Catalytic Site of Glycogen Phosphorylase: Structural Changes during Activation and Mechanistic Implications†

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ABSTRACT: The structure of the activated form of glycogen phosphorylase has been probed at two different levels: in the crystalline state, by X-ray crystallography, and in solution, by kinetic studies on a modified enzyme using substrate analogues. Addition of a good, nondegradable substrate analogue, glucose cyclic 1,2-phosphate, to crystals of phosphorylase *a*, previously washed to remove glucose, results in partial activation. Changes occur in the lattice constants, and considerable structural rearrangement is observed upon difference Fourier analysis. The analogue is found at essentially the same site as glucose but with a 1-Å translation within the active site. Several protein conformational changes occur in response to this binding and activation, the most prominent being an order → disorder transition affecting a β-hairpin loop (residues 282–286) at the entrance to the active site. This movement creates a substrate phosphate binding pocket within the catalytic site and would increase the accessibility to oligosaccharide substrate. The phosphorus atom of GCP is 6.8 Å away from the phosphorus of the pyridoxal phosphate (PLP) coenzyme. Kinetic studies with pyridoxal-reconstituted phosphorylase, which is active in the presence of phosphate or a suitable analogue as activator, were undertaken to test the hypothetical role of the PLP as a nucleophile in the catalytic mechanism and to elucidate the disposition of the substrate and PLP phosphate groups in the active conformer of the enzyme. The studies with β-D-glucose 1-phosphate are inconsistent with a mechanism in which the PLP phosphate participates in a backside (with respect to the glucosyl C1–O1 bond) attack on the glucosyl carbon atom to yield a covalent intermediate or to stabilize a glucosyl carbonium ion. The observation of good competitive inhibition by methylenediphosphate, but poor competitive inhibition by ethylene- and propylenediphosphonate, suggests that the phosphate of the coenzyme and that of the substrate may be closer together in the fully activated structure than observed in the X-ray structure of the GCP complex. Possible catalytic mechanisms based upon the direct or protein-mediated contact between the substrate and PLP phosphates are discussed in light of these and other recent data.

Reasonable speculations upon the catalytic mechanism of glycogen phosphorylase based on X-ray crystallographic evidence require some knowledge of the configuration of the enzyme's active site in its activated state and the binding mode of the substrate therein. However, the high-resolution data described to date for phosphorylase *a* have been obtained with the glucose-inhibited, T-state, enzyme form (Sprang & Fletterick, 1979) and those for phosphorylase *b* with a partially activated enzyme (Johnson et al., 1980). Indeed, in the latter case, it was suggested that the binding mode observed for glucose-1-P is, in fact, a nonproductive mode and the proposed catalytic mechanism was based on an alternative substrate binding mode deduced by model building. No fully activated, R-state structure has yet been described.

This paper describes, by X-ray diffraction analysis, the partial activation of crystalline phosphorylase *a* by a substrate analogue, GCP.[1] Local changes at the catalytic site with respect to the parent, T-state enzyme [discussed in the preceding paper (Sprang et al., 1982)] are described with emphasis upon possible mechanisms of activation and catalysis. The conformation changes associated with GCP binding alone do not characterize the full T → R transition. Previous studies (Madsen et al., 1978) have demonstrated that, in the presence of substrates that can form a ternary E·S complex, e.g., oligosaccharide and glucose-1-P, phosphorylase *a* crystals can shatter or become seriously disordered. Similar observations were made with GCP plus oligosaccharide.

Results obtained by solution techniques have also been used as a basis for speculations upon the catalytic mechanism of the enzyme. The strong inhibition of phosphorylase by D-gluconolactone was taken as evidence for a reaction mechanism involving a glucosyl cation (Tu et al., 1971). However, the absence of measurable secondary kinetic isotope effects (Firsov et al., 1974) would seem to argue against this. Initial velocity (Engers et al., 1970a; Gold et al., 1970) and isotope exchange (Engers et al., 1970b) studies indicate a rapid equilibrium, random kinetic mechanism for phosphorylase *a*, suggesting that the interconversion of the substrate ternary complex and the product ternary complex is rate limiting in the reaction. Accordingly, no exchange of [^{32}P]orthophosphate into glucose-1-P is observed in the absence of glycogen (Cohn & Cori, 1948), nor does orthophosphate exchange into the coenzyme pyridoxal phosphate, even in the presence of substrates (Illingworth et al., 1958). Phosphorolysis proceeds through cleavage of the C–O bond of glucose-1-P (Cohn, 1949), but no exchange could be detected between the bridging and nonbridging phosphoryl oxygen atoms of glucose-1-P in the absence of glycogen (Gold & Osber, 1972). However, such exchange has been observed with potato phosphorylase in the presence of cyclodextrin (Kokesh & Kakuda, 1977). There is thus circumstantial evidence for the involvement of a glucosyl enzyme intermediate in the mechanism. Use of ^{31}P NMR spectroscopy to study the phosphate group of the essential

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[1] Abbreviations: DTT, dithiothreitol; PLP, pyridoxal phosphate; glucose-1-P, α-D-glucose 1-phosphate; GCP, α-D-glucose cyclic 1,2-phosphate; UDP-Glc, uridine diphosphoglucose; PLPP-α-Glc, pyridoxal(5′)diphospho(1)-α-D-glucose; EDTA, ethylenediaminetetraacetic acid.

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pyridoxal phosphate residue led to the speculation that in the active form of the enzyme, this phosphate was fully deprotonated (Feldman & Hull, 1977). However, recent studies using the ternary complex enzyme·maltopentaose·substrate analogue, with glucose cyclic 1,2-phosphate as the analogue, have demonstrated that this may not be the case and that the phosphate is most likely either monoprotonated or a tightly bound constrained dianion (Withers et al., 1981a). Suggested roles of the PLP phosphate as a base (Helmreich & Klein, 1980) or as a nucleophile (Johnson et al., 1980) are therefore rendered less likely on this basis.

Pyridoxal-reconstituted phosphorylase provides an interesting enzyme form with which to investigate the role of PLP in the catalytic mechanism and the relative dispositions of the coenzyme and the substrate glucose-1-P. This enzyme was originally thought to be inactive (Kastenschmidt et al., 1968) but was later shown to exhibit activity in the presence of a noncovalently bound anion activator such as phosphate, fluorophosphate, or phosphite (Parrish et al., 1977). This activator is thought to occupy the phosphate binding pocket normally occupied by the coenzyme phosphate. Pyridoxal phosphorylase has been shown to bind AMP some 3-fold more tightly than native enzyme and to exhibit homo- and heterotropic cooperativity of substrate and activator binding (Kastenschmidt et al., 1968) and as such behaves in a manner very similar to native enzyme. Pyrophosphate is a potent inhibitor of pyridoxal phosphorylase, showing competitive kinetics with both the activator anion and the substrate glucose-1-P and a binding stoichiometry of 1:1 with the enzyme monomer (Parrish et al., 1977). This result suggests that the pyrophosphate can compete simultaneously at the two subsites and that possibly these two subsites are close together in the activated form as suggested previously (Parrish et al., 1977; Withers et al., 1981b).

A kinetic scheme that fits the data obtained for this enzyme is proposed, kinetic studies with substrate analogues and partial "coenzyme–substrate" analogues are described and rationalized on this basis, and speculations are made on possible catalytic mechanisms for this enzyme.

Materials and Methods

Crystallographic Analysis. Crystals of rabbit muscle phosphorylase *a* were grown as previously described (Fletterick et al., 1976). The preparation of native phosphorylase *a* crystals in which glucose is absent is described in the preceding paper (Sprang et al., 1982). Crystals of the GCP–phosphorylase *a* complex were prepared by soaking native crystals in 5 mM GCP for 12–24 h in standard buffer at 22 °C. The GCP solution was stored frozen and used over a 3-week period. The intensities of approximately 11 400 unique reflections were measured from 12 crystals of the GCP–phosphorylase *a* complex and represent 50% of the observable unique data to 3 Å. The merging *R* factor

$$R = \sum_{hkl}\sum(I_{hkl}^{i} - \bar{I}_{hkl})/\sum_{hkl}\bar{I}_{hkl}$$

is 4.8%, where I is the mean intensity of reflection h for i data sets. This sum is taken over all contributors to a common set consisting of 50 scaling reflections in addition to 30 reflections that overlap with the previous data set. The latter are measured at the onset of data collection for one crystal and the conclusion for the succeeding crystals. The correction for radiation damage never exceeded 30%.

The position of GCP was determined from a difference electron density map calculated with Fourier coefficients: $F_{GCP} - F_{native}$. The measurement of data from the native crystals has been described (Sprang et al., 1982). Crystallographic phases were calculated from the current atomic coordinates for phosphorylase *a*. These were originally derived from a 2.5-Å X-ray diffraction analysis of the glucose-inhibited (T-state) structure (Sprang & Fletterick, 1979) and have subsequently been partially refined with 2.1-Å resolution data to a crystallographic *R* factor of 0.30. Coordinates for the GCP model were generated from the atomic coordinates of α-D-glucose (Brown & Levi, 1979) by positioning a phosphorus atom so that the dihedral angles formed by the atoms P–O2–C2–H2 and P–O1–C1–O5 are approximately 180°. The two remaining phosphate oxygen atoms were positioned to lie in the perpendicular bisecting plane formed by the P, O1, and O2 atoms. P–O bond distances were assumed to be 1.55 Å, and the endo- and exocyclic O–P–O angles were taken to be 109° and 115°, respectively. A previous NMR study (O'-Connor et al., 1979) has shown that the pyranose ring of GCP is not distorted from its normal $^4C_1$ conformation.

Active-site coordinates for GCP were derived from the model structure by a rigid body rotation–translation which gave the best fit to the difference electron density. Structural changes that accompany the binding of GCP were interpreted from a difference electron density map calculated with Fourier coefficients $F_{GCP} - F_{parent}$. The model-electron density fitting was done with the UCSF Computer Graphics System (an E&S Picture System II color display linked to a DEC 11/70) using PIG, a program written by O. Jones.

The model-building study to position oligosaccharide at the catalytic site was carried out with the coordinates for the parent phosphorylase *a* structure and a regular oligosaccharide helix constructed from α-D-glucose units (Brown & Levi, 1979). The model-building procedure was accomplished with the aid of a computer graphics program, MIDS, written by M. Pensak, C. Huang, and T. Ferrin.

Kinetic Studies. Buffer chemicals were obtained from Sigma Chemical Co., except for DTT which was obtained from Bio-Rad Laboratories. Potassium phosphite was obtained from ICN Pharmaceuticals. Sodium pyrophosphate, methylenediphosphonic acid, UDP-Glc, and glucose-1-P were from Sigma Chemical Co. and ethylene- and propylenediphosphonic acid from Alfa. Glucosyl fluoride was synthesized according to Micheel & Klemer (1961). Glucose cyclic 1,2-phosphate was synthesized and purified as described previously (Withers et al., 1981a).

Rabbit muscle phosphorylase *b* (EC 2.3.1.1) was prepared by the method of Fischer & Krebs (1962), using DTT instead of cysteine, and recrystallized at least 3 times before use. Phosphorylase *a* was prepared from phosphorylase *b* with phosphorylase kinase (EC 2.7.1.38) (Krebs et al., 1964). Protein concentration was determined from absorbance measurements at 280 nm by using an absorbance index, $E_{1cm}^{1\%}$ of 13.2 (Buc & Buc, 1968). Rabbit liver glycogen (type III) purchased from Sigma Chemical Co. was purified on a Dowex 1-Cl column and assayed by the method of Dische (Ashwell, 1957). The concentration of glycogen is expressed as the molar equivalent of its glucose residues.

Apophosphorylase *b* was prepared by an adaptation of the method described by Shaltiel et al. (1966). Phosphorylase *b* was kept at room temperature in resolution buffer (0.4 M imidazole and 0.1 M cytsteine–citrate, pH 6.2) for 1.5 h and then passed down a Sephadex G-25 column equilibrated with resolution buffer. This protein was then desalted by passage down a second Sephadex G-25 column equilibrated with 50 mM glycerophosphate/50 mM mercaptoethanol buffer, pH 6.8. Assays of this apoenzyme routinely showed less than 0.1% activity and could be reconstituted with PLP to normal activity.

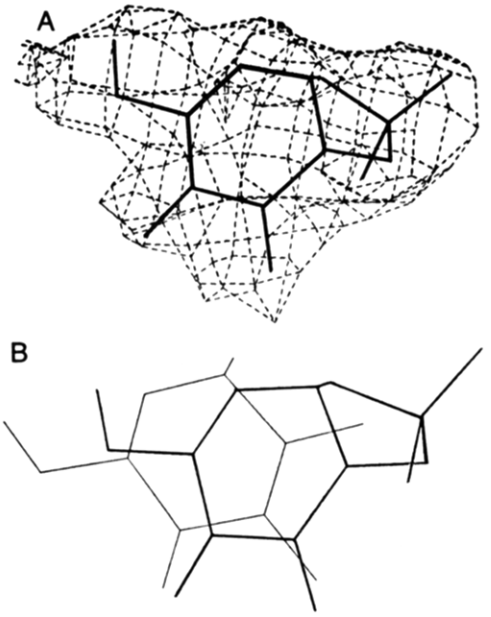

FIGURE 1: (A) Difference electron density; $F_{native} - F_{GCP}$ contour at two standard deviations above the mean density of the map. (B) Comparison of binding mode for GCP (dark line) in phosphorylase *a*–GCP complex with α-D-glucose (lighter line) in parent structure.

Apophosphorylase was reconstituted with pyridoxal as described previously (Parrish et al., 1977). Pyridoxal phosphorylase thus prepared was used for kinetics within 3 days and then discarded.

Pyridoxal phosphorylase was assayed by using the Fiske–Subbarow phosphate analysis in the direction of glycogen synthesis as described by Engers et al. (1970a,b), except that potassium phosphite was added as an essential activator. Reaction mixes were 0.2 mL, and reactions were performed at 30 °C, pH 6.8, in a buffer containing 50 mM triethanolamine hydrochloride, 100 mM KCl, 1 MM EDTA, and 1 mM DTT. Concentrations of substrates and activators employed in the inhibition studies were 1 mM AMP, 20–30 μg of pyridoxal phosphorylase per reaction, 1% glycogen, and 10–50 mM glucose-1-P when substrate concentration was varied; otherwise, it was maintained at 16 mM. In those experiments where phosphite was varied, the concentrations employed ranged from 0.75 to 7.5 mM; otherwise, it was maintained at a concentration of 7.5 mM. Inhibitor concentrations used ranged from $0.2K_i$ to $5K_i$, or as shown in the data. β-D-Glucose-1-P was tested as a substrate and as an inhibitor at concentrations up to 10 mM.

Results

Crystallographic Studies. Binding Mode of GCP. The structure of the active site of phosphorylase in the T state with the inhibitor glucose bound at the catalytic site is described in the preceding paper (Sprang et al., 1982). Binding of the substrate analogue GCP to a crystal of phosphorylase results in a moderate amount of disorder in the crystal lattice and small but significant changes in the lattice constants (a = 128.1→128.5 Å; c = 116.4→116.7 Å; both ±0.1 Å). The root mean square (rms) change in scattering amplitudes (rms $\Delta F/F$) with respect to native crystals is 7.5%. A strong peak of positive density is observed in the active-site region of the $F_{GCP} - F_{native}$ map, and the model of GCP in the $^4C_1$ (O'Connor et al., 1979) conformation can be fit unambiguously into this difference electron density (Figure 1A). The position at which GCP binds is nearly identical with that found for glucose in the parent crystals. Accordingly, the $F_{GCP} - F_{parent}$ difference map clearly shows a phosphate peak near the glucosyl binding locus (Figure 2).

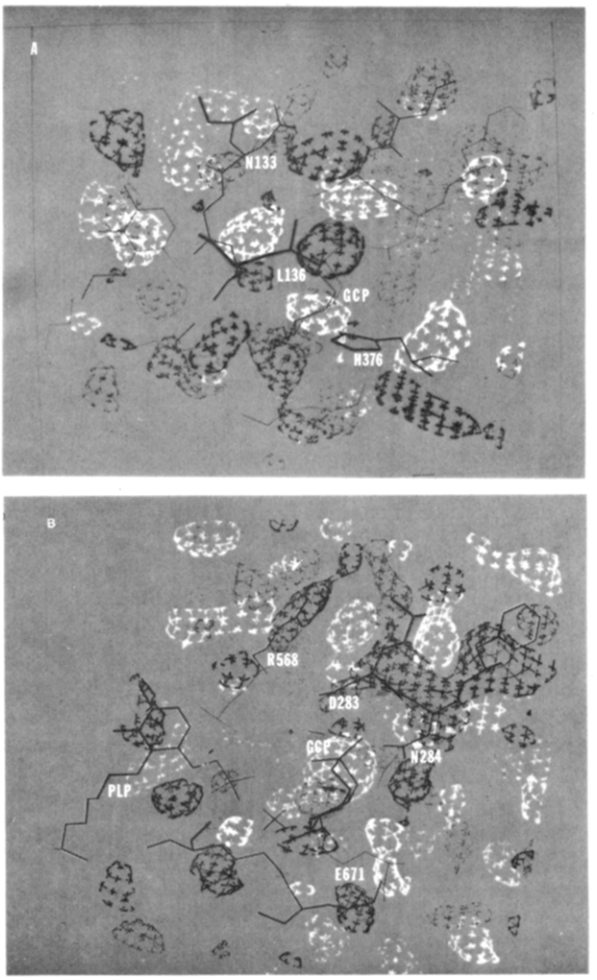

FIGURE 2: Two overlapping, serial sections through $F_{GCP} - F_{parent}$ difference electron density map. Both positive (white) and negative (black) contour level as in Figure 1A.

By superimposing the GCP model onto that of glucose (Figure 1B) we observe that the two molecules are oriented similarly in the active site. However, with respect to the glucose position in the parent crystals, GCP is translated about +1 Å along the y axis toward the entrance of the active-site cavity. Further, the C2–C5 axis of GCP is rotated by about 15° relative to that of glucose. The difference in orientation of the two ligands is significant only in the crystallographic y–z plane since the shift of corresponding atoms from their glucose to their GCP postions is less than 0.2 Å in the x direction.

Conformational Changes on GCP Binding. Structural changes have been determined by difference Fourier analysis of GCP bound crystals with respect to both native and parent data. Although the distribution of positive and negative difference density is qualitatively similar for the two experiments, the magnitude of the structural changes appears to be greater (rms $\Delta F/F$ = 12.5%) if the GCP-bound crystals are compared with parent. The positive and negative electron density contours for GCP-parent experiment are shown in Figure 2.

Conformational changes are numerous but largely localized at the GCP binding site and include polypeptide main-chain shifts, side-chain reorientations, and translations of solvent molecules. It is not possible in all cases to unambiguously assign difference electron density to the reorientation of a

specific residue or solvent, nor can the magnitude of the shifts be accurately determined. The most significant features of the difference electron density with respect to the parent enzyme structure (Figure 2) are summarized as follows:

(a) Difference density is associated with residues 133–136, a polypeptide loop at the N terminus of a short helix (136–142) which caps the active site [see Figure 2 of Sprang et al. (1982)]. The affected residues appear to move away from the GCP binding site.

(b) Residues 282–286 are associated with strong negative difference electron density encompassing both side- and main-chain atoms. The positive density near these residues is poorly connected but may indicate that this polypeptide loop moves away from the protein surface. These residues appear to undergo a transition to a relatively disordered state.

(c) H376 moves into the active-site cavity, maintaining hydrogen bond contact with the glucosyl moiety of GCP (see below). Paired peaks of positive and negative electron density near the imidazole ring opposite D338 suggests the reorientation of a solvent molecule bridging the two residues.

(d) The guanidinium moiety of R568 shifts to a position near that occupied by D283 in the parent structure.

(e) The side chain of K573 has no associated negative density, but a path of positive density stretching between the PLP and GCP phosphate positions may indicate disordered ion pairing to both groups.

(f) Scattered density along the β-ribbon 671–675 may represent translation of solvent molecules (viz., near carbonyl oxygen of A672 and Oγ of S673) or minor side-chain rotations (S673 and T675).

(g) The PLP coenzyme is associated with scattered density. A pair of positive and negative peaks near the PLP phosphate could correspond to a rotation of the phosphate slightly away from the GCP phosphate or a change in its amplitude of vibration.

(h) A water molecule within the catalytic site surrounded by Y572, K573, and E671 shifted from its position in the parent enzyme. This solvent is hydrogen bonded to the 3-hydroxyl group of glucose in the T state, and its translation may reflect the slightly different binding mode of GCP.

The preceding observations emphasize the observed conformation changes at the active site and do not comprise a complete catalog of structural rearrangements within the enzyme. Simultaneous addition of maltopentaose and GCP to washed crystals results in massive reordering of the crystal packing to the extent that $\Delta F/F$ approaches 0.4 (R. F. Fletterick, unpublished results). Such data are not easily interpretable but are evidence for the incomplete nature of the activation process observed with GCP.

GCP–Active-Site Interactions. The contacts between GCP and the enzyme have been calculated by using the coordinates for the parent nonactivated structure and are summarized in Table I. In view of the protein conformation changes induced by the binding of GCP, these must be interpreted with caution. They do point out, however, that GCP cannot be accommodated by a purely T-state catalytic site conformation observed in the parent structure (Sprang et al., 1982). A GCP phosphate oxygen would be in steric conflict with the side chain of N284 and subject to charge repulsion by the carboxylate group of D283 (Table I). Otherwise, the ligand–protein interactions involving the 3-, 4-, 5-, and 6-glucosyl oxygen atoms appear similar to those between the parent enzyme and the corresponding atoms of α-D-glucose. Despite the apparently weak contacts between the glucosyl O5 and O6 atoms and H376, a conformation change in the GCP complex involving

Table I: α-D-Glucose Cyclic 1,2-Phosphate-Phosphorylase *a* Nonbonded Contacts[a]

GCP atom	protein atom	distance (A)	glucose-1-P[b]
	Possible Hydrogen Bonds		
OP2	K573 N	2.8	N133, H570, K573
O2	Y573 Oη	2.8	T377, E671
	T377 Oγ	3.5	
O3	E671 Oϵ1	3.1	S673 (O, N)
	G674 N	3.3	
O4	G674 N	2.4	T675 (N)
	T675 N	3.5	
O5	H376 Nδ1	3.5	H376
O6	H376 Nδ1	3.1	H376
	G135)		
	Steric Conflicts, Charge Repulsion		
OP1	N294 Cγ	2.1	
	N294 Oδ	1.3	
	D283 Oδ1	3.1	
O2	N284 Oδ	2.6	
O4	G674 Cα	2.7	

[a] Polar contacts less than 3.5 Å are determined with respect to the parent coordinate set. [b] Phosphorylase *b* residues determined by Johnson et al. (1980) to be in contact with glucose 1-phosphate.

that residue shifts it closer to the pyranose ring. The 3-hydroxyl may hydrogen bond directly to the carboxylate group of E671 or, as in the parent structure, through an intervening water molecule. The phosphate group of GCP lies 6.8 Å (P to P) from the phosphate group of PLP. It is also within 3.0 Å of the K573 Nζ atom, thus forming a buried charge pair interaction. The side chain of K567 is located within the binding pocket but is not within hydrogen-bonding distance of the GCP phosphate.

The contacts made by GCP with active-site residues differ somewhat from those observed by Johnson et al. (1980) for glucose-1-P in phosphorylase *b* as indicated in Table I. This may be due in part to differences in conformation between the phosphorylase *a* and *b* forms. In contrast to the case reported here, no significant structural changes are noted for phosphorylase *b* upon addition of glucose-1-P (Johnson et al., 1980). Thus, the native state within phosphorylase *b* crystals may represent a partially activated conformation. The phosphate group of glucose-1-P is possibly within contact distance of H570 in phosphorylase *b* while no such interaction is observed for GCP, although this may be a consequence of 1,2 cyclization, which draws the phosphorus toward the pyranose ring. In a separate experiment (R. J. Fletterick, unpublished results) it was shown by a 4.5-Å resolution difference Fourier analysis that phosphite binds to phosphorylase *a* at a site ca. 1.5 Å away from the GCP phosphate position, between the positions taken by the D283 and N284 side chains in the parent enzyme and less than 4 Å from the Nϵ2 atom of H570.

Interaction of Oligosaccharide at the Catalytic Site: A Model Building Study. Attempts to observe an oligosaccharide–active-site complex in crystals of either phosphorylase *a* or phosphorylase *b* have proved unsuccessful, possibly due to the deleterious effects of saccharides on crystal integrity. Nevertheless, it is of interest to speculate on the binding mode of oligosaccharide on the basis of that observed for α-D-glucose in the parent structure (Sprang et al., 1982a) and GCP as discussed in this report.

We propose that the nonreducing terminal residue of a glycogen A chain (Madsen & Cori, 1958; Goldsmith et al., 1982) binds to phosphorylase in a mode similar to that of GCP.

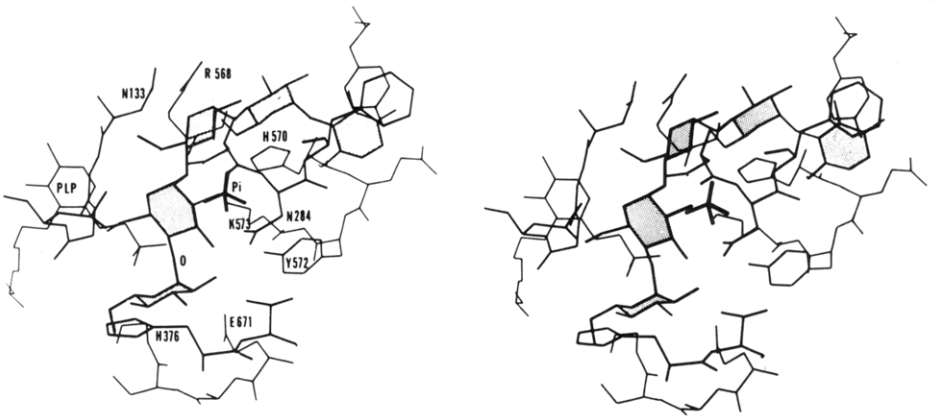

FIGURE 3: Result of model-building study of oligosaccharide bound at active site. Torsion angle between glucose 1 and glucose 2 (that for which bridge oxygen is labeled): ϕ ($O4_i$–$C1_i$–$O1_i$–$C4_{i+1}$ = 0 when cis) = −40° and ψ ($C1_i$–$O1_i$–$C4_{i+1}$–O_{1i+1} = 0 when cis) = −35°. Torsion angle between remaining pairs: ϕ = −15° and ψ = −35°. Position of orthophosphate (P_i) determined from 4.5-Å resolution difference Fourier study with HPO_3^{2-} (R. J. Fletterick, unpublished results).

Consequently, by superimposing the nonreducing end of a model of an oligosaccharide fragment onto the corresponding atoms of GCP it should be possible to approximate the active-site–oligosaccharide complex. The range of possible binding modes is limited by the allowed conformations for the oligosaccharide as well as the steric restrictions imposed by the binding site. A plausible model which satisfies both requirements is illustrated in Figure 3.

With the exception of the torsion angles about the bridge oxygen linking the first two residues (numbering from the nonreducing terminus), the ϕ and ψ angles (see legend to Figure 3) define a regular oligosaccharide helix. The structure lies within the broad potential energy minimum described by Rees & Scott (1971) and is similar to the conformation observed for oligosaccharide bound at the glycogen storage site (Goldsmith et al., 1982). It is stabilized by interresidue O–H···O hydrogen bonds linking the O3′ hydroxyl to that of the O2 hydroxyl of the previous residue. Steric constraints require an alternative conformation about the linkage between the first two residues (ϕ = −40°, ψ = −35°) in which the O2–O3′ hydrogen bond is broken. Nevertheless, this conformation has been observed in maltoheptaose bound at the glycogen storage site (Goldsmith et al., 1982) at a "kink" between two helical segments and in the crystal structure of 6-iodophenyl α-maltoside (Tanaka et al., 1976). This more extended conformation might be reasonable on chemical grounds, destabilizing the linkage between the glucose moiety and the polysaccharide leaving group prior to phosphorolysis. The postulated oligosaccharide–catalytic site model would predict that, as a result of the depth of the binding pocket and the helical conformation of the bound saccharide, phosphorylase would cleave only up to the fifth glucose unit from the 1→6 bond at which the A chain is attached to the remainder of the glycogen particle. Previous analysis of the limit dextrins resulting from phosphorylase action (Brown & Cori, 1961) has shown this to be the case.

The model reveals few steric conflicts between protein and polysaccharide that cannot be relieved by minor conformation changes in the protein. No protein atom is required to move by more than 1.5 Å from it position in the parent enzyme. Certain of these conformation changes, e.g., the order → disorder transition of the β-turn sequence 282–286 and the shift of the strand 133–135 toward the protein interior, are indeed observed for GCP binding alone. This saccharide binding mode would require rotations about the Cα–Cβ bonds of Y612 and H570 to avoid steric conflicts. The putative complex might be stabilized by hydrogen bonds with the residues of the long active-site loop 567–573 and the prehelical sequence 131–135. Oligosaccharide binding could possibly result in reordering the β-turn sequence 285–285 through side-chain oligosaccharide hydrogen bonds.

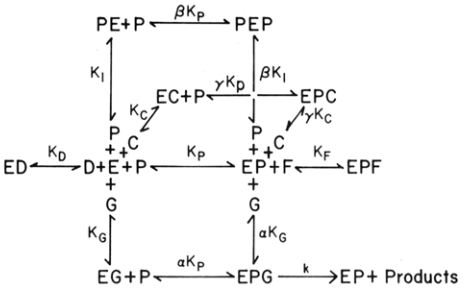

FIGURE 4: Kinetic scheme for pyridoxal-reconstituted phosphorylase *b* where E = pyridoxal enzyme·glycogen·AMP complex, G = glucose-1-P, P = phosphite, D = diphosphate inhibitor (pyrophosphate, methylenediphosphonate, etc.), C = inhibitor competitive with glucose-1-P such as glucose cyclic 1,2-phosphate, and F = inhibitor such as glucose which can bind only to the EP complex. The complete velocity equation is $V_{max}/v = \alpha K_G K_P/([G][P]) + \alpha K_G K_P[D]/([G][P]K_D) + \alpha K_G/[G] + \alpha K_G K_P/([G]K_I) + \alpha K_G K_P[C]/([G][P]K_C + \alpha K_P/[P] + \alpha K_G[P]/(\beta[G]K_I) + \alpha K_G[C]/(\gamma[G]K_C) + \alpha K_G[F]/([G]K_F) + 1$.

Kinetic Studies. (*A*) *Pyridoxal Phosphorylase b.* The dependence of this form of the enzyme upon two obligate activators, i.e., not only AMP but also phosphate or a suitable analogue, required formulation of a new kinetic model to allow analysis of kinetic data determined with this system. This model was further complicated by the fact that the anion activator could also act as a competitive inhibitor with substrate at higher concentrations. However, since the dissociation constant for phosphite as an activator is much smaller than that for phosphite as a competitive inhibitor of substrate binding (Parrish et al., 1977), studies of the activation properties of phosphite should be possible, in the absence of any significant inhibition.

The model derived to describe this system is shown in Figure 4. This scheme is similar to one described previously by Segel (1975) but is extended to incorporate additional ligands. Such a model allows certain predictions to be made as to the kinetic behavior of the system as follow:

(i) In the absence of inhibitors, C, D, and F, this model is described by the rate equation

$$\frac{1}{v} = \frac{\alpha K_G}{V_m}\left(1 + \frac{K_P}{[P]} + \frac{K_P}{K_I} + \frac{[P]}{\beta K_I}\right)\frac{1}{G} + \frac{1}{V_m}\left(1 + \frac{\alpha K_P}{[P]}\right)$$

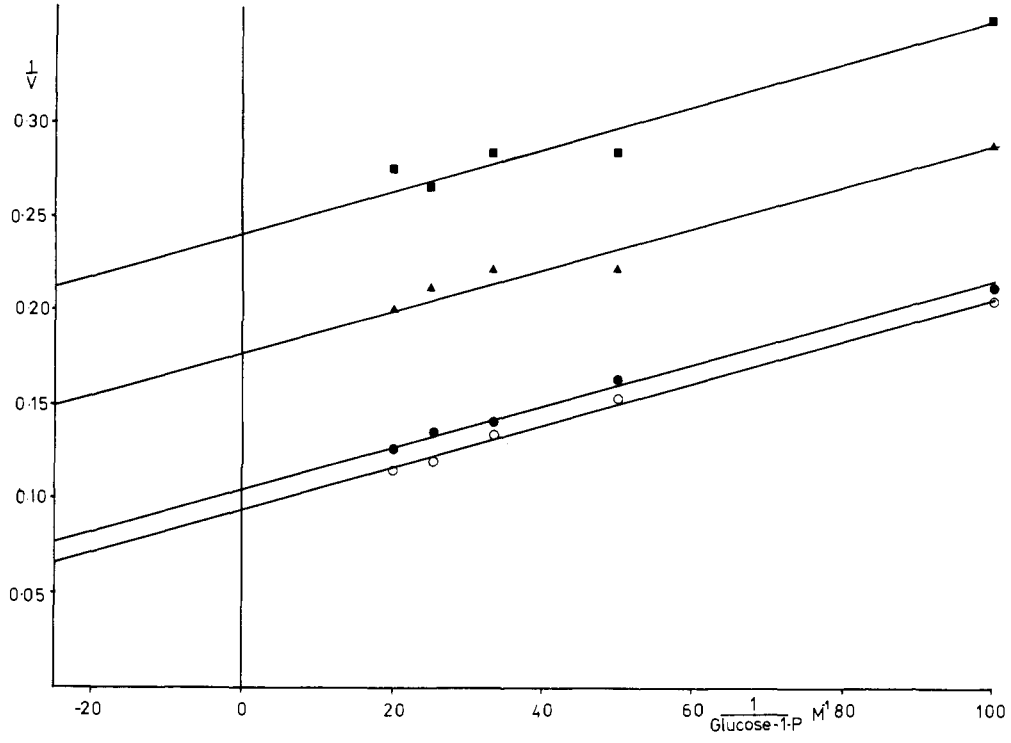

FIGURE 5: Lineweaver–Burk plot for pyridoxal phosphorylase *b* as a function of varied phosphite concentrations, as follows: (■) 0.75, (▲) 1.25, (●) 3.75, and (○) 7.5 mM phosphite.

Plots of $1/v$ vs. $1/[\text{glucose-1-P}]$ should give straight line plots, but these are unconventional in that at concentrations of phosphite between K_P and βK_I the lines should appear parallel.

(ii) The kinetics of inhibition exhibited by inhibitors C, D, and F should fall into three separate classes. Inhibitors D should show competitive inhibition against both substrate, glucose-1-P, and activator, phosphite. A replot of the slopes of their Lineweaver–Burk plots should yield, from the intercepts on the abscissa, values for the apparent dissociation constants

$$K_D(\text{app}) = \left(\frac{[G]}{K_G} + 1\right)K_D$$

and

$$K_D(\text{app}) = \left(\frac{[P]}{K_P} + 1\right)K_D$$

assuming that $[P] \ll K_I$ and $K_P \ll K_I$.

Different apparent K_D's would thus be anticipated in the two cases. Inhibitors C should show competitive kinetics with glucose-1-P but noncompetitive kinetics with phosphite. The intercept in the slope replot in the case in which glucose-1-P is varied should give a value approaching γK_c, assuming saturating phosphite concentration. Inhibitors F should show competitive inhibition with glucose-1-P but uncompetitive kinetics with phosphite since they cannot bind to "free" enzyme. A third prediction, based on the model, but not required by it, would be that in comparison with native phosphorylase *b*, the R-type inhibitors, e.g., GCP, should bind relatively more effectively than T-type inhibitors, e.g., glucose, since pyridoxal enzyme is known to bind AMP more tightly than does native enzyme.

These predictions were tested as follows: (i) The results of an investigation of the effects of simultaneously varying the concentrations of glucose-1-P and phosphite within the lower range of phosphite concentrations are given in Lineweaver–Burk format in Figure 5. Parallel lines are observed, as predicted for phosphite concentrations between K_P and βK_I, indicating an increase in V_m and apparent K_m with increasing concentrations of phosphite. It should be noted, however, that while the kinetic pattern is consistent with the model, it does not prove it since parallel lines are an insensitive test, and for technical reasons, we do not have an estimate of K_P so that we can be sure that $[P] > K_P$ as well as αK_P. A replot of the intercepts a value for αK_P of 1.75 mM which represents the dissociation constant for phosphite to the enzyme–substrate complex.

(ii) All inhibitors tested showed competitive inhibition against glucose-1-P as shown in Table II and as demonstrated graphically for GCP in Figure 6. Inhibitors D indeed also showed direct competitive inhibition against phosphite as shown in Table II, yielding different apparent K_i values when measured against glucose-1-P than against phosphite, as predicted. All four compounds in fact showed smaller apparent K_i values against phosphite than against glucose-1-P.

Inhibitors C, e.g., GCP, showed competitive kinetics against glucose-1-P but noncompetitive kinetics against phosphite, as predicted by the model, as shown in Figure 6 and Table II. The K_i value indicated in Table II for GCP against glucose-1-P represents γK_c, assuming saturating phosphite concentration. The value obtained vs. phosphite is simply an apparent K_i measured in the presence of 16 mM glucose-1-P.

Inhibitors F, e.g., glucose and glucosyl fluoride, indeed showed competitive kinetics against glucose-1-P and uncompetitive kinetics against phosphite as predicted. This suggests that glucose can only bind significantly when phosphite is present, which in turn suggests that phosphite is responsible for the formation of part of the glucose binding site. This has since been confirmed (S. G. Withers, unpublished results) by a ^{31}P NMR study of the effect of glucose on nucleotide binding. Caffeine, however, shows noncompetitive kinetics under these conditions, showing that it is able to bind to phosphite-free enzyme. This is consistent with the fact that caffeine binds at a locus some 10 Å away from the PLP.

Table II: Inhibition of Pyridoxal Reconstituted Phosphorylase *b* and Native Enzyme by Active Site Directed Compounds

compound	inhibition type	pyridoxal reconstituted enzyme: K_I(app) (mM) (vs. glucose-1-P)	pyridoxal reconstituted enzyme: K_I(app) (mM) (vs. phosphite)	native enzyme, K_I(app) (mM) (vs. glucose-1-P)
caffeine	T	8.5 (comp.)[a]	16.5 (noncomp.)	1 (comp.)
glucose	T	52 (comp.)	100 (uncomp.)	2 (comp.)
α-glucosyl fluoride	T	12 (comp.)	12 (uncomp.)	0.6 (comp.)
glucose cyclic 1,2-P	R	6 (comp.)	4 (noncomp.)	0.9 (comp.)
UDP-Glc	R	8 (comp.)	7 (noncomp.)	1 (comp.)
pyrophosphate[b]	R	0.3 (comp.)	0.03 (comp.)	no inhibition
methylenediphosphonate	R	1.5 (comp.)	0.5 (comp.)	
ethylenediphosphonate	R	9.0 (comp.)	2.8 (comp.)	
propylenediphosphonate	R	19 (comp.)	9 (comp.)	
β-glucose-1-P		>>10		

[a] Comp., uncomp., and noncomp., refer to competitive, uncompetitive, and noncompetitive inhibition patterns observed on reciprocal plots. [b] Determined by Parrish et al. (1977).

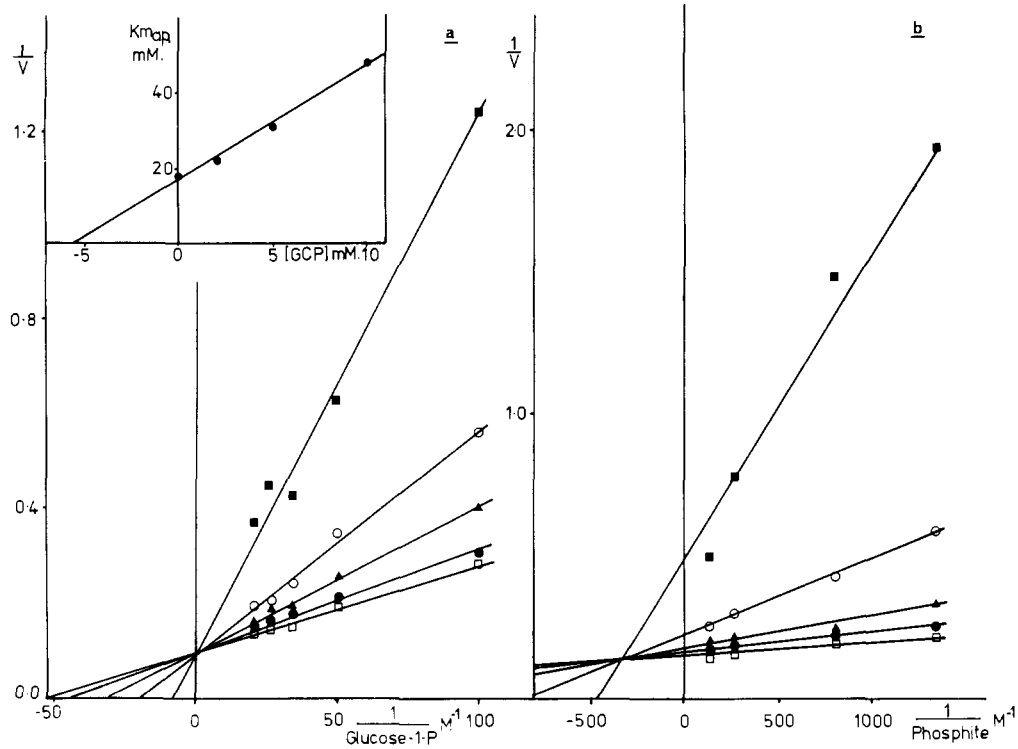

FIGURE 6: (a) Lineweaver–Burk plot for pyridoxal phosphorylase *b* as a function of varied concentrations of GCP, as follows: (■) 20, (○) 10, (▲), 5, (●) 2, and (□) 0 mM GCP. Phosphite concentration was maintained at 7.5 mM. (Inset) Replot of K_m(app) vs. GCP concentration. (b) Lineweaver–Burk plot for pyridoxal phosphorylase at a fixed concentration of glucose-1-P (16 mM) as a function of varied GCP concentrations, as follows: (■) 20, (○) 10, (▲) 5, (●) 2, and (□) 0 mM.

Significantly, these three T-type inhibitors all showed weaker inhibition against phosphite than against glucose-1-P in contrast to the R-type inhibitors where the reverse situation obtained. This difference is borne out in other sets of data in Table II, as follows:

All the R type inhibitors bind some 7–8-fold more weakly to pyridoxal phosphorylase than to native phosphorylase. This is consistent with the 7–8-fold weakening of the substrate binding constant from native to pyridoxal phosphorylase (K_m = 15 mM for glucose-1-P, at 7.5 mM phosphite with pyridoxal phosphorylase; K_m = 2 mM for glucose-1-P with native phosphorylase). Parrish et al. (1977) reported a K_m value of 28 mM for pyridoxal phosphorylase *b*, but differences in assay conditions could be responsible for this discrepancy. This 7–8-fold weakening is in some ways surprising in light of the tighter binding of AMP to pyridoxal phosphorylase and, therefore, presumably its more R-type conformation. However, examination of the inhibition constants for the T-type inhibitors shows that their binding is weakened even more on going from native to pyridoxal phosphorylase as both glucose and glucosyl fluoride bind some 25 times more weakly. However, phosphite alone at these low concentrations is not capable of stabilizing the enzyme in a full R state, since it has been shown that glucose and caffeine can both bind simultaneously with phosphite. Apparent K_i values for caffeine do not fit this pattern for T-type inhibitors perfectly, but this may be due to the fact that it is binding at a different site and is thus able to bind to the free enzyme.

(B) Absence of Inhibition by β-Glucose 1-Phosphate. A mechanism of action of phosphorylase has recently been proposed, (Johnson et al., 1980) which involves the participation of phosphate of the PLP in a nucleophilic attack on the backside of the substrate glucose-1-P molecule at C1 to facilitate displacement of P_i and produce some form of intermediate β-glucosyl-PLP compound. By use of the observed activity of the pyridoxal enzyme this hypothesis was tested as follows:

Assuming this mechanism to hold true for the pyridoxal

enzyme, β-glutose-1-P would necessarily be formed as an intermediate in the reaction (using P_i as activator instead of phosphite), in place of the proposed β-glucosyl-PLP formed with native enzyme. This mechanism would therefore predict that β-glucose-1-P would act as a substrate for the pyridoxal enzyme, if a full covalent β-glucosyl-PLP is invoked, or at least as a tight inhibitor of the "transition-state" type, if the PLP were postulated to act as a stabilizing group of the carbonium ion formed.

Our results show *no* action of β-glucose-1-P as a substrate or as an inhibitor up to concentrations of 10 mM. This result therefore argues against the proposed mechanism and agrees with results recently reported (Takagi et al., 1981) showing that apophosphorylase *b* can be reconstituted with β-glucosyl-PLP but that this new enzyme form is not degraded to native enzyme upon addition of glycogen as would be required in the postulated mechanism.

(*C*) *Inhibition by Alkanediphosphonates.* Knowledge of the distance separating the substrate phosphate and that the coenzyme in the activated form of phosphorylase is crucial to an understanding of the mechanism of the enzyme. The X-ray-derived crystal structure indicates a separation of some 6–7 Å, yet the kinetic studies with pyridoxal phosphorylase and pyrophosphate might suggest that the two phosphates are much closer. As a means of further probing the interphosphate distances, a series of diphosphonate compounds with different numbers of bridging methylene groups have been tested as inhibitors of pyridoxal phosphorylase. It might be anticipated that the diphosphonate having a phosphorus–phosphorus separation corresponding to that in activated phosphorylase would be the best inhibitor. An X-ray crystallographic study (Yount, 1975) shows that the P–C bond is slightly longer (1.79 Å) than the P–O bond (1.61 Å) but that the PCP bond angle (117°) is less than the POP bond angle (130°). This results in a P–P distance of 3.05 Å in methylenediphosphonate and a P–P distance of 2.92 Å in pyrophosphate. The two phosphates are therefore some 0.13 Å further apart. In addition, methylenediphosphonate has pK_3 and pK_4 values, respectively, 1 and 2 units higher than pyrophosphate and would therefore bear the equivalent of at least one full charge less (S. G. Withers, unpublished data). Nevertheless, it is still a good inhibitor (Table II), competitive with both glucose-1-P and phosphite, and as such is analogous to pyrophosphate. The subtle differences in structural and particularly electronic parameters could easily account for the 5–15-fold difference in K_i values. Moreover, the observation of slight differences between the binding constants for methylenediphosphonate and pyrophosphate is not crucial to the central argument; it is only important that methylenediphosphonate bind in the same general mode as pyrophosphate since comparisons of K_i values are only made within the series of diphosphonates.

The interphosphorus distances in these compounds in their preferred, fully extended conformations have been estimated by model building and are approximately 3.0, 5.0, and 6.0 Å for the methylene-, ethylene- and propylenediphosphonates, respectively. The results given in Table II demonstrate that the apparent K_i values for the diphosphonates increase with the number of bridging methylene groups, thus the interphosphorus separation. Methylenediphosphonate therefore binds tighter than either of its more extended analogues. The observation of lower K_i values for the diphosphonates when measured against phosphite than when measured against glucose-1-P is consistent with the data obtained with pyrophosphate and fits the predicted pattern described earlier. An analogous kinetic scheme has previously been elucidated by Purich & Fromm (1972) to explain the kinetic patterns observed for the inhibition of adenylate kinase by $P^{1'},P^{4'}$-diadenosine tetraphosphate.

Discussion

The conformation changes induced in phosphorylase *a* by the binding of the R-state inhibitor, GCP, appear to characterize a subset of the complex changes that take place in the structural transition toward an active (R) state. Much more extensive conformation changes are observed in phosphorylase crystals upon simultaneous addition of GCP and maltoheptaose; thus, the results presented here represent only a qualitative description of the effect of substrate binding. However, the changes which are observed to occur are significant, since they do allow binding of a substrate analogue, and can be considered to represent a stage in the overall T → R transition.

The most dramatic of the structural changes affects a sequence of residues, 282–286, that form a β-turn loop at the entrance to the catalytic site. The immediate effect of this conformation change is to render the active site accessible to its second substrate, a terminal A chain of glycogen. Due to its poor binding constant at the active site (K_m = 22 mM) (Kasvinsky et al., 1978), oligosaccharide has never been observed to bind at this site in X-ray crystallographic analyses, probably as it is unable to bind to an inactive or intermediate conformation of the enzyme. Our own model-building studies now suggest that the observed disordering of the hairpin loop upon activation is sufficient to admit a helical oligosaccharide into the catalytic site cavity and indicate a structural basis for interaction between the protein and its substrate.

There is a possible "inverse" symmetry in the order → disorder transitions that accompany the allosteric response of phosphorylase. Binding of T-state inhibitors such as glucose and caffeine leads to a disordering of the N terminus of phosphorylase (Withers et al., 1981a) and a stabilization of the β-hairpin turn (282–286) (Sprang et al., 1982). Binding of activators (AMP) and substrate analogues (GCP) has the opposite effect.

A significant consequence of the observed changes at the active site is the creation of a phosphate binding site containing R568, H570, and K573 and an N-terminal helix dipole, to accommodate the phosphate of GCP or orthophosphate. Indeed, this group is probably the prime agent of the local conformational response and may trigger structural changes elsewhere in the enzyme. The phosphate cannot be accommodated sterically or electrostatically in the T conformer due to a potential steric clash with N284 and a repulsive electrostatic interaction with D283. In the T structure D283 interacts with R568 and might be considered to occupy a "virtual" phosphate binding site, as has been observed previously (Withers et al., 1981a) and used to rationalize ^{31}P NMR spectra.

Role of the Pyridoxal Phosphate Coenzyme in Catalysis. The demonstration that the PLP phosphate is probably dianionic in an activated AMP complex of phosphorylase *b* and in phosphorylase *a* appeared consistent with proposals that the coenzyme serves as a nucleophile (Johnson et al., 1980) or a base (Helmreich & Klein, 1980). The former mechanism involves a backside attack of the PLP phosphate at the anomeric carbon of glucose-1-P or glycogen and thus requires an inverted binding mode of the substrate in the activated complex. The binding mode of GCP presented in this work as well as that of glucose-1-P in crystals of phosphorylase *b* is such that the C1–O1 bond of the substrate is directed toward the PLP phosphate. Johnson et al. (1980) have proposed that the observed complex is in fact nonproductive and argue, on

steric grounds, that oligosaccharide cannot bind to the active site in a favored conformation such that the nonreducing terminus takes up the position observed for glucose-1-P in the phosphorylase complex. The study presented here shows that it is indeed possible to construct a model for the interaction of an oligosaccharide in the favored left-handed helical conformation (Rees & Scott, 1971; Goldsmith et al., 1982) with phosphorylase *a* in a manner consistent with the observed binding mode of GCP (Figure 3). The results obtained with β-glucose 1-phosphate argue against a role of the coenzyme phosphate either as a nucleophile displacing the substrate phosphate in a backside attack on glucose-1-P or as a counter ion stabilizing the glycosyl carbonium ion. This is also consistent with the demonstration by Takagi et al. (1981) that apophosphorylase *b* reconstituted with β-glucosyl-PLP is not degraded to native enzyme. These observations have led us to consider a direct role for the PLP phosphate in labilizing the phosphate group of glucose-1-P to increase its leaving group ability. The problem is that of understanding, first, the nature of the interaction between the two phosphate groups and, second, how it might contribute to the labilization of the glycoside bond.

There is little evidence leading to a definitive description of the phosphate–phosphate interaction in the tertiary E·S complex or the transition state. The crystallographic data presented here demonstrate a center to center distance of 6.8 Å for the locally activated GCP bound complex. Similar results were obtained by Johnson et al. (1980) in their analysis of the partically activated glucose-1-P–phosphorylase *b* complex. However, neigher of these analyses was performed in the presence of oligosaccharide substrate when further conformational changes are known to occur.

A recent proposal has been made by Withers et al. (1981b) that the phosphate groups are in close contact within the E·S complex. This suggestion is consistent with the competitive inhibition of pyridoxal phosphorylase by pyrophosphate (Parrish et al., 1977) and also with the rapid rate of reconstitution of apophosphorylase *b* by pyridoxal pyrophosphate (Shimomura & Fukui, 1978). Most recently, Withers et al. (1981b) demonstrated that the enzyme catalyzed the cleavage of the glycosidic bond of the covalently bound coenzyme–substrate analogue PLPP-α-Glc upon addition of maltopentaose. More recent studies (Takagi et al., 1982) show that this glucose unit may be transferred to glycogen, forming an α-1,4 linkage. These findings indicate that an enzyme complex in which the phosphate groups are covalently linked produces both an active E·S conformation and a labile glucosyl linkage, indicating that, in the normal reaction, the two phosphates are brought close together but that a full pyrophosphate intermediate is not formed, since the enzyme–pyrophosphate is stable over a period of several days.

The inhibitory effect of the diphosphonate inhibitors is shown to decrease monotonically with the number of methylene groups separating the phosphorus atoms. Further, these inhibitors are known to bind to R-state phosphorylase on the basis of their kinetic behavior described earlier and their allosteric improvement of the nucleotide binding constant (S. G. Withers, unpublished results). The most obvious and reasonable interpretation of this experiment would therefore indicate a very close phosphate–phosphate approach within the active site in its activated form. Nevertheless, it is possible that binding of the inhibitors to a fairly polar site could be compromised by a loss of hydrogen bonding capacity due to the hydrophobic nature of the intervening carbon chain. The contribution of this effect would be expected to increase with

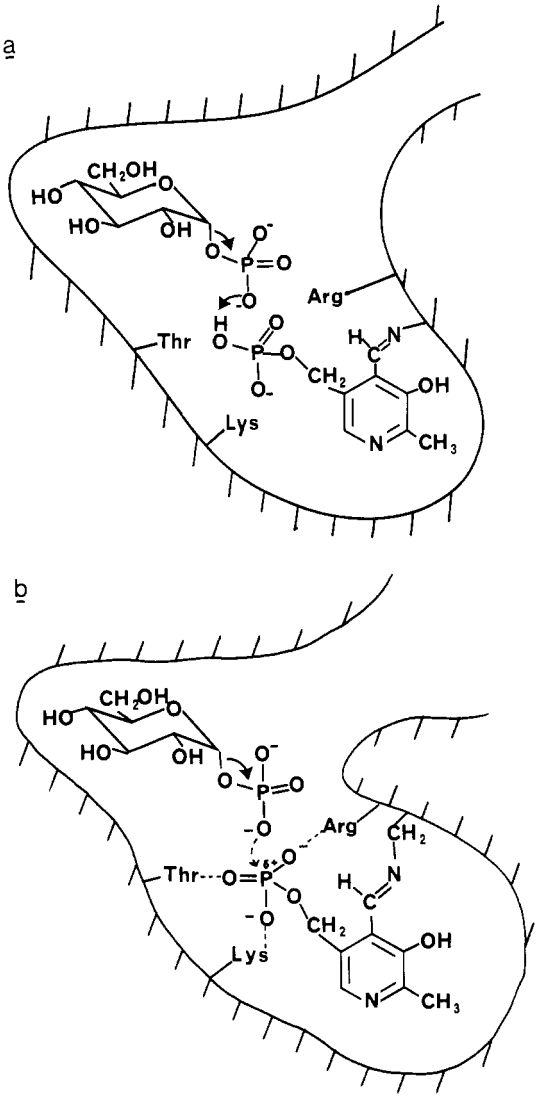

FIGURE 7: (a) Role of monoanionic PLP as an acid catalyst. (b) Role of dianionic PLP as an electrophile.

the length of the carbon chain but would have to be very pronounced to completely annul and reverse any increased binding affinity due to the correct positioning of the two phosphorus atoms.

A close approach of the phosphate group of glucose-1-P to that of the coenzyme suggests at least two possible distinct mechanistic roles for the latter. First, it could be acting as an acid catalyst, as suggested previously (Pfeuffer et al., 1972), if the close association results in an enforced proton transfer from the coenzyme phosphate to that of glucose-1-P (Figure 7a). Proton transfer could be effected directly between the phosphate groups, or as suggested by Miller et al. (1980, 1981) and by Dreyfus et al. (1980), by an intervening basic residue of the enzyme. Both R568 and K573 could be important in this context. A monoprotonated state for PLP is consistent with ^{31}P NMR studies of phosphorylase in the presence of both substrates (Withers et al., 1981a) and also with the findings that both pyridoxal 5′-phosphate monomethyl ester (Pfeuffer et al., 1972) and pyridoxal 5′-phosphorofluoridate (S. G. Withers, unpublished results), which cannot deprotonate in the pH range studied, are inactive. However, the observation of a similar activation of pyridoxal phosphorylase by phosphite (pK = 6.6) and fluorophosphate (pK = 4.8) (Parrish et al., 1977) may indicate that acid catalysis by the PLP phosphate is unimportant.

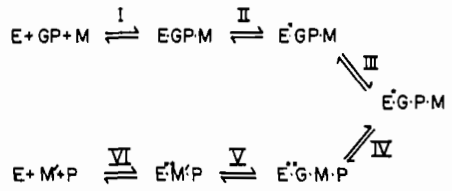

FIGURE 8: Catalytic mechanism of glycogen phosphorylase. E = phosphorylase + AMP, GP = glucose-1-P, P = inorganic phosphate, G = "activated" glucosyl unit, M = oligosaccharide, M' = lengthened oligosaccharide, and E and E* represent different enzyme conformations while E and E** may represent one and the same enzyme conformation.

A second possibility for the role of PLP proposed recently by Withers et al. (1981b) is that the phosphate of the coenzyme remains dianionic but that in response to activation, it becomes tightly coordinated by neighboring basic groups and in this process is constrained toward a trigonal bipyramidal configuration, with the empty apical position pointing toward the substrate phosphate. This apical position would have some electrophile nature, and as such the PLP phosphate could act as an electrophile, withdrawing electron density from the substrate and thereby labilizing the glucosidic bond (Figure 7b).

Mechanisms in which the electrophilicity of a substrate phosphate group is enhanced through trigonal coordination to polar groups on the enzyme are implicated for several enzyme-catalyzed reactions. In the cases of both staphylococcal nuclease (Cotton et al., 1979) and ribonuclease (Deakyne & Allen, 1979), a distortion of the phosphate to a trigonal bipyramidal configuration has been proposed as a means of stabilizing a transition state and increasing the electrophilicity of the phosphate. A similar mechanism has been envisaged for adenylate kinase in which two ADP molecules approach "head to head" and exchange a phosphate (Pai et al., 1977).

The formation of a pseudo pyrophosphate bond as a catalytic step would be analogous to an abortive phosphate transfer reaction. This event would require tight coordination of the PLP phosphate oxygen atoms, not only to facilitate a pyramidal configuration as discussed above but also to prevent the expulsion of water or pyridoxal as a result of a complete in-line attack by the substrate phosphate. The ^{31}P NMR results discussed above (Withers et al., 1981a) are also consistent with an immobilized but dianionic PLP phosphate. Within the phosphorylase active site, it is probable that the PLP phosphate makes at least two hydrogen bonds with the main-chain amides of residues G674 and T675 (Sprang et al., 1982). The present crystallographic evidence suggests that K573 may participate in an ion pair interaction with both phosphate groups. Additional stabilization could be achieved by small conformation changes effecting the reorientation of K567 and R568.

In each of the models considered above, the coenzyme enhances the lability of the phosphate of substrate glucose-1-P which, once cleaved, could act as a base either directly or via an enzymic group, deprotonating the incoming 4-hydroxyl, in the direction of glycogen synthesis. A similar mechanism has been proposed for aldolase, where the substrate phosphate has been implicated as the base that abstracts a proton during the catalytic event (Periana et al., 1980). There is some evidence (T. Fukui, unpublished results) for a protein group of pK_a = 8.2 in the neighborhood that could be involved in such a process. Whichever the precise role of the PLP phosphate, its catalytic effect would be the same. The overall mechanism for phosphorylase shown in Figure 8 is proposed to follow the sequence of events broadly outlined (in the direction of glycogen synthesis).

The binding of glucose-1-P and oligosaccharide (I) is followed by a conformational change (II), which brings the two substrate and PLP phosphate groups into close proximity, resulting in the distortion and enhanced electrophilicity of the latter, thus labilizing the glucosyl phosphate linkage. This reorientation might be brought about by a shift or rotation of the N-terminal domain, from which most contacts to the glucosyl moiety originate, with respect to the C-terminal domain, to which the PLP is anchored. Bond cleavage (III) then ensues, with the coenzyme phosphate and its neighboring basic groups essentially sequestering the released inorganic phosphate. The glucosyl carbonium ion generated may be stabilized as an intermediate by a negatively charged enzymic group, attacked directly in a concerted reaction by the oligosaccharide, or trapped by an enzymic group as a glucosyl enzyme intermediate. Recent estimates of lifetimes in the order of 10^{-15} s or less for glycosyl cations in free solution (Jencks, 1980) cast doubt on the carbonium ion as a stable intermediate. The concerted reaction, while unattractive for steric reasons, should be considered since such reactions have been observed in studies of spontaneous glycosyl transfer reactions (Sinnott & Jencks, 1980) when a very open transition state simultaneously involving both attacking and leaving groups is invoked. Probably the most likely process would involve the formation of the glucosyl enzyme intermediate most likely with a carboxylate by analogy with studies on β-glucosidase (Bause & Legler, 1974) and sucrose phosphorylase (Voet & Abeles, 1970) and with recent results on potato phosphorylase (Klein et al., 1981). This has been suggested previously (Koshland, 1953). The carboxylate of E671, after a translation of the glucosyl moiety into the active-site pocket (see II), could occupy a suitable position for such an attack.

The glucosyl unit now reacts with the nonreducing end of the oligosaccharide chain which must occupy approximately the same space as the original phosphate moiety since the reaction proceeds with retention of configuration at the glucose moiety. This apparent steric problem could be solved in one of two ways; either the phosphate diffuses away, allowing the oligosaccharide to slide into place, or a conformation change occurs (IV), possibly a reversal of the original conformation change (II), to pull the pyridoxal phosphate and sequestered inorganic phosphate out of the way and place the activated glucosyl unit adjacent to the nonreducing end of the oligosaccharide. Diffusion of phosphate from the active site is unlikely since it would allow the influx of a water molecule, resulting in hydrolysis, rather than phosphorolysis, of glycogen. Moreover, it is observed that the glucose unit of the PLPP-α-Glc phosphorylase derivative is transferred to glycogen (Takagi et al., 1982), and here the phosphate "leaving group" is covalently linked into place and therefore unable to diffuse away. A mechanism involving a second or restorative conformation change to reposition the reactants and intermediates is consistent with the overall reaction rate ($\sim$150–160 s^{-1}), similar to that observed for other enzymes whose rate is thought to be limited by a conformation change accompanying product release (Rose et al., 1974). In either event, the 4-hydroxyl group of the terminal sugar residue would then attack the glucosyl moiety at the C1 position (V), possible aided by a basic group on the enzyme, or the cleaved orthophosphate, which could deprotonate the 4-hydroxyl group facilitating attack. The lengthened oligosaccharide would then be released (VI), with or without inorganic phosphate.

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