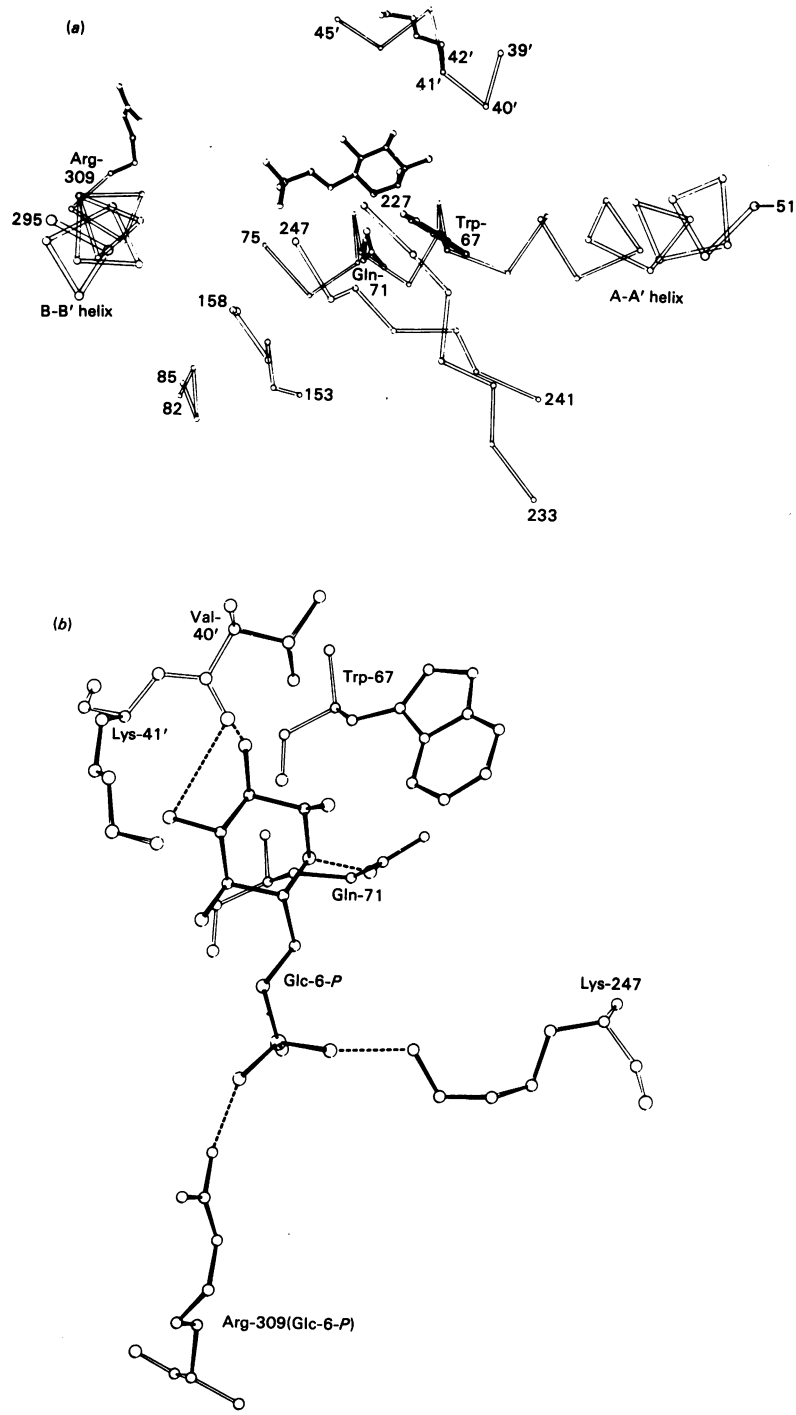


Fig. 3. *(a) View of the structural elements and some of the side chains involved in the binding of Glc-6-P at site N, and (b) details of the interaction between Glc-6-P and phosphorylase b at site N*

(*a*) The site is formed where the *C*-terminal ends of the A–A′ and B–B′ helices come together and is lined below by β-sheet and above by the cap region 39′–45′ from the symmetry-related subunit. Arg-309 is in the position occupied in the native enzyme. The view is down the crystallographic *y* axis as in Fig. 1. (*b*) Interactions include ionic contacts between the phosphate groups and Lys-247 and Arg-309 (after a shift from its native position), and hydrogen bonds between O-2 and O-3 and the carbonyl oxygen atom of Val-40′ and between O-5 and NE2 of Gln-71. Trp-67 is in Van der Waals contact with the C-1 and C-6 atoms.

tryptophan interaction is different to that observed for *N*-acetylglucosamine binding to lysozyme (Blake *et al.*, 1967).

The solvent contact area changes calculated on forming the Glc-6-*P*–phosphorylase complex show that for the Glc-6-*P* molecule the change in area is 82% of that of the solvent contact area of the unbound molecule. If we take an area of 0.03 nm^2 (3 $Å^2$) as representative of an atom that could be in contact with water, then only the sugar hydroxy O-4 atom and the phosphate O-9 oxygen atoms are accessible to water in the complex. The remainder of the molecule is entirely buried. The shielded residues on the protein, in addition to those already discussed (Val-40′, Gln-71, Lys-247, Arg-309, Trp-67), include Pro-194 and Tyr-155. The contributions of Pro-194 and Tyr-155 are of interest. Their separations from Glc-6-*P* [0.5 nm (5 Å) and 0.6 nm (6 Å) respectively] are such that their contribution to Van der Waals binding energy must be very small. Nevertheless these residues are close enough to the Glc-6-*P* molecule so that water must be excluded, and hence they experience a relatively large charge in solvent contact area.

The Glc-6-*P* molecule is comprised of a polar periphery of hydroxy groups, the charged phosphate group and non-polar core of carbon atoms of the glucopyranose ring. On forming the complex with phosphorylase, there is an almost equal change in the solvent contact areas for the non-polar atoms and the polar atoms of the protein [−0.262 nm^2 (−26.2 $Å^2$) and −0.295 nm^2 (−29.5 $Å^2$) respectively]. Thus the enzyme site provides the right contributions in terms of polar, non-polar and ionic interactions that complement the structure of the Glc-6-*P* molecule.

Comparison with AMP

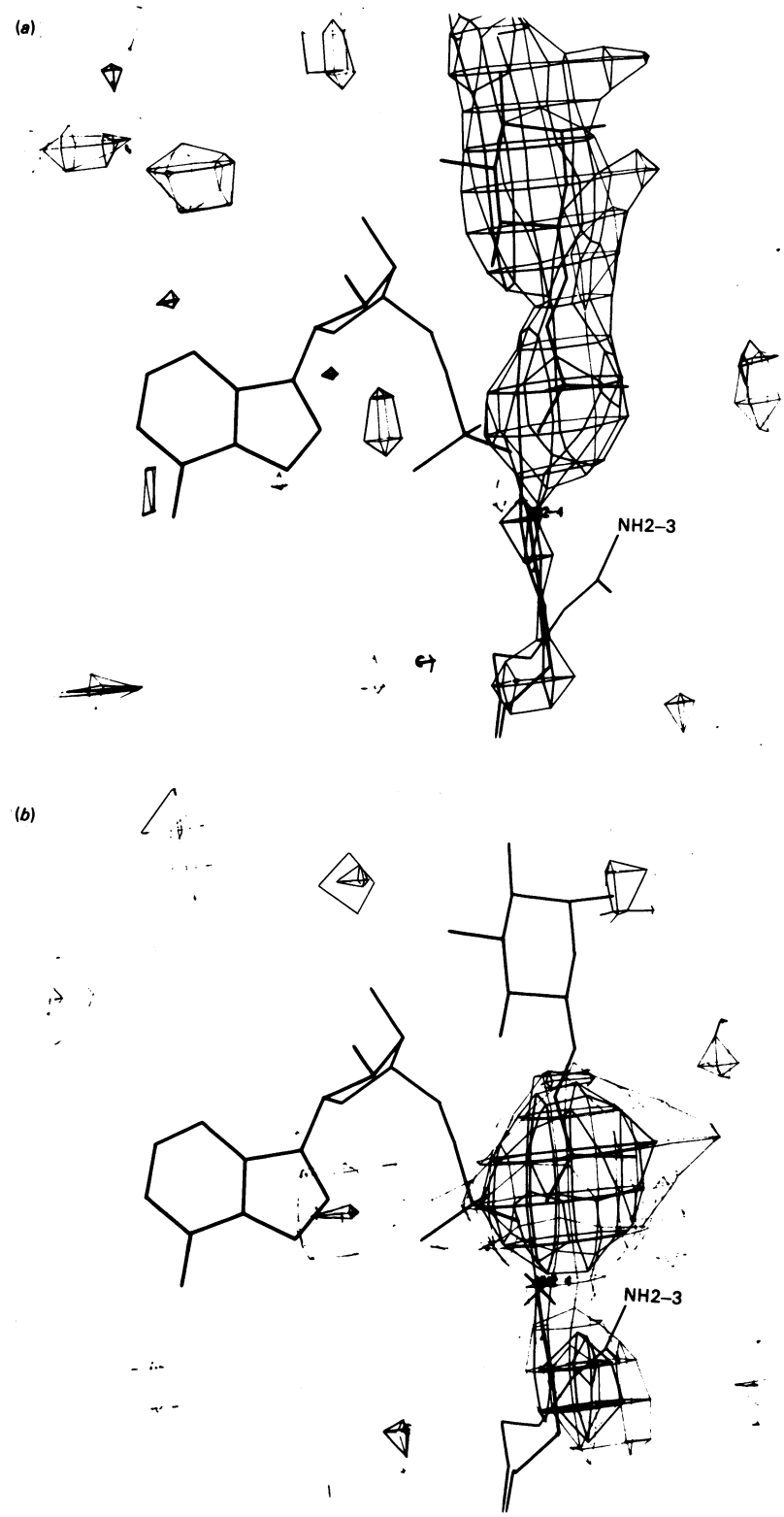
A comparison of the positions of AMP (Stura *et al.*, 1983) and Glc-6-*P* (Figs. 4 and 5) shows that the sites are adjacent and non-overlapping. The closest the atoms come together is at the phosphate-binding site, where the separation P (AMP) to P (Glc-6-*P*) is 0.27 nm (2.7 Å). The adenine ribose position of the AMP is more than 0.6 nm (6 Å) from the glucose of Glc-6-*P*. The contacts to the enzyme are quite different. Those for AMP involve the carbonyl oxygen atom of Asn-44′ and the side chains of Val-45′, Gln-72, Tyr-75 and Arg-309; those for Glc-6-*P* involve the carbonyl oxygen atom of Val-40′ and the side chains of Trp-67, Gln-71, Lys-247 and Arg-309. Depite these differences, it is unlikely that the two molecules bind simultaneously at their respective sites because of the proximity of the phosphate groups. Both phosphate-binding sites utilize the guanidinyl group of Arg-309, but this residue shifts to different positions in the two complexes (Figs. 4 and 5). In AMP the shift from the native structure (CZ atom to CZ atom) is 0.19 nm (1.9 Å), whereas in Glc-6-*P* the shift is considerably larger, namely 0.45 nm (4.5 Å). The separation between Arg-309 in the AMP complex and Arg-309 in the Glc-6-*P* complex is 0.4 nm (4 Å).

Phosphate binding

Allosteric site. The difference Fourier synthesis for 0.5 M-phosphate binding showed a major peak (height some 320 arbitrary units) that was over 4 times the 'noise' in the synthesis. In position and in peak height this peak was identical with that observed for the phosphate group of Glc-6-*P* (Fig. 4). There was also evidence from lower contours (at about 100 arbitrary units) that the side chain of Arg-309 from the CD atom onwards moved to a similar position to that occupied in the binding of Glc-6-*P*. The major interactions that stabilize P_i in this position are therefore the same as for the phosphate group of Glc-6-*P*, namely interaction with the guanidinium group of Arg-309 and the NZ atom of Lys-247. There was a slight tail of the P_i peak towards the position occupied by the phosphate group in AMP, indicating that this site may be weakly occupied (Fig. 4). There was no indication for movement of the side chain of Val-40′ or any of the other associated cap residues, and so we assume that the movement of these seen in the Glc-6-*P* difference map are a consequence of Glc-6-*P* binding and not of P_i binding or DMM cross-linking. Although the enzyme–P_i crystals had been cross-linked, there was no evidence for the major DMM cross-linking peak, but the secondary peak that represents a small disturbance at the *N*-terminal end of the A–A′ helix was present.

Catalytic site. P_i difference Fourier synthesis showed several positive and negative features at the catalytic site that were only just above the 'noise' level. (The maximum peak height in this region is 150 arbitrary units.) It is assumed that these features represent some disturbance of atoms at this site, but their precise interpretation awaits crystallographic refinement of the complex structure. These features were not observed in the Glc-6-*P* difference map. Residues for which there is some indication of movement include Asn-284 (main chain), Glu-381 (side chain), Arg-568 (side chain), His-570 (side chain), Arg-574 (side chain) and Ala-609–Pro-610 (main chain).

None of the features appeared strong enough (in comparison with the P_i binding site at site N) to indicate definite P_i binding at the catalytic site. This supports our view (Johnson *et al.*, 1980; Jenkins *et al.*, 1981) that in the present crystal form of the enzyme, which is close to the T state, the phosphate-recognition site at the catalytic site is


(a)
NH2–3
(b)
NH2–3

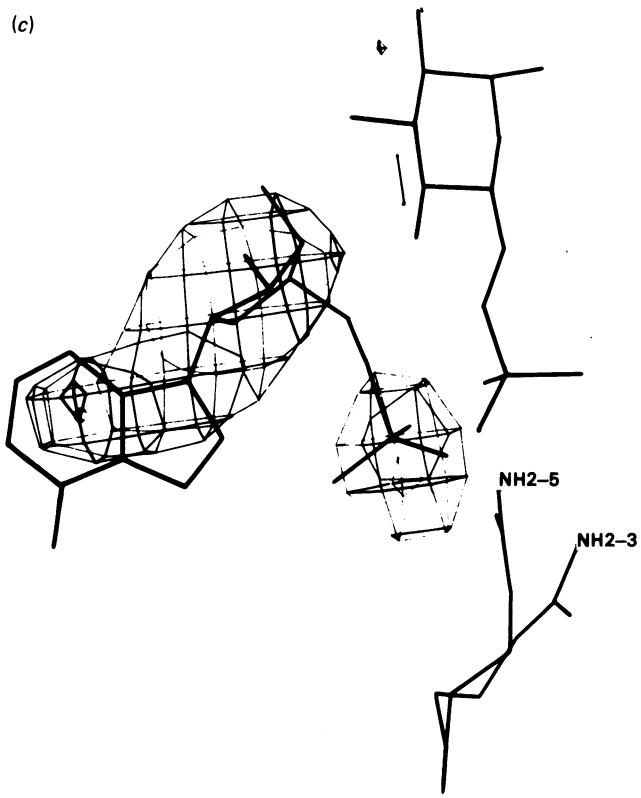


Fig. 4. *Comparative views from the Evans and Sutherland Picture System II of the difference Fourier syntheses for (a) Glc-6-P, (b) P_i and (c) AMP*

(*a*) View for Glc-6-*P*, showing the fit of the molecule and the density indicating the shift of Arg-309 from its native position (NH2–3) to its new position (NH2–4). Contour level is 100 arbitrary units. (*b*) View for P_i, showing the major peak in the position of the Glc-6-*P* phosphate position but with a slight tail towards the AMP phosphate position and the movement of Arg-309. Contour level is 100 (light contours) and 150 (dark contours). (*c*) View for AMP from the AMP- and glucose 1,2-cyclic phosphate-binding study (Stura *et al.*, 1983), showing the fit of the molecule and the movement of Arg-309 from NH2–3 (native) to NH2–5 (AMP). The contour level (150 arbitrary units) has been chosen to emphasise the phosphate position. The peak heights of the phosphate group and adenine group are 384 and 488 arbitrary units. The movement of the arginine residue and the continuity between the lobes of density of the AMP are apparent at a contour level of 100 arbitrary units.

not fully formed. Even at 0.5 M concentrations of P_i it is not occupied.

Other sites. There was no indication for phosphate binding at the serine-residue phosphate-binding site observed in phosphorylase *a* (which involves Arg-69 and Arg-43′), nor at any other site on the enzyme.

Discussion

DMM successfully stabilizes phosphorylase *b* crystals without producing significant conformational changes. At present it is not possible to assign cross-links to specific lysine residues. In view of the fact that the reaction in the crystal is probably incomplete and that at full substitution only five non-hydrogen atoms among approx. 7000 protein atoms would be added, the failure to define unambiguously the reaction sites in the difference Fourier synthesis is not surprising. It is hoped that as the refinement approaches completion a clearer interpretation of the modifications produced by cross-linking may be apparent.

The present results show that the allosteric inhibitor Glc-6-*P* binds to a site adjacent to the allosteric activator site with its phosphate moiety partially overlapping but distinct from phosphate-binding site for AMP [P-atom–P-atom separation 0.27 nm (2.7 Å)]. The glucose moiety Glc-6-*P* and the adenine ribose moiety of AMP do not overlap. The phosphate-binding site for Glc-6-*P* is close to that observed for the β-phosphate group of ATP

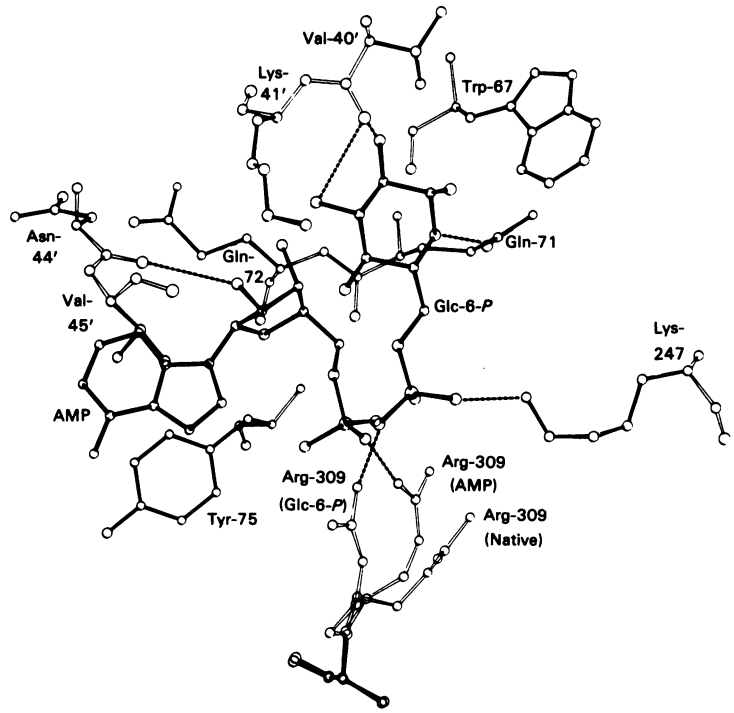


Fig. 5. *Comparison of the interactions made by AMP and Glc-6-P at site N of glycogen phosphorylase b*
Although the two molecules are shown together, it is unlikely that they would bind simultaneously because the P–P separation of their phosphate groups is only 0.27 nm (2.7 Å). Arg-309 is shown in its native position, and in its new positions for Glc-6-*P* and for AMP. The contacts to AMP involve Tyr-75 and Val-45' (to the base), CO group of Asn-44' and Gln-72 (to the ribose) and Arg-309 to the phosphate as described in Stura *et al.* (1983). The contacts to Glc-6-*P* are given in Fig. 4(*b*) and in the text.

(Johnson *et al.*, 1979). The results are consistent with the allosteric effects observed in solution studies. Thus partial competition between the activator AMP and the inhibitor Glc-6-*P* (Wang *et al.*, 1970) can be explained because both metabolites utilize distinct but mutually exclusive phosphate-binding sites while the remaining parts of the two molecules occupy non-overlapping sites. The two phosphate-binding sites are too close for both to be occupied simultaneously unless they are covalently bonded, as in ATP or ADP. Thus a ternary AMP–enzyme–Glc-6-*P* complex is unlikely because both metabolites must use their appropriate enzyme phosphate-binding sites in order to bind. Adenosine is not an activator of phosphorylase *b* (Okazaki *et al.*, 1968; Morange *et al.*, 1976) and does not bind at the allosteric effector site (in phosphorylase *a*) (Kasvinsky *et al.*, 1978). Likewise Glc-6-*P* needs both its phosphate and its glucose components in order to bind. Glucose by itself binds only at the catalytic site some 3.2 nm (32 Å) away (E. A. Stura, M. S. P. Sansom & L. N. Johnson, unpublished work).

The binding of the α-anomer of Glc-6-*P* is preferred in the crystal. This result is consistent with the observations made by Battersby & Radda (1976), who demonstrated in a ^{31}P-n.m.r. study that the binding of Glc-6-*P* is stereospecific for the α-anomer to the exclusion of observable interaction with the β-anomer. In the crystal structure it is clear that the β-anomer could not bind in the same position as that observed for the α-anomer, because this would result in bad contacts between the β-hydroxy O-1 atom and the side chain of Trp-67. Trp-67 is relatively buried and is in Van der Waals contact with Gln-71, Thr-228, Pro-229 and Leu-243. It would be difficult for the Trp-67 residue to move without disrupting the structure. It is possible to fit the β-anomer in another conformation with reasonable contacts to the enzyme, but this fit is not supported by the difference electron density. It must be assumed that the favourable contacts made by the binding of the α-anomer are sufficient to result in the preferred binding. Battersby & Radda (1976) note that the α-anomer is produced by the phosphoglucomutase reaction

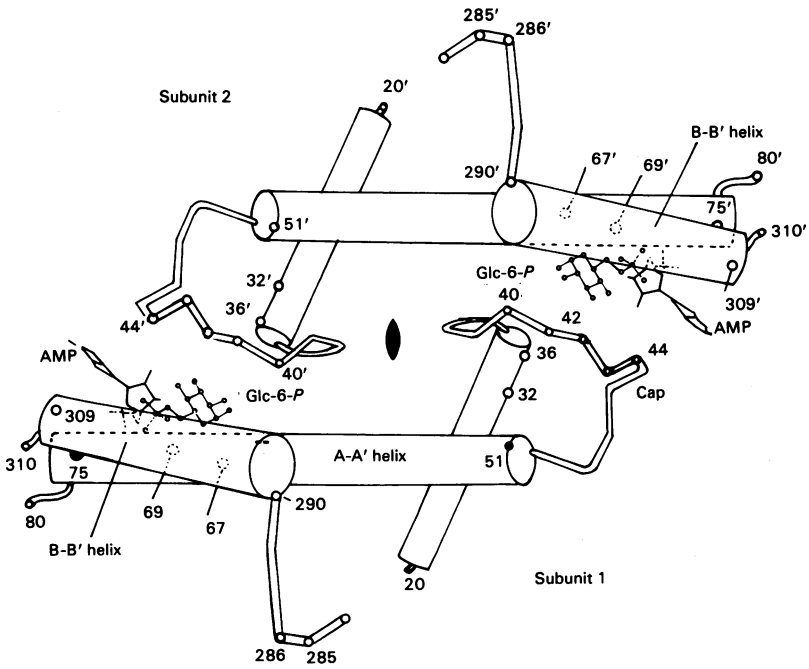


Fig. 6. *Schematic diagram of the phosphorylase b dimer in the region of the allosteric effector site*
The view is down the twofold-symmetry axis of the dimer and shows the way in which the two sites may communicate with one another through the A–A′ helix and the cap region. The B–B′ helix is in Van der Waals contact with the A–A′ helix only at the *C*-terminal end. It is not in contact with the cap region. The space between the A–A′ helix and the B–B′ helix is filled with other structural elements. AMP- and Glc-6-*P*-binding positions are shown, but it is unlikely that both could bind simultaneously (see the text).

(Gadian *et al.*, 1974) and that the correspondence in stereospecificity of the two consecutive enzymes in glycogenolysis may be important for the efficiency of feedback control.

The dissociation constant for α-Glc-6-*P* binding to phosphorylase *b* ($K_D = 19\,\mu M$) is smaller than that for AMP ($K_D \simeq 80\,\mu M$ in the absence of other ligands). The high affinity for Glc-6-*P* can be explained by the large number of specific interactions made with the enzyme, such that 82% of the Glc-6-*P* molecule is buried. All but one of the hydroxy oxygen atoms are involved in hydrogen bonds. With AMP only 66% of the molecule is buried, and only two polar groups (the ribose hydroxy O-2′ atom and the phosphate oxygen atom) are involved in contacts (Stura *et al.*, 1983). Moreover, the phosphate-specificity site for Glc-6-*P* involves both Arg-309 and Lys-247 side chains whereas that for AMP involves only Arg-309.

Binding studies with 0.5M-phosphate support the notion that the phosphate-binding site for Glc-6-*P* is the high-affinity binding site. P_i by itself binds almost exclusively at this site with only a very weak indication of binding at the phosphate-binding site for AMP and no definite indication for binding at the catalytic site. Why is this high-affinity site not occupied by AMP? Model building with the computer graphics shows that, when the AMP molecule is moved so that its phosphate group occupied this site, there are no bad contacts with the enzyme, but both the favourable interaction with Tyr-75 and the hydrogen bond to the Asn-44′ carbonyl oxygen atom were destroyed. Evidently these and other favourable interactions must be sufficient to displace the phosphate group some 0.27nm (2.7Å) from the high-affinity site.

The increase in affinity for AMP is one of the key features of the homotropic and heterotropic responses of phosphorylase. The enzyme has an absolute requirement for a nucleotide with a dianionic phosphate group (Withers & Madsen, 1980). Binding at the phosphate moiety is therefore a key factor. At the present stage there appear to be two possibilities for the conformational response. Either the AMP remains bound to its present position and the other basic groups (e.g. Lys-247 and Arg-193) move so as to tighten the phosphate-binding site, or the AMP shifts [0.27nm (2.7Å)] so that its phosphate group occupies the present existing high-affinity phosphate-binding site and other groups on the enzyme (e.g. Tyr-75 and Asn-44′) move so as to interact with the new position of

AMP. In the absence of information on the R state we cannot distinguish these possibilities, but we note that movements are already observed for Tyr-75 and Asn-44′ on binding AMP.

Comparison of the interactions made by AMP and Glc-6-*P* to the enzyme indicate a route by which the homotropic and heterotropic effects might be mediated. Both metabolites make contact with residues from (1) the A–A′ helix (Gln-72 and Tyr-75 for AMP; Gln-71 for Glc-6-*P*), (2) the B–B′ helix (Arg-309 for both AMP and Glc-6-*P*) and (3) the cap region of the symmetry-related subunit (Val-45′ and the main-chain carbonyl group of Asn-44′ for AMP; the main-chain carbonyl group of Val-40′ for Glc-6-*P*). In both studies there are indications for movement of Arg-309 and for residues in the cap region. The *N*-terminal end of the A–A′ helix (Tyr-51) is in contact with its own cap region (Asn-42). Thus conformational changes induced by the binding of either AMP or Glc-6-*P* to one subunit will be felt by the other subunit. Fig. 6 gives a diagrammatic representation of these effects. The specific interaction of the 2-hydroxy group of Glc-6-*P* with the main-chain carbonyl group of Val-40′ is of significance. 2-Deoxy-Glc-6-*P* is an inhibitor of phosphorylase *b* ($K_i = 0.45$ mM) but does not exhibit allosteric properties (Bailey *et al.*, 1981). Likewise the 2′-hydroxy group of the adenine ribose (which interacts with the main-chain carbonyl oxygen atom of Asn-44′) is important for both activation and binding of AMP (Okazaki *et al.*, 1968; Black & Wang, 1970). Thus the interactions across the subunit–subunit interface for both compounds are of importance for the allosteric response.

The route by which these changes are communicated to the catalytic site is more obscure. An overall movement of the B–B′ helix (residues 291–312) is likely to affect the loop carrying residues Phe-285 and Phe-286, which forms part of the nucleoside-inhibitor-binding site and the entrance to the catalytic site. Communication of these changes to some of the catalytic-site residues (e.g. His-570, Arg-568 and Glu-381) could modulate activity. Changes in the loop region at the start of the B–B′ helix have been observed when crystals of glucose-inhibited phosphorylase *a* are exposed to substrates (Madsen *et al.*, 1978). Disturbances at the catalytic-site residues are observed in the present study on binding P_i at the allosteric-effector-binding site.

The problem of why one metabolite (AMP) leads to activation and the other (Glc-6-*P*) to inhibition remains to be elucidated. It may be that Glc-6-*P*, by virtue of its position and contacts, is effective in keeping the 'jaws' of the A–A′ and B–B′ helices apart, whereas AMP, which is situated closer to the 'hinge' region of the two helices, is more effective in bringing the helices together (Fig. 6). The importance of the two helices, the A–A′ and the B–B′ helices, in the transmission of the allosteric response is of interest in view of current ideas that a favoured route for transmission of conformational changes involves adjustment of side-chain rotations by relative displacement of packed α-helices (Baldwin & Chothia, 1979; Chothia *et al.*, 1983; Levine *et al.*, 1983).

The role of the *N*-terminal residues (1–16) in the control of phosphorylase is intriguing. Localization or removal of the *N*-terminal tail results in loss of Glc-6-*P* inhibition. In phosphorylase *a*, significant inhibition by Glc-6-*P* is not observed unless some degree of T-state stabilization is achieved by the binding of glucose (Melpidou & Oikonomakos, 1983). With phosphorylase *b*′ [the limited tryptic digest of phosphorylase *b*, lacking residues 1–16 (Graves *et al.*, 1968)] no inhibition by Glc-6-*P* is observed. Conversely Glc-6-*P* is able to influence the conformation of the *N*-terminal tail. Phosphorylation of Ser-14 by phosphorylase kinase is inhibited by Glc-6-*P* by virtue of its effects on the substrate, phosphorylase *b* (Tu & Graves, 1973). The present crystallographic results give some indication as to how these effects might be mediated. In phosphorylase *b* the first 18 residues from the *N*-terminus are not located in the electron-density map and are assumed to be flexible (Weber *et al.*, 1978). In phosphorylase *a* the *N*-terminal tail is anchored by interaction of the Ser-14 phosphate group with Arg-69 and Arg-43′ and by a salt link between Arg-10 and Asp-32′, i.e. by interactions across the subunit–subunit interface (Sprang & Fletterick, 1980). On binding Glc-6-*P* to phosphorylase *b* in the crystals we see conformational changes in the region of residues 40′–41′ that extend to the adjacent segment of chain involving His-36′. His-36 is located on the outer face of the *N*-terminal helix (residues 21–36; Fig. 6), and is adjacent to Asp-32. Thus the conformational changes are in the region of the subunit–subunit interface and are also related to those residues involved in the localization of the *N*-terminal tail in phosphorylase *a*.

The activation of phosphorylase *b* by AMP was the first example of allosteric enzyme (Cori & Cori, 1936). The kinetics of this and other responses of the enzyme have been extensively investigated in terms of the various models proposed to account for allosteric response, often with complex results (as discussed by Graves & Wang, 1972). Because the present crystallographic results have been obtained by diffusion of metabolites into cross-linked preformed crystals, it is unlikely that the observed conformational changes represent the full manifestation of the conformational response of the enzyme. Nevertheless the present studies indicate

that there must be at least four different conformational states of phosphorylase *b*. Our starting crystals were obtained in the presence of the weak activator IMP: the crystals are active but have low affinity for the substrate. Removal of IMP leads to no well-defined changes in the crystal, yet subtle changes must occur that lead to loss of activity. Hence we characterize the state of the enzyme in the present crystal form as a T′ state to distinguish it from the nucleotide-free state, the T state. On addition of the strong activator AMP to the native crystals, the crystals crack and the initiation of the T→R conformational transition is seen in the crystals (Stura *et al.*, 1983). On addition of the inhibitor Glc-6-*P* to the native-enzyme crystals, the crystals again crack, but different conformational changes are observed, which must represent a transition from the T′ (weakly active) to an I (inhibited) state. Co-crystallization studies are needed in order to characterize the full extent of these conformational changes.

The results confirm our previous observation that, in the form of the enzyme present in the crystal, the phosphate-recognition site at the catalytic site is not fully formed. Even at 0.5M-phosphate concentrations, binding of P_i at the catalytic site is not observed. When either glucose 1-phosphate (Johnson *et al.*, 1980) or glucose 1,2-cyclic phosphate (Jenkins *et al.*, 1981) are bound, the specificity is mainly for the glucose moiety. The phosphate groups make only limited contacts to the enzyme (to the helix dipole of the helix beginning at Gly-135 in the case of glucose 1-phosphate; to the pyridoxal phosphate oxygen atom and possibly His-570 in the case of glucose 1,2-cyclic phosphate). It is to be expected that, in the ternary enzyme–substrate complex in the R state, certain basic residues must move in order to stabilize the transition state and promote catalysis. Arg-568, His-570, Lys-573 and Arg-574 are possible candidates. The results described in the present paper show that conformational transitions that lead to an increase in affinity at phosphate-binding sites are key factors in the allosteric response of phosphorylase *b* at both the allosteric and the catalytic sites.

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