PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Phosphorylase: Control and Activity [and Discussion]

J. A. Jenkins, Louise N. Johnson, D. I. Stuart, E. A. Stura, K. S. Wilson, G. Zanotti and D. R. Wilkie

Phil. Trans. R. Soc. Lond. B 1981 **293**, 23-41
doi: 10.1098/rstb.1981.0057

Phil. Trans. R. Soc. Lond. B **293**, 23–41 (1981)
Printed in Great Britain

Phosphorylase: control and activity

By J. A. Jenkins,† Louise N. Johnson, D. I. Stuart, E. A. Stura, K. S. Wilson and G. Zanotti‡

Laboratory of Molecular Biophysics, Department of Zoology, South Parks Road, Oxford OX1 3PS, U.K.

Recent results from the crystallographic studies on glycogen phosphorylase *b* at 3 Å resolution are reviewed with special reference to other themes of the meeting. The structural similarity of the fold of 150 residues in phosphorylase to that observed in lactate dehydrogenase is discussed and the binding sites for NADH in phosphorylase are described. The binding of the potent inhibitor glucose-1,2-cyclic phosphate to phosphorylase *b* in the crystal has been studied at 3 Å resolution. The results are compared with those previously obtained for glucose-1-phosphate and discussed with reference to proposals for a mechanism of catalysis that involves the essential cofactor pyridoxal phosphate.

1. Introduction

Under anaerobic conditions, the ATP required to sustain muscle contraction is supplied by the conversion of glycogen to lactic acid via glycolysis and the rephosphorylation of the hydrolysed ATP via the creatine kinase reaction. Phosphorylase catalyses the first step in glycogen degradation and, since the fundamental work of the Coris (Cori & Cori 1940), is one of the best understood examples of a regulatory enzyme. Phosphorylase was the first enzyme whose activity was shown to be regulated by phosphorylation–dephosphorylation reactions (Krebs & Fischer 1956) and was one of the first eukaryotic enzymes for which regulation by allosteric effectors was recognized (Cori & Cori 1936; Helmreich & Cori 1964; Monod *et al.* 1965). Phosphorylase exists in two interconvertible forms. Phosphorylase *b*, found in resting muscle, is inactive except in the presence of AMP, IMP or certain analogues of AMP, and is inhibited by ATP or glucose-6-phosphate. Thus the enzyme's activity is regulated by the intracellular concentrations of these metabolites. In response to nervous or hormonal signals, phosphorylase *b* is converted to phosphorylase *a* by the action of phosphorylase kinase which results in the incorporation of a single phosphate per monomer at Ser 14. Phosphorylase *a* is always active, although its activity may be increased by AMP and inhibited by glucose. Inactivation of phosphorylase *a* is catalysed by phosphorylase phosphatase which in its turn is regulated by an inhibitor which also exists in phosphorylated and dephosphorylated forms. Evidence is accumulating that, in addition to the well recognized hormonal response of the cyclic AMP dependent protein kinase, other proteins involved in regulation of glycogen metabolism, such as the phosphatase, may also be integrated into a network of regulatory pathways which allow the activities of other functionally related cellular processes to be controlled in a synchronous manner (see review by Cohen *et al.* (1979)).

† Present address: Department of Structural Biology, Biozentrum der Universität Basel, CH-4056 Basel, Switzerland.

‡ On leave of absence from Centro di Studi Biopolimeri, Istituto di Chimica Organica, 35100 Padua, Italy.

Phosphorylase *b* is a single polypeptide chain (M_r = 97333) containing 841 amino acids. The physiologically active form of the enzyme is the dimer. The elucidation of the complete amino acid sequence of this large molecule by Titani *et al.* (1977) was a major achievement that has revolutionized phosphorylase studies, as will be apparent in the description of the crystallographic results. The enzyme contains one mole of pyridoxal phosphate per mole (Baranowski *et al.* 1957) linked to the enzyme via a Schiff base to Lys 679. The Schiff base can be reduced with sodium borohydride with no loss of activity (Fischer *et al.* 1958). The

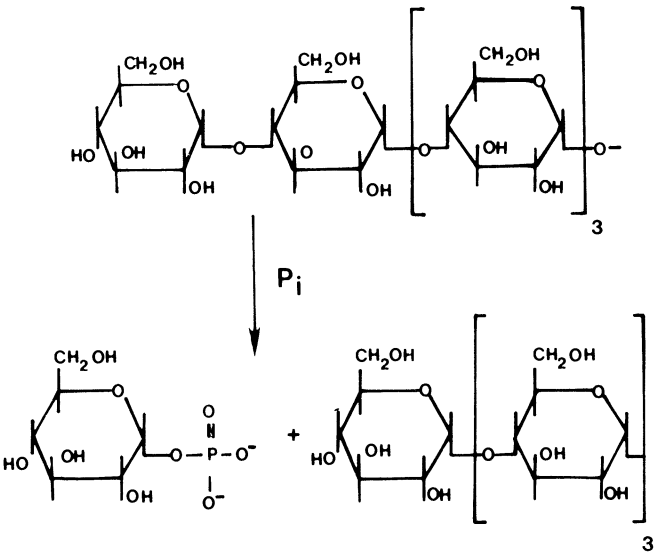

FIGURE 1. The reaction catalysed by phosphorylase; $K_{eq}(P_i/G1P)$ = 3.6.

role of the cofactor is therefore quite different from its role in conventional vitamin B_6 dependent enzymes. Yet because of the high concentration of phosphorylase in muscle (5–7 % of the soluble protein), as much as 60 % of the vitamin present in rat muscle and as much as 75–96 % of the total vitamin B_6 content of murine muscle is accounted for by phosphorylase (see review by Krebs & Fischer (1964)). The role of the cofactor in phosphorylase has long remained a mystery, but recent evidence (summarized by Helmreich & Klein (1980)) strongly supports the proposal that the 5′-phosphate group plays an important role in catalysis.

The crystal structures of rabbit muscle phosphorylase *a* and phosphorylase *b* have been solved at 2.5 and 3 Å resolution respectively (Sprang & Fletterick 1979; Weber *et al.* 1978). Phosphorylase *b* crystals are obtained in the presence of IMP, a weak activator that is only effective at high concentrations (2 mM), and which in contrast to AMP produces no increase in affinity of the enzyme for the substrate (Black & Wang 1968). Although the crystals have been shown to be active (Kasvinsky & Madsen 1976), both crystallographic and solution studies indicate that the enzyme in the crystal is close to the T state (Johnson *et al.* 1979). Phosphorylase *a* crystals are obtained in the presence of 50 mM glucose, a strong inhibitor. They are nearly isomorphous to those for phosphorylase *b* and again the enzyme is in the T state. So far no suitable crystals of the R form of the enzyme have been obtained, and this lack of knowledge of the active, high-affinity structure is a severe limitation to the interpretation of our results. Efforts to obtain such crystals are continuing.

In order to describe our crystallographic studies on the interactions between phosphorylase

b and its substrates, we summarize briefly what was known about the mechanism in advance of the crystallographic work. Phosphorylase catalyses the sequential phosphorylation from the non-reducing end of α(1–4) linked glucose residues of glycogen or linear oligosaccharides (figure 1). The enzyme reaction has the following properties.

TABLE 1. ALLOSTERIC ACTIVATION OF GLYCOGEN PHOSPHORYLASE *b* BY NUCLEOTIDES

(From Black & Wang (1968).)

nucleotide	concentration	Hill coefficient, *n*, for G1P	concentration of G1P for half maximum velocity	Hill coefficient, *n*, for nucleotide
AMP	0.052 mM	1.6	9.0 mM	1.4‡
	2.08 mM	1.2	4.5 mM	
IMP		2.0†	32–37†	2.0 ([G1P] = 10.4 mM) 1.2 ([G1P] = 83.3 mM)

† Independent of [IMP]. ‡ Independent of [G1P].

1. Muscle phosphorylase exhibits a much greater affinity for branched polysaccharides than for corresponding linear polysaccharides (Brown & Cori 1961), an observation that could be explained by an entropic effect arising from the presence of a tight binding site for glycogen, the glycogen storage site, distinct from the catalytic site. In contrast, potato phosphorylase has a high affinity for linear polysaccharides and amylopectin but a low affinity for glycogen, and glucans bind preferentially at the catalytic site (Shimomura & Fukui 1980).

2. Phosphorylase will digest up to four sugar residues from an α(1–6) branch point and the smallest oligosaccharide that will serve as an acceptor in the reverse reaction is maltotetraose (Brown & Cori 1961).

3. The affinity of phosphorylase *b* for G1P† is increased in the presence of AMP, a strong activator, but not in the presence of IMP (table 1).

4. The reaction proceeds via breaking of the α-C1–O1 glycosidic bond with retention of configuration (Hassid *et al.* 1943).

5. The enzyme exhibits rapid equilibrium bi-bi kinetics, although this observation needs to be re-examined in the light of the crystallographic evidence for a strong glycogen-binding site independent of the catalytic site (Kasvinsky *et al.* 1978*a*). The rate-limiting step involves the interconversion of the ternary complex.

6. Rabbit muscle phosphorylase will not catalyse exchange between G1P and inorganic phosphate (Cohn & Cori 1948). However, in the potato enzyme it has been shown that, in the presence of cyclodextrin as a pseudo second substrate, positional isotope exchange rates for the ester and phosphoryl oxygens of G1P are similar to those observed in catalysis (Kokesh & Kakuda 1977). This observation provides the major evidence for the existence of a glucosyl intermediate in the enzyme reaction.

7. There is a change in the state of ionization of the 5′-phosphate group of pyridoxal phosphate from a monoanion to a dianion following conversion of phosphorylase from the inactive to the active state. On binding of substrates to the potato enzyme there is an apparent increase in p*K* of the 5′-phosphate group (see review by Helmreich & Klein (1980)).

† Abbreviations: G1P, α-D-glucopyranose-1-phosphate; G12P, α-D-glucopyranose-1,2-cyclic phosphate.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

8. pH rate profile studies (Kasvinsky & Meyer 1977) indicate a pK 6.56, which has been assigned to the phosphate substrate, and two groups involved in catalysis with pK_{1es} = 6.12 and pK_{2es} = 7.0 at 30 °C. At 15 °C, pK_{1es} is very much less than 6.1.

9. *In vitro*, acid hydrolysis of G1P below pH 4 proceeds via the formation of a carbonium ion intermediate (Bunton *et al.* 1958).

10. Early work on the secondary isotope effect for phosphorylase gave a ratio of rate constants k_H/k_D = 1.1 consistent with a carbonium ion intermediate (Tu *et al.* 1971). More recent studies show a ratio of 1.0 consistent with a covalent intermediate (Firsov *et al.* 1974).

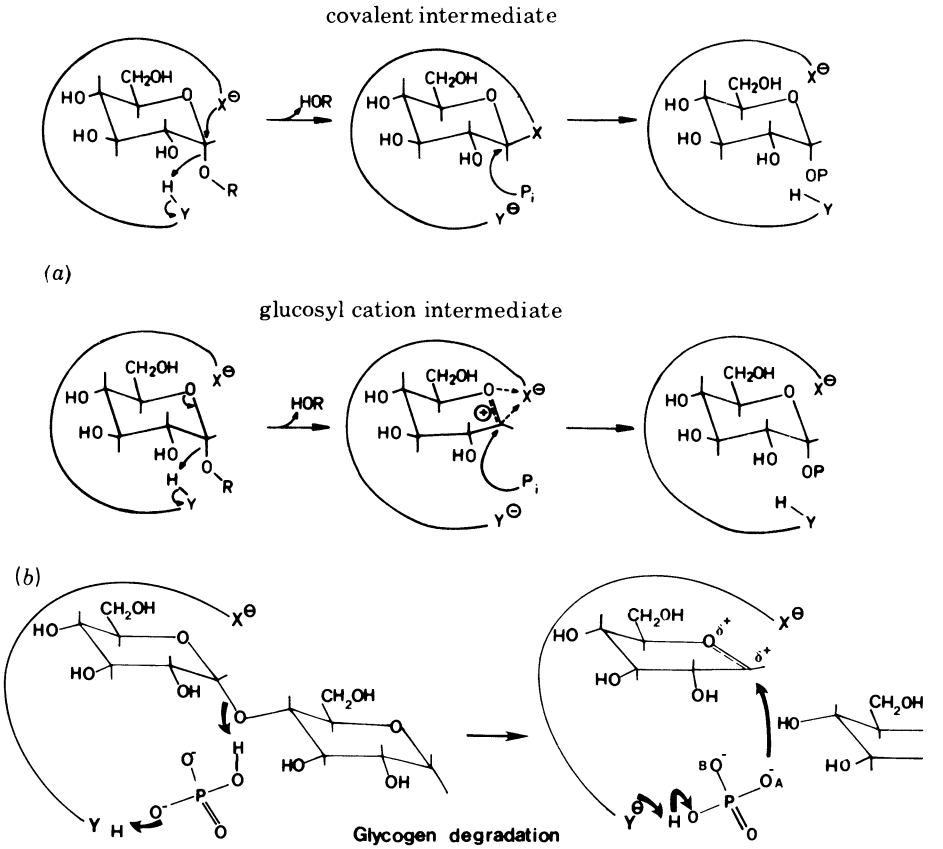

FIGURE 2. Possible double displacement mechanisms for phosphorylase. (*a*) The reaction involves general acid catalysis in which an acid group Y donates a proton to the glycosidic oxygen (R = (glucosyl)$_n$) leading to the formation of a carbonium–oxonium ion intermediate which is favoured and stabilized by a nucleophilic group X. The ion pair intermediate may collapse to a covalent β-glucosyl–enzyme intermediate. In the second step, Y acts as a base to promote attack by phosphate (from Feldmann *et al.* 1978). (*b*) The same as (*a*) except that the acid group Y acts through the phosphate ion to promote acid attack on the glycosidic bond.

Because the reaction proceeds with retention of configuration, a glucosyl-enzyme intermediate has been invoked in the reaction pathway, although, as outlined above, direct evidence for such an intermediate is difficult to obtain. The mechanisms proposed therefore involve a double displacement reaction (figure 2*a*) in which an acid group donates a proton to the glycosidic oxygen with the formation of either a β-glucosyl-enzyme covalent intermediate or a carbonium–oxonium ion stabilized by a nucleophilic group on the enzyme. These mechanisms do not explain why the reaction involves phosphorylation rather than

hydrolysis, although such an explanation could of course be provided by steric properties, a specific recognition site for phosphate and the exclusion of water from the catalytic site. Phosphorylysis could also be explained by a modified mechanism in which the acid group did not act directly on the substrate but served to promote acid attack by the phosphate (figure 2*b*). Such a mechanism imposes severe restraints on the positions of the substrates to avoid the attacking group and leaving groups occupying the same position at the same time, and implies that the acid group on the enzyme must be a stronger acid than phosphate.

In our recent crystallographic work, we have directed our studies towards a search for potential transition state analogues. α-D-Glucopyranose-1,2-cyclic phosphate (G12P) partially fulfils this requirement. G12P is a potent inhibitor of phosphorylase, competitive with G1P and non-competitive with glycogen (Kokesh *et al.* 1977; Hu & Gold 1978). For potato phosphorylase, the affinity is 26 times greater than that for G1P, while for rabbit muscle phosphorylase *a* the affinity is approximately 5 times greater ($K_i \approx 0.3$ mM and 0.5 mM in the absence and presence of AMP, respectively). For phosphorylase *b*, similar results are obtained in the presence of AMP, but in its absence equilibrium dialysis experiments suggest a dissociation constant greater than 1 mM (Dreyfus *et al.* 1980). Two, not necessarily exclusive, proposals have been put forward to account for the high affinity of the cyclic phosphate (Kokesh *et al.* 1977). First, it may arise simply from better contacts with the enzyme. Thermodynamic calculations show that an increase of the order of 5–26 times corresponds to a change in Gibbs free energy of 4–8 kJ/mol equivalent to one or two hydrogen bonds. Secondly, and perhaps more interesting, it was recognized that the cyclic ester could represent a rigid analogue of one of the rotational isomers of G1P, and hence might mimic the conformation of the enzyme-bound substrate.

2. The structure of phosphorylase *b*

2.1. *Crystallography*

Crystals of phosphorylase *b*, grown in the presence of 10 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethane sulphuric acid (BES), 10 mM magnesium acetate, 2 mM IMP, pH 6.7, are tetragonal space group $P4_32_12$ with unit cell dimensions $a = b = 128.5$, $c = 115.9$, and with one monomer (M_r 97333) per asymmetric unit. The two subunits of the physiologically active dimer are related by the crystallographic twofold axis at $z = \frac{1}{2}$. Since the original interpretation of the fold at 3 Å resolution (Weber *et al.* 1978), an improved electron density map has been calculated based on a nominal four heavy atom derivatives with an overall figure of merit of 0.64. Interpretation at 3 Å resolution has been made possible through knowledge of the complete amino acid sequence (841 residues) of phosphorylase (Titani *et al.* 1977). A molecular model on a scale of 2 cm/Å has been built by using a Richards box (Richards 1968). Model building has proved enormously instructive (although laborious) and has facilitated interpretation of the structure. The coordinates (measured with a new sound-ranging coordinate-measuring device (Marsh 1981)) have been subjected to a constrained least-squares refinement (Konnert 1976) with the use of the Science Research Council's Cray 1 computer at Daresbury. The refinement is still in progress, but results to date are encouraging with a current R value of 34 %. Data to 2 Å resolution have been collected by using the synchrotron radiation facility at Lure on the D.C.I. storage ring at Orsay. The quality of the 2 Å data is as good as that of the 3 Å data and extension of refinement to 2 Å resolution

is in progress. The results described in this paper are based on the model-built coordinates, but it is expected that a more precise structure will be available shortly.

2.2. *The structure and metabolite binding sites*

The spatial distribution of the four metabolite binding sites that have been established from crystallographic experiments is shown schematically in figure 3. The activators AMP or IMP bind at the allosteric effector site (site N) which is close to the subunit–subunit inter-

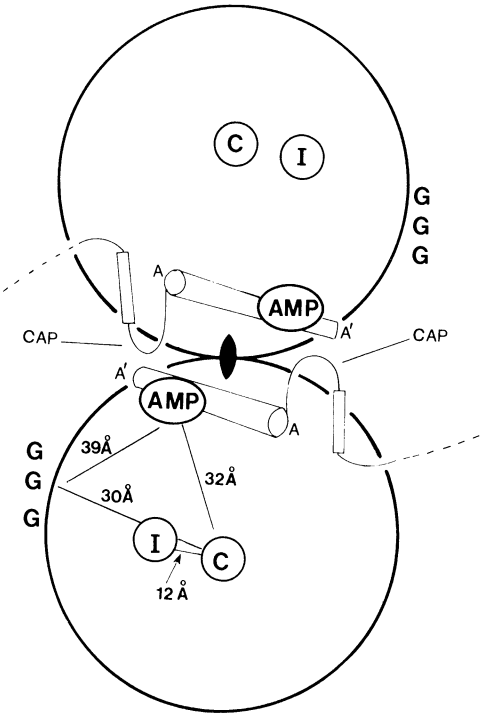

FIGURE 3. A schematic diagram of the phosphorylase dimer showing the four metabolite binding sites. AMP, C, G-G-G and I are the allosteric effector site, the catalytic site, the glycogen storage site and the nucleoside inhibitor site, respectively.

face of the physiologically active dimer. This site also binds the allosteric inhibitors ATP and glucose-6-phosphate. The catalytic site, site C, which binds the substrate and the inhibitor glucose, is some 32 Å from the allosteric effector site and is at the centre of the molecule. Some 30 and 39 Å away from the catalytic site and the allosteric site (respectively) is a tight binding site for glycogen analogues such as maltotriose, maltopentose and maltoheptaose (site G). This site is almost certainly utilized in the attachment of phosphorylase to the glycogen particles *in vivo* (Meyer *et al.* 1970). Finally there is a nucleotide or nucleoside inhibitor site, I, which is some 12 Å from the catalytic site. This site also shows specificity for adenosine, adenine and a number of other purines or methylated oxypurines (Kasvinsky *et al.* 1978*b*). The affinity for AMP at site I is considerably less than the affinity at site N, the major regulatory site (Wang *et al.* 1970). On the other hand, IMP binds to site I with an affinity comparable to that of site N (Uhing *et al.* 1979) a phenomenon that may partly explain the different kinetic behaviour of the activators. The separation between allosteric sites of the two subunits is 45 Å, between the catalytic sites on the two subunits is 58 Å, and the separation between the allosteric site on one subunit to the catalytic site on the other subunit is 49 Å.

Figure 4 shows a schematic drawing of the fold of one subunit, which can be described in terms of three domains.

The N-terminal domain (upper part of figure 4; residues 19–311) includes the allosteric effector site and all the contacts between the subunits of the physiologically active dimer. The two helices AA′ (51–76) and BB′ (291–311) flank the nucleotide allosteric site and lie over

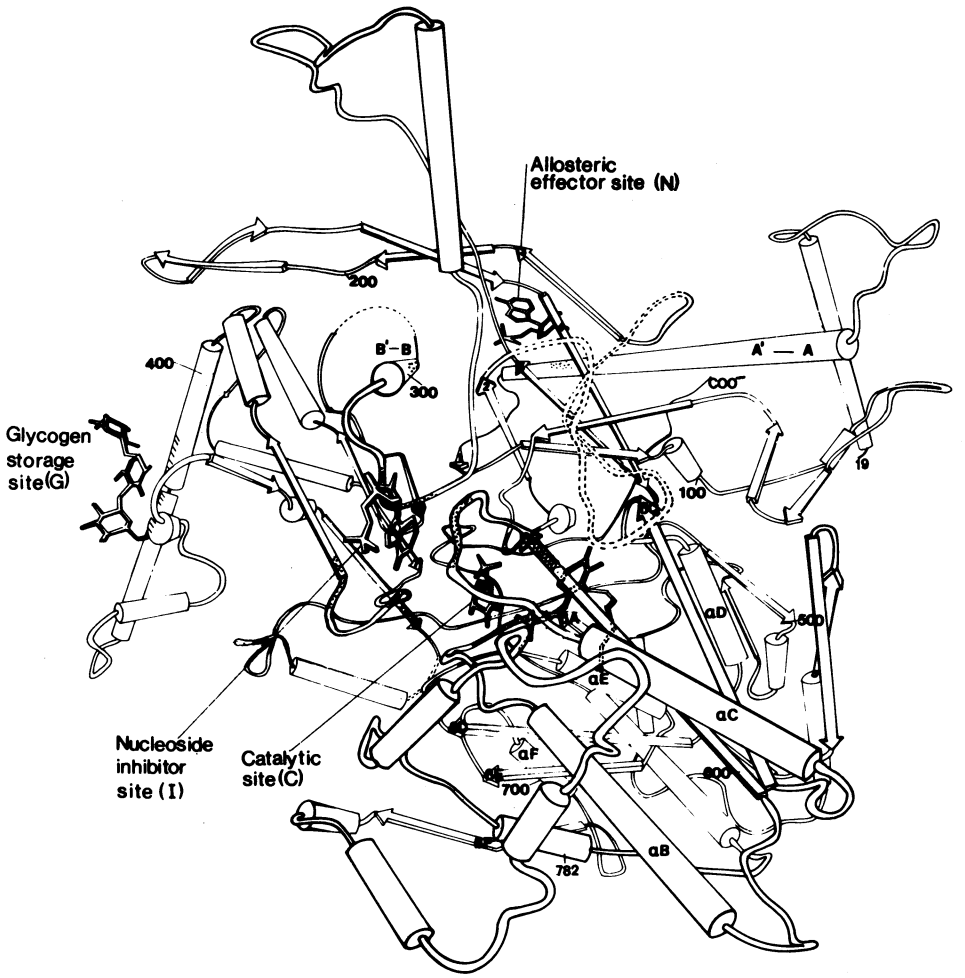

FIGURE 4. A schematic perspective drawing of the phosphorylase *b* monomer viewed down the crystallographic *y* axis. α-Helices and β-strands are represented by cylinders and arrows, respectively. AMP is shown binding at the allosteric effector site (N) and the nucleoside inhibitor site I. G1P and the nearby pyridoxal phosphate are shown at the catalytic site and the major route of access to the catalytic site is shown by the thin line from the label. Maltotriose is shown bound to the glycogen storage site. (From Johnson *et al.* (1979).)

a five-stranded β sheet containing both parallel and antiparallel strands. The interactions of AMP, ATP and glucose-6-phosphate at site N have been described elsewhere (Johnson *et al.* 1979; Lorek *et al.* 1981). The main contacts between subunits involve the cap region (residues 36–45), which folds over the allosteric site of the symmetry related molecule, and the tower (residues 251–277). The helix of one tower (266–277) interacts with the antiparallel helix of the tower of the other subunit, while the top of the tower (254–266) and part of the helix interact with the loop 280–292 and the region 176–182 of the other subunit.

A major difference between phosphorylase *b* and *a* has been noted for the first 19 residues

(Weber *et al.* 1978). In phosphorylase *b*, these residues are not located in the electron density map and it has been concluded that they are mobile. In phosphorylase *a*, residues 6–16 are defined and the seryl-phosphate group at position 14 interacts with Arg 69 on the AA′ helix and Arg 43 and His 36 from the cap region of the symmetry related molecule (Sprang & Fletterick 1979). The significance of these results in relation to the solution properties of the enzyme has been discussed (Weber *et al.* 1978).

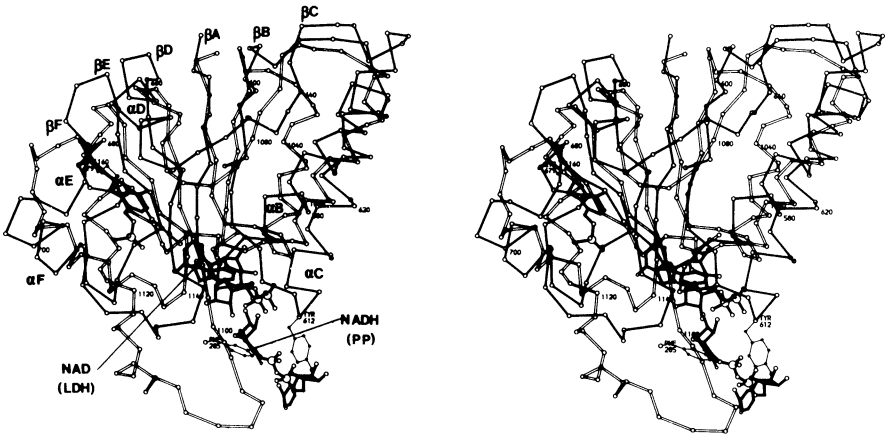

FIGURE 5. A stereo diagram of the α carbon positions of the nucleotide binding domain of phosphorylase *b* (residues 562–711; thick lines) superimposed on the nucleotide binding domain of lactate dehydrogenase (residues 22–170 with residue number increased by 1000; thin double lines). The positions of the pyridoxal phosphate (linked to Lys 679) and NADH bound to phosphorylase are shown, together with the position of NAD^+ bound to lactate dehydrogenase. The adenine ring of NADH(PP) is seen almost edge-on and, in the present interpretation of the model, is very close to the side chain of Phe 285.

There is a break in the continuity of the electron density between residues 311 and 322, presumably because of disturbance from heavy atom positions. The glycogen binding domain (residues 322–485) contributes two further parallel strands to the sheet of the N-terminal domain, making a seven-stranded sheet in all. The strong glycogen binding site on the surface involves contacts mostly to the long and slightly bent α-helix between residues 396 and 416. With maltoheptaose, the left-handed oligosaccharide helix fits in the groove of the right-handed α-helix and forms contacts with side chains from four turns. Maltoheptaose also interacts with Lys 436 (near the non-reducing end) and the main chain in the region of 355–360 (at the reducing end). Details of these interactions will be published elsewhere (Stura *et al.* 1981).

The remainder of the molecule, the C-terminal domain (residues 486–841) forms the lower right part of figure 4. In the middle of the C-terminal domain there is a region (first noted in phosphorylase *a* (Fletterick *et al.* 1976)) of six parallel strands of β-sheet and five α-helices characteristic of the 'nucleotide binding domain' found in lactate dehydrogenase and related enzymes (Rossmann *et al.* 1974) (figure 5). In phosphorylase *b* the domain corresponds to residues 561–711. A comparison of the two nucleotide binding domains by using the methods described by Stuart *et al.* (1979) and Rossmann & Argos (1976) gave a root-mean-square deviation for C_α atom positions of 3.3 Å for 114 out of 150 residues. This value compares well with those obtained for comparisons of different dehydrogenases. There are no similarities in sequence. The major differences appear to be the longer loop between

βD and αE in lactate dehydrogenase and the longer loop between βA and αB in phosphorylase. In lactate dehydrogenase, the coenzyme NADH is in contact with the βA–αB loop and in phosphorylase the cofactor pyridoxal phosphate is bound to a lysine on the αE helix. So the major structural changes occur in the functional regions of both molecules.

Following an observation by G. van de Werve that NADH inhibits phosphorylase activity, we have studied the binding of NADH in the crystal (Zanotti *et al.* 1981), NADH binds tightly at site N in the 'folded' conformation, which is rather similar to that observed in n.m.r. studies in solution (see McDonald *et al.* (1972) and references therein), and in contrast to the 'extended' conformation observed in single crystal studies (Saenger *et al.* 1977) and in complexes with lactate dehydrogenase (Chandrasekhar *et al.* 1973). The major contacts with the enzyme involve the adenosine moiety and are similar, but not identical, to those observed for AMP. NADH also binds at site I, but less tightly. In this discussion it is site I that is of interest because it utilizes contacts from the 'nucleotide binding domain'. At site I the specificity is provided for the adenosine moiety, mostly by the aromatic rings of Tyr 612 from the βB–αC loop and the Phe 285 from the N-terminal domain (figure 5). The density for the nicotinamide is less well defined. Our interpretation suggests that the dinucleotide is in the folded conformation but that stacking between the adenine and nicotinamide rings is prevented by the contacts of the adenine to the enzyme. It can be seen that the NADH site in phosphorylase is distinct from the NAD^+ site in lactate dehydrogenase (the separation between adenines is about 15 Å). The pyrophosphate binding site for NADH in lactate dehydrogenase is also distinct from the phosphate binding site observed for the substrate G1P in phosphorylase (separation approximately 17 Å). Despite a similarity in topology, the nucleotide binding domains have generated two distinct nucleotide binding sites; that for lactate dehydrogenase is of crucial importance for catalysis, while the physiological significance, if any, for phosphorylase remains to be established. The structural and evolutionary significance of such results are discussed by others at this symposium (Sternberg *et al.* and Rossmann).

2.3. *The catalytic site*

The catalytic site has been identified by the tight binding of the substrate, G1P, (Johnson *et al.* 1980) and is located in the centre of the molecule where the three domains come together. Most obvious access to the site is through a narrow cylinder 15 Å long indicated by the catalytic site arrow in fig. 4. The proximity of G1P to the pyridoxal phosphate provides direct support for the role of the cofactor in catalysis although the closest approach of the 5′-phosphate oxygen to O1 of G1P is long, 5.3 Å. The pyridoxal phosphate is bound by a Schiff base linkage to Lys 679, which is located on the short αE helix of the nucleotide binding domain. The coenzyme is buried in the interior of the enzyme and shielded from bulk water, consistent with observations from solution studies. The phosphate moiety is stabilized partly by interaction with a helix dipole (Hol *et al.* 1978) from the αE helix and partly by the ε-amino group of Lys 567 from the βA strand.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

3. The binding of glucose-1,2-cyclic phosphate

3.1. *Experimental*

The binding of the substrate, G1P, has been described in a previous paper (Johnson *et al.* 1980). In this section we give brief experimental details for the binding of G12P and compare the results with those for G1P.

G12P was generously given to us by O. Bensaud and H. Buc (Paris) and by E. J. M. Helmreich (Wurzburg). As a precaution it was decided to stabilize the crystals by cross-linking them with malonicdimethyldiimidate because many metabolites disrupt phosphorylase *b* crystals. Later work showed that this precaution was unnecessary because G12P does not in fact crack the crystals. Previous experiments (Lorek *et al.* 1981) have shown that the diimidate produces no changes in the crystal apart from a cross-link between two lysine residues at the subunit–subunit interface and a small disturbance at the amino end of the AA′ helix.

For data collection, cross-linked crystals of phosphorylase *b*, already mounted in their capillary tubes, were soaked in a solution containing approximately 10 mM G12P, 0.04 M ammonium bicarbonate, 0.11 M triethanolamine hydrochloride, 10 mM magnesium acetate, pH 7.5, for 2 days. Some crystals were subsequently soaked in solutions containing 100 mM AMP in BES buffer pH 6.8 for 18 h. G12P did not diffuse out under these conditions. Data to 3 Å resolution for G12P both in the presence and absence of AMP were collected on an Arndt–Wonacott oscillation camera with $\Delta\phi = 2°$. Results will be described only for the AMP–G12P complex. A total of 34620 reflexions were measured, which reduced to a set of 14660 independent reflexions, representing some 80% of the data to 3 Å resolution, with a merging R value on intensities of 0.134. After scaling to native the fractional change in structure factor amplitude was 0.173.

The three-dimensional difference Fourier synthesis for G12P in the presence of AMP showed three significant peaks, which represent strong binding of G12P at the catalytic site, strong binding of AMP at the allosteric site, and weak binding of AMP at the nucleoside inhibitor site. The interpretation of the map was made with molecular models on a scale of 2 cm/Å by using a Richards box (Richards 1968).

3.2. *Results*

The structure of G12P poses interesting conformational problems. Examination of molecular models shows that the five-membered cyclic phosphate ring can only be achieved with certain deviations from standard stereochemistry. The tetrahedral angle at the phosphorus must be closed to about 100° and the angles at the phosphate ester oxygens also decreased so that the cyclic phosphate ring resembles that observed in the single-crystal studies of methylethylene phosphate (Steitz & Lipscombe 1965). Such a distortion at the phosphorus is consistent with observations on the ^{31}P resonance of G12P (H. W. Klein, personal communication). With these constraints on the cyclic phosphate ring the glucopyranose moiety can adopt its normal chair conformation. The approximate planes of the two rings are inclined by about 120°.

In the difference electron density map for G12P the position of the phosphate is clear. With this constraint, and the rigid structure deduced from model building, there is only one interpretation for the position of the glucopyranose moiety that satisfactorily accounts for the density (figure 6*b*). However, at 3 Å resolution subtle differences between a chair and half-

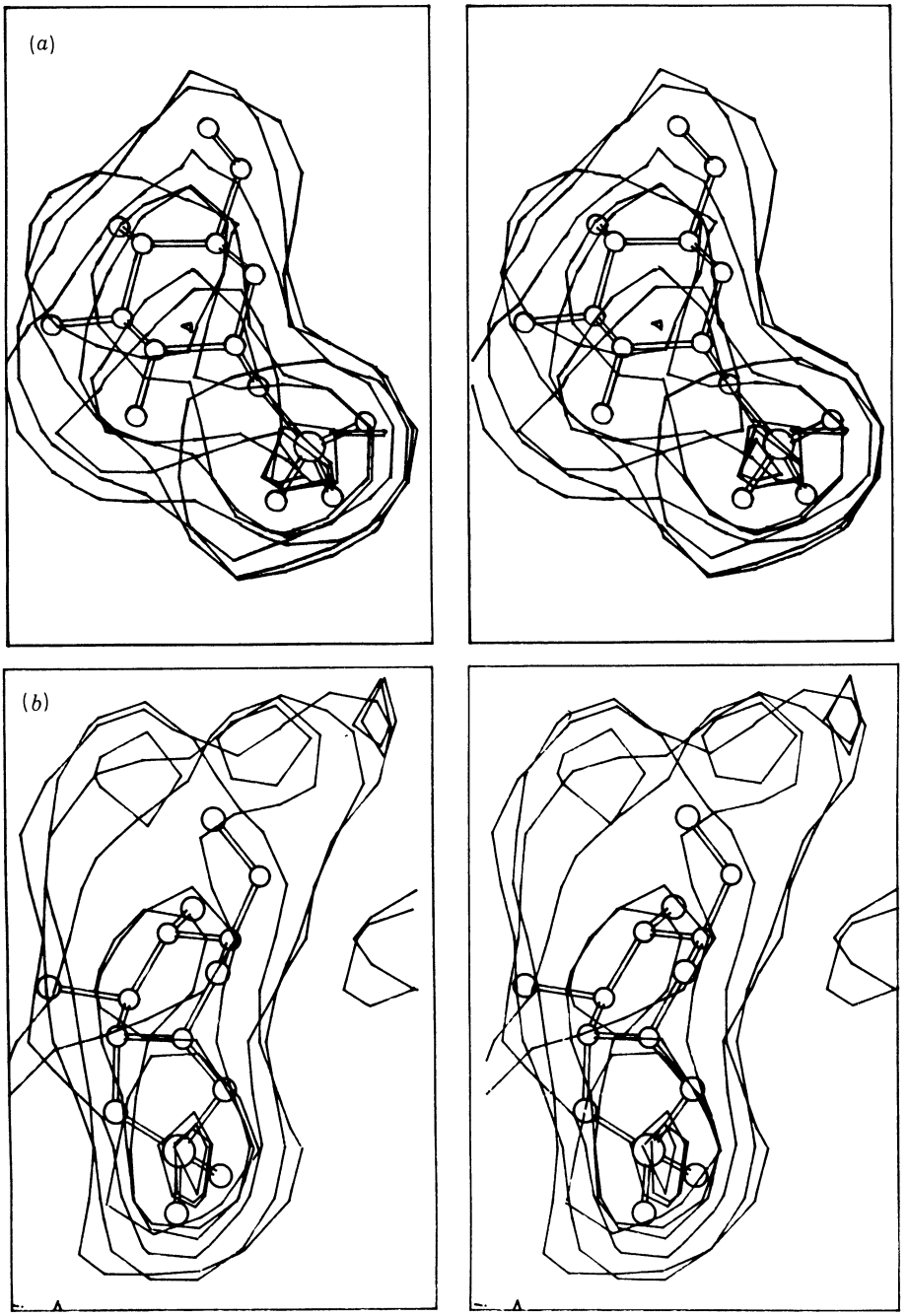

FIGURE 6. Stereo diagrams of the difference Fourier syntheses projected down the crystallographic x axis for (*a*) G1P sections 31/128–35/128 and (*b*) G12P sections 30/128–35/128. For clarity only three contour levels are shown at 15, 45 and 75 arbitrary units.

chair conformation for the glucopyranose cannot be distinguished. Thus, although the chair conformation is consistent with the density and is preferred from stereochemical considerations, alternative conformations cannot be excluded.

A comparison with G1P shows that the glucopyranose ring is in approximately the same position (figure 6*a*) but rotated by about 20° about the x axis. The phosphate positions are

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

markedly different. The phosphate of G12P is shifted 2.4 Å from the position in G1P, mostly in the z direction.

The contacts of G12P to the enzyme are compared with those for G1P in figure 7. In general the polar contacts to the sugar moiety are similar, with perhaps rather fewer good hydrogen bonds (according to our present interpretation of the structure) in G12P. The major difference lies in the contacts to the phosphate oxygens. In G1P the phosphate moiety appears to be

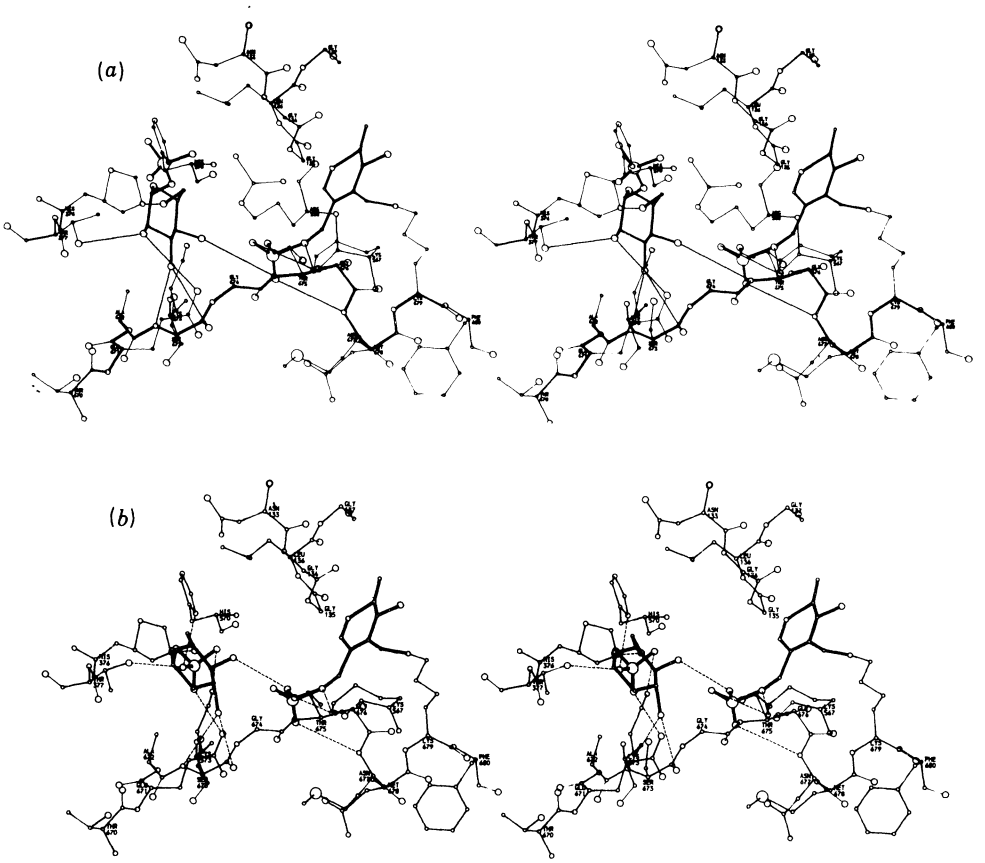

FIGURE 7. Stereo diagrams of the catalytic site of phosphorylase b projected down the crystallographic y axis, showing the positions of the pyridoxal phosphate and (*a*) G1P and (*b*) G12P as observed in the crystal structure.

stabilized by an interaction with the helix dipole of the distorted α-helix from residues 132–137 and by an interaction with His 570. In G12P, the phosphate is further away from the helix dipole but can now make more convincing interactions with His 570 and Lys 573. In addition, one of the phosphate oxygens and the glucopyranose oxygen O4 of G12P are close (about $3{\cdot}8 \pm 0.5$ Å) to the O5 and O6 atoms of the 5′-phosphate of pyridoxal phosphate, respectively – an arrangement that could be stabilized if the pyridoxal phosphate were in its protonated monoanion state. In G1P the closest approach of the phosphate oxygens to the 5′-phosphate group of pyridoxal phosphate is greater than 5 Å.

The results for nucleotide binding will be discussed in detail elsewhere.

No large conformational changes are observed in the electron density for the cross-linked phosphorylase–AMP–G12P complex, although small shifts may be revealed by refinement.

3.3. *Discussion of G12P result*

The G12P molecule is highly constrained. In binding to the catalytic site of phosphorylase, the glucose moiety is in almost the same position as in G1P while the phosphate is some 2.4 Å away from the phosphate position of G1P. Our present results suggest that the improved binding of G12P arises from the better contacts with the phosphate, especially the interaction with Lys 573. The proximity to the 5′-phosphate of pyridoxal phosphate suggests that binding is likely to lead to some perturbation in the apparent state of ionization. It is interesting that this second phosphate site does not appear to be a high-affinity site. At 0.5 M concentrations, inorganic phosphate binds preferentially at the allosteric site and no strong binding is observed at the catalytic site (Lorek *et al.* 1981). An increase in the reactivity of Arg 568 towards arginine-specific reagents has been observed in chemical modification studies when phosphorylase is in the active conformation (Dreyfus *et al.* 1980). In the present structure, Arg 568 is relatively inaccessible, consistent with this result, but a small change in conformation would allow the guanido group to contribute interactions to the 5′-phosphate of pyridoxal phosphate and/or to the phosphate of the substrate or inhibitor, thus providing a higher affinity site for phosphate. No changes are seen, however, in the G12P difference Fourier synthesis.

G12P is not a very effective transition state analogue in terms of its affinity for the enzyme. It binds only one order of magnitude tighter than the substrate, whereas in a good transition state analogue an increase in affinity compared with the substrate should be as great as the catalytic rate enhancement of the enzyme (i.e. of the order of 10^{10}) (Pauling 1946; Wolfenden 1969). Nevertheless, it is interesting that a possible interpretation of the density, although not one preferred by us, is one in which some of the strain in the molecule is taken up by distortion of the glucopyranose ring to the half chair $^4H_3$, a conformation similar to that as observed in the single crystal studies for D-gluconolactone. D-Gluconolactone is a potent inhibitor of several glycosidases, for which it seems likely that it mimics a transition state carbonium–oxonium ion intermediate (see discussion by Leaback (1968)) and it also inhibits phosphorylase ($K_i \approx 1$ mM for phosphorylase *b* in the presence of 1 mM AMP) (Tu *et al.* 1971). At our present stage of analysis, it appears that both the chair and half-chair conformations can be accommodated at the G1P site for phosphorylase and there are no definite interactions that favour one conformation over the other. Thus in the low-affinity T state of the enzyme we observe no constraints that favour the putative transition state analogue over the substrate in the binary complex. Of course such constraints may be too subtle to distinguish at 3 Å resolution or only become apparent in the R state or in the ternary complex. With these limitations, the present crystallographic results suggest that G12P binds well because it can make better contacts with the enzyme via its cyclic phosphate moiety.

4. Catalytic mechanism

In a previous paper (Johnson *et al.* 1980) we put forward proposals for a mechanism for phosphorylase based on the G1P binding studies and results from molecular model building. We summarize briefly the arguments that led to these proposals and review them in connection with the new result for G12P.

Our attempts to derive a catalytic mechanism from the present structure of phosphorylase *b* suffer from two limitations. First, the structure is known only for the less-affine form of the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

enzyme. No significant conformational changes have been observed on binding substrates or inhibitors. Secondly, binding of the second substrate, glycogen or its analogues, has not been observed at the catalytic site. The reasons for this are obscure but may be associated with the relatively high K_m for oligosaccharide substrates.

From the studies with G1P the following were observed.

1. With the substrate bound as observed for G1P, the relative orientation of the 5′-phosphate group of pyridoxal phosphate is such that it cannot participate in the initial step of catalysis as a dianion. In fact, the relative orientations are such that the 5′-phosphate group would be in a good position to function as a general acid and donate a proton to the glycosidic bond, except that the separation (5.3 Å) is rather too large. In the potato enzyme, an apparent increase in the pK of the 5′-phosphate group has been observed on binding the substrates arsenate and maltoheptaose (see review by Helmreich & Klein (1980)). For the rabbit muscle enzyme, a perturbation to a pK of about 7.0 would be necessary to account for the pH rate profile data of Kasvinsky & Meyer (1977). A 'phosphate mechanism' of the type envisaged in figure 2*b* appears unlikely because the relevant distances are too large.

2. With the substrate bound as observed for G1P, the only group which is close enough to function as a nucleophile, in our present interpretation of the structure, is His 376. The importance of a good nucleophilic group for the promotion and stabilization of the transition state in glycoside hydrolysis has been emphasized (Vernon 1967). His 376 is close to the C1 and O5 atoms of G1P but its involvement in hydrogen bonds via its Nδ2 atom to the O6 hydroxyl of G1P and via its Nδ1 atom to the carboxylate of Asp 338 results in an orientation that makes it unfavourable for nucleophilic attack. The side chain of Asp 338 also appears to be involved in a hydrogen bonding network that includes neutral residues Asn 375 and Tyr 373. It is difficult to predict what effect this internal hydrogen bonding arrangement might have on the pK of His 376. Certainly in chymotrypsin and related enzymes, the environments and hydrogen bonding between Asp 102 and His 57 confer unusual properties on the imidazole group.

The other potential nucleophilic group in the vicinity is Glu 671. The carboxylate side chain of this group is within hydrogen bonding distance of the O2 and O3 hydroxyls of G1P but it is too far (more than 5 Å) to attack the C1 or O5 atoms of G1P.

3. With the terminal glucose of a glycogen substrate bound as observed for G1P, it is impossible to direct the polysaccharide along the major crevasse leading to the active site without severe distortion. When a second sugar is attached to the glucosyl moiety of G1P in either the preferred conformation for α(1–4) linked oligosaccharides or the secondary minima conformation defined by Rees (1977), bad contacts are made especially in the region of the loop 132–135 from the N-terminal domain and the βA strand (in the region of 568–570) of the nucleotide binding domain. Both these regions are potential candidates for conformational changes. The 132–135 loop because of its high content of glycine residues and the Arg 568 strand because of the chemical evidence already cited (Dreyfus *et al.* 1980). If this bottle-neck is relieved by conformational changes, the polysaccharide could have access to solvent through a long channel some 25 Å long flanked by the N-terminal domain on one side and the C-terminal domain on the other and emerge close to the acidic groups in the region of residue 124. However, this route to the surface is so long that it would be difficult to account for the observation that phosphorylase can digest to within four residues of a branch point. On the other hand, if glycogen could be directed along the preferred route (the major crevasse leading

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

to the active site (figure 4)) an explanation for this observation is apparent. The crevasse is just long enough to admit four or five sugars but is too narrow to admit branched oligosaccharides.

None of these arguments taken separately could rule out a mechanism. For example, it is just possible to envisage a mechanism in which the 5′-phosphate of pyridoxal phosphate acted as a general acid through a rather large distance and with a significant perturbation in pK on binding substrate, His 376 acted as a nucleophile to form a transient covalent

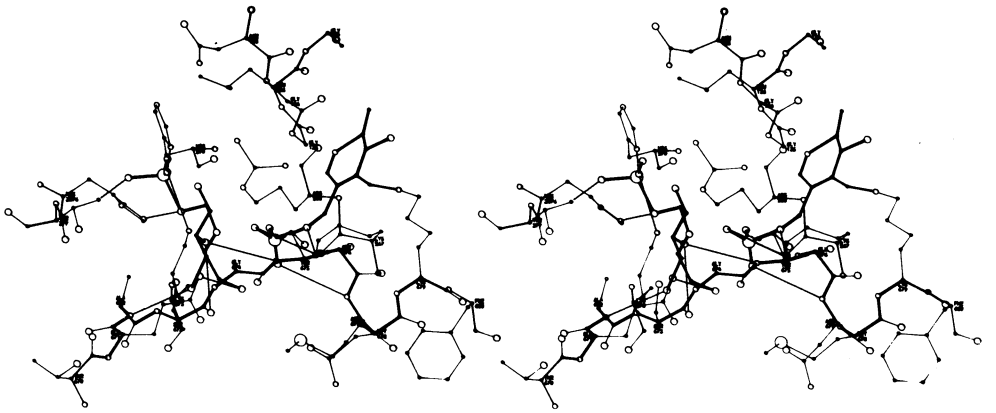

FIGURE 8. A stereo diagram of the catalytic site of phosphorylase *b*, showing an alternative binding mode for G1P deduced from model building studies. The position of the side chain of His 376 has been altered from the position observed in the crystal structure. (From Johnson *et al.* (1980).)

glucosyl intermediate but only after a conformational change, and that glycogen is accommodated either by severe strain on the polysaccharide conformation or by a very large conformational change in the protein. Taken together, it is apparent that at each step there are difficulties in reconciling the X-ray evidence with known biochemistry and stereochemistry. We therefore considered the possibility that the substrate binding to the less-affine form represented non-productive binding, and alternative modes of binding were explored. It was found that if the G1P were rotated through 180°, it could still be accommodated at the catalytic site with good contacts to the enzyme (figure 8). In this confirmation of G1P, the essential features of a mechanism are readily apparent. The C1–O1 bond is now directed along the major active site crevasse so that glycogen in its preferred conformation can be accommodated without difficulty. The 5′-phosphate of pyridoxal phosphate is in the correct position to function as a dianion to promote the formation and stabilization of a carbonium–oxonium ion intermediate, while His 376 can function as a general acid after a small conformational change. Accordingly it was proposed (Johnson *et al.* 1980) that in the presence of AMP and the second substrate, glycogen, conformational changes take place at the catalytic site that direct the productive binding of G1P, possibly by creating a more specific phosphate binding site. The essential features of the catalytic mechanism in the proposed productive binding mode are shown in figure 9. The apparent shift in pK of the pyridoxal phosphate on binding substrate could be explained by the proximity to the carbonium ion.

The result for G12P has shown that with the constraints provided by the cyclic phosphate ring there is a second phosphate binding site at the catalytic site, in addition to that demonstrated by G1P, and that, although there is some flexibility for the orientation of the glucopyranose ring, its orientation is similar to that observed for the X-ray result on G1P.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The proximity of this new phosphate site to the 5′-phosphate of the pyridoxal phosphate caused us to review again a possible phosphate mechanism (figure 2*b*) in which the 5′-phosphate acts as an acid to promote attack by inorganic phosphate. Such a mechanism appears plausible in terms of the stereochemical constraints on the two phosphates and the sugar, but, as noted above, so long as the glycosyl moiety is in the same orientation as that observed for G1P in the X-ray structure there is no good nucleophilic group for stabilization of the intermediate

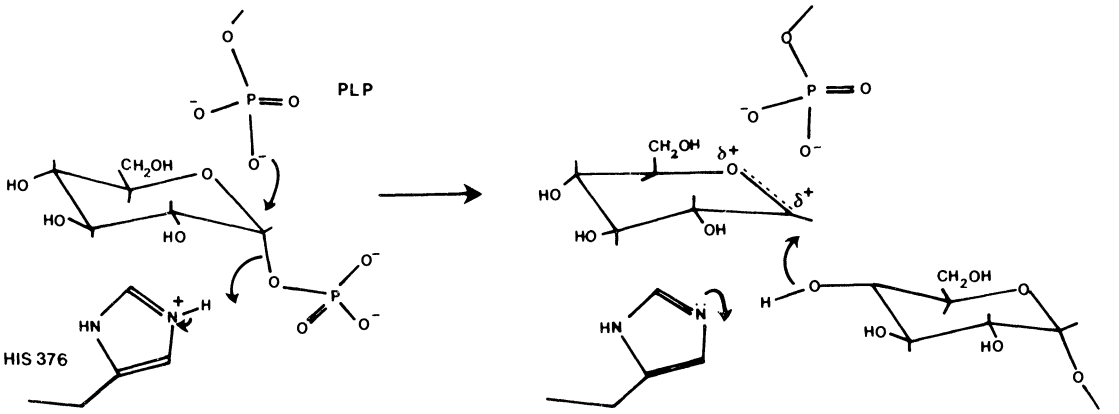

FIGURE 9. Proposed mechanism for phosphorylase *b* based on the crystal structure and the alternative binding mode for G1P deduced from model-building studies.

and there is no easy way to build a glycogen substrate along the active site crevasse. On the other hand, in the alternative conformation of G1P proposed from model building, the new phosphate position as defined by G12P binding would be entirely consistent with our hypothesis. It is only 1.2 Å from the position proposed and is in the right orientation to allow attack at the C1 atom with retention of configuration after cleavage of the C1–O1 bond.

In conclusion, the study of G12P binding in the crystal has allowed us to compare enzyme contacts to the inhibitor with those to the substrate. Although important differences are noted, especially with respect to the phosphate, their significance in terms of mechanism is difficult to assess without more detailed knowledge on the relation of G12P to the intermediates in the reaction pathway. With the stereochemical constraints imposed by our present interpretation of the structure at 3 Å resolution, our original proposals for the mechanism appear plausible. They have the advantage of simplicity and economy of basic principles. However, more detailed crystallographic experiments and further solution studies are required to test these proposals and to provide a deeper understanding of the molecular basis of catalysis.

It is a pleasure to acknowledge Sir David Phillips's continuing interest and support for this project. We thank the Medical Research Council for financial support of J.A.J. and E.A.S., and the Science Research Council for support of D.I.S. G.Z. was supported by a NATO Scholarship. We also wish to thank Dr O. Bensaud and Dr H. Buc and Professor E. J. M. Helmreich for generous gifts of glucose-1,2-cyclic phosphate and Dr G. K. Radda and Dr S. Arnott for useful discussions.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

References (Jenkins *et al.*)

Baranowski, T., Illingworth, B., Brown, D. H. & Cori, C. F. 1957 *Biochim. biophys. Acta* **23**, 16–21.

Black, W. J. & Wang, J. H. 1968 *J. biol. Chem.* **243**, 5892–5898.

Brown, D. H. & Cori, C. F. 1961 In *The enzymes* (ed. P. D. Boyer, H. Lardy & K. Myrback), 2nd edn, vol. 5, pp. 207–228. New York: Academic Press.

Bunton, C. A., Llewellyn, D. R., Oldham, K. G. & Vernon, C. A. 1958 *J. chem. Soc.*, pp. 3588–3594.

Chandrasekhar, K., McPherson, A., Adams, M. J. & Rossmann, M. G. 1973 *J. molec. Biol.* **76**, 503–518.

Cohen, P., Embi, N., Foulkes, G., Hardie, G., Nimmo, G., Hylatt, D. & Shenolikar, S. 1979 In *From gene to protein: information transfer in normal and abnormal cells* (ed. T. R. Russell, K. Brew, H. Faber & J. Schultz), pp. 463–481. Academic Press.

Cohn, M. & Cori, G. T. 1948 *J. biol. Chem.* **175**, 88–93.

Cori, C. F. & Cori, G. T. 1936 *Proc. Soc. exp. Biol. Med.* **34**, 702–705.

Cori, G. T. & Cori, C. F. 1940 *J. biol. Chem.* **135**, 733–756.

Dreyfus, M., Vandenbunder, B. & Buc, H. 1980 *Biochemistry, Wash.* **19**, 3634–3642.

Feldman, K. Horl, M., Klein, H. W. & Helmreich, E. J. M. 1978 In *Proc. 11th FEBS Meeting, Copenhagen*, (ed. V. Esmann), vol. 42, pp. 205–218. Oxford: Pergamon.

Firsov, L. M., Bogacheva, T. I. & Breslev, S. E. 1974 *Eur. J. Biochem.* **42**, 605–609.

Fischer, E. H., Kent, A. B., Snyder, E. R. & Krebs, E. G. 1958 *J. Am. chem. Soc.* **80**, 2906–2907.

Fletterick, R. J., Sygusch, J., Semple, M. & Madsen, N. B. 1976 *J. biol. Chem.* **251**, 6142–6146.

Graves, D. J. & Wang, J. H. 1972 In *The enzymes* (ed. P. D. Boyer), 3rd edn, vol. 7, pp. 435–482. New York: Academic Press.

Hackert, M. L. & Jacobson, R. A. 1971 *Acta crystallogr.* B**27**, 203–209.

Hassid, W. Z., Cori, G. T. & McReady, R. M. 1943 *J. biol. Chem.* **148**, 89–96.

Helmreich, E. J. M. & Cori, C. F. 1964 *Proc. natn. Acad. Sci. U.S.A.* **51**, 131–138.

Helmreich, E. J. M. & Klein, H. W. 1980 *Angew. Chem. int. Edn Eng.* **19**, 441–455.

Hol, W. G. J., van Duijnen, P. T. & Berendsen, H. J. C. 1978 *Nature, Lond.* **273**, 443–446.

Hu, H.-Y. & Gold, A. M. 1978 *Biochim. biophys. Acta* **525**, 55–60.

Johnson, L. N., Stura, E. A., Wilson, K. S., Sansom, M. S. P. & Weber, I. T. 1979 *J. molec. Biol.* **134**, 639–653.

Johnson, L. N., Jenkins, J. A., Wilson, K. S., Stura, E. A. & Zanotti, G. 1980 *J. molec. Biol.* **140**, 565–580.

Kasvinsky, P. J. & Madsen, N. B. 1976 *J. biol. Chem.* **251**, 6852–6859.

Kasvinsky, P. J. & Meyer, W. L. 1977 *Arch. Biochem. Biophys.* **181**, 616–631.

Kasvinsky, P. J., Madsen, N. B., Fletterick, R. J. & Sygusch, J. 1978*a* *J. biol. Chem.* **253**, 1290–1296.

Kasvinsky, P. J., Madsen, N. B., Sygusch, J. & Fletterick, R. J. 1978*b* *J. biol. Chem.* **253**, 3343–3351.

Kokesh, F. C., Stephenson, R. K. & Kakuda, Y. 1977 *Biochim. biophys. Acta* **483**, 258–262.

Kokesh, F. C. & Kakuda, Y. 1977 *Biochemistry, Wash.* **16**, 2467–2473.

Konnert, J. H. 1976 *Acta crystallogr.* A **32**, 614–617.

Krebs, E. G. & Fischer, E. H. 1956 *Biochim. biophys. Acta* **20**, 150–157.

Krebs, E. G. & Fischer, E. H. 1964 *Vitamins Hormones* **22**, 399–410.

Leaback, D. H. 1968 *Biochem. biophys. Res. Commun.* **32**, 1025–1030.

Lorek, A. L., Wilson, K. S., Stura, E. A., Jenkins, J. A., Zanotti, G. & Johnson, L. N. 1981 *J. molec. Biol.* (Submitted.)

Marsh, D. J. 1981 (In preparation.)

McDonald, G., Brown, B., Hollis, D. & Walter, C. 1972 *Biochemistry, Wash.* **11**, 1920–1930.

Meyer, F., Heilmeyer, L. M. G., Haschke, R. H. & Fischer, E. H. 1970 *J. biol. Chem.* **245**, 6642–6648.

Monod, J., Wyman, J. & Changeux, J.-P. 1965 *J. molec. Biol.* **12**, 88–118.

Morange, M., Kolb, A., Buc, H., Chachaty, C. & Langlet, G. 1977 *Eur. J. Biochem.* **74**, 99–106.

Pauling, L. 1946 *Chem. Engng News* **24**, 1375–1377.

Rees, D. A. 1977 *Polysaccharide shapes*. London: Chapman Hall.

Richards, F. M. 1968 *J. molec. Biol.* **37**, 225–230.

Rossmann, M. G., Moras, D. & Olsen, K. W. 1974 *Nature, Lond.* **250**, 194–199.

Rossmann, M. G. & Argos, P. 1976 *J. molec. Biol.* **105**, 75–95.

Saenger, W., Reddy, B. S., Mühlegser, K. & Weiman, G. 1977 *Nature, Lond.* **267**, 225–229.

Shimomura, S. & Fukui, T. 1980 *Biochemistry, Wash.* **19**, 2287–2294.

Sprang, S. & Fletterick, R. J. 1979 *J. molec. Biol.* **131**, 523–551.

Steitz, T. A. 1965 *J. Am. chem. Soc.* **87**, 2488–2489.

Stuart, D. I., Levine, M., Muirhead, H. & Stammers, D. K. 1979 *J. molec. Biol.* **134**, 109–142.

Stura, E. A. *et al.* 1981 (In preparation.)

Titani, K., Koide, A., Hermann, J., Ericsson, L. H., Kumar, S., Wade, R. D., Walsh, K. A., Neurath, H. & Fischer, E. H. 1977 *Proc. natn. Acad. Sci. U.S.A.* **74**, 4762–4766.

Tu, J.-I., Jacobson, G. R. & Graves, D. J. 1971 *Biochemistry, Wash.* **10**, 1229–1236.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Uhing, R. J., Jansky, A. M. & Graves, D. J. 1979 *J. biol. Chem.* **254**, 3166–3169.
Vernon, C. A. 1967 *Proc. R. Soc. Lond.* B **167**, 389–401.
Wang, J. H., Kwok, S.-C., Wirch, E. & Susuki, I. 1970 *Biochem. biophys. Res. Commun.* **40**, 1340–1347.
Weber, I. T., Johnson, L. N., Wilson, K. S., Yeates, D. G. R., Wild, D. L. & Jenkins, J. A. 1978 *Nature, Lond.* **274**, 433–437.
Wolfenden, R. 1969 *Nature, Lond.* **223**, 704–705.
Zanotti, G. *et al.* 1981 (In preparation.)

Discussion

D. R. Wilkie, F.R.S. (*Department of Physiology, University College London, U.K.*). I wish to begin with a comment from the point of view of a physiologist on the abstract provided for the meeting notice by Dr Johnson and her coworkers. In it is the statement, 'Glycogen represents the major energy source for muscle contraction once supplies of creatine phosphate have been exhausted.' This implies that glycolysis begins only when phosphocreatine (PCr) has been depleted, which is certainly not so. Glycolysis and lactic acid formation begin very soon after stimulation, while the concentration of PCr is almost as high as it is at rest. For example, glycolysis begins within 10–15 s after stimulation even in oxygenated frog muscles at 0 °C (Gilbert *et al.* 1971). More recent studies on fatigue under anaerobic conditions with the use of ^{31}P n.m.r. (Dawson *et al.* 1978, 1980*a*, *b*) have shown that until fatigue has become severe, glycolysis and hydrolysis of PCr contribute almost equally to the replenishment of the ATP that had been hydrolysed during contraction.

Returning to the present elegant paper, I was puzzled by the values given in the first slide for the concentrations in resting muscle of free ADP (0.2 mM) and of free AMP (3–6 μM). These are the concentrations that arise in muscles so fatigued as to be scarcely capable of contraction. The correct value for the concentration of free ADP is one-tenth, and of free AMP one-hundredth or less, of these. All theories that we may hear about at this Meeting that assume that enzyme activities are 'modulated by concentration of metabolites' (quoting again from the meeting notice abstract) should take due account of these facts. A factor of 100, or even of 10, makes an appreciable difference to every quantitative theory about the effect of the affinities of binding sites for substrates on enzymes. I realize that only a small part of Dr Johnson's presentation was concerned with regulation, and my main purpose is to set realistic physiological limits within which all ideas about enzyme regulation in muscle must operate.

Since time for discussion was limited, it was only in a later conversation with Dr Johnson that I could explain the justification for my assertions.

1. The resting $[ADP]_{free}$ is approximately 20 μM rising to perhaps 0.3 mM only in extreme fatigue. This conclusion rests essentially on the many measurements of the concentrations of phosphocreatine and of creatine (Cr) in muscles at rest and after contraction.

There can be little doubt about the equilibrium constant of the reaction

$$H^+ + MgADP^- + PCr^{2-} \rightleftharpoons MgATP^{2-} + Cr^0. \qquad (1)$$

It is between 1.5 and 2 nM^{-1} (see, for example, Lawson & Veech 1979). Thus, if one knows the concentration of MgATP, of H^+, and the ratio of PCr to Cr, there is little room for manoeuvre about the concentration of $MgADP^-$ when the reaction is at or close to equilibrium. The high activity of the enzyme creatine phosphokinase (CPK) together with other considerations have made it seem likely for many years that reaction (1) was indeed always at, or

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

close to, equilibrium. The most direct proof that this is so has been given recently by the technique of saturation transfer n.m.r. (Brown *et al.* 1980, 1981). There is no evidence that CPK is an enzyme that is switched on and off.

2. The assertion that the resting $[AMP]_{free}$ is approximately 0.1 μM, rising to 22.5 μM in extreme fatigue, depends merely on the knowledge that the equilibrium constant for the reaction

$$2\ ADP \rightleftharpoons ATP + AMP \qquad (2)$$

is close to unity (see Lawson & Veech 1979) and that the necessary enzyme, adenylate kinase, is present to keep the reactants close to their equilibrium concentrations. I know of no good evidence that adenylate kinase is a switched enzyme. If it can indeed be switched off, the concentration of free AMP is probably even lower than has been indicated above.

This is because of the almost ubiquitous presence in muscle of a third enzyme, AMP deaminase, which converts AMP to inosine monophosphate (IMP). It is likely that this enzyme is switched (see, for example, Atkinson 1977, p. 211) as would seem necessary if all the adenine is not to become degraded to inosine. The precise mechanism of this switching is not clear. Certainly AMP deaminase remains active – as does adenylate kinase – in the presence of high concentrations of PCr (Dydynska & Wilkie 1966).

References

Atkinson, D. E. 1977 *Cellular energy metabolism and its regulation*. Academic Press.

Brown, T., Chance, E. M., Dawson, M. J., Gadian, D. G., Radda, G. K. & Wilkie, D. R. 1980 The activity of creatine kinase in frog skeletal muscle studied by saturation transfer nuclear magnetic resonance. *Proc. physiol. Soc., Camb.* **305**, 84–85.

Brown, T., Chance, E. M., Dawson, M. J., Gadian, D. G., Radda, G. K. & Wilkie, D. R. 1981 The activity of creatine kinase in frog skeletal muscle studied by saturation transfer nuclear magnetic resonance. *Biochem. J.* (In the press.)

Dawson, M. J., Gadian, D. G. & Wilkie, D. R. 1978 Muscular fatigue investigated by phosphorus nuclear magnetic resonance. *Nature, Lond.* **274**, 861–866.

Dawson, M. J., Gadian, D. G. & Wilkie, D. R. 1980*a* Mechanical relaxation rate and metabolism studied in fatiguing muscle by phosphorus nuclear magnetic resonance. *J. Physiol., Lond.* **299**, 465–484.

Dawson, M. J., Gadian, D. G. & Wilkie, D. R. 1980*b* Studies of the biochemistry of contracting and relaxing muscle by the use of ^{31}P n.m.r. in conjunction with other techniques. *Phil. Trans. R. Soc. Lond.* B **289**, 445–455.

Dydynska, M. & Wilkie, D. R. 1966 The chemical and energetic properties of muscles poisoned with fluorodinitrobenzene. *J. Physiol., Lond.* **184**, 751–769.

Kretzschmar, G. C., Wilkie, D. R. & Woledge, R. C. 1971 Chemical change and energy output during muscular contraction. *J. Physiol., Lond.* **218**, 163–193.

Lawson, J. W. R. & Veech, R. L. 1979 Effects of pH and free Mg^{2+} on the K_{eq} of the creatine kinase reaction and other phosphate hydrolyses and phosphate transfer reactions. 1979 *J. biol. Chem.* **254**, 6528–6537.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES