Catalytic site of glycogen phosphorylase: Structure of the T state and specificity for α-D-glucose

ARTICLE *in* BIOCHEMISTRY · NOVEMBER 1982

Impact Factor: 3.02 · DOI: 10.1021/bi00264a038 · Source: PubMed

Catalytic Site of Glycogen Phosphorylase: Structure of the T State and Specificity for α-D-Glucose†

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ABSTRACT: α-D-Glucose inhibits glycogen phosphorylase *a* by binding at the catalytic site of the inactive conformer (T state) at the same position as does the substrate α-D-glucose 1-phosphate to the active (R state) enzyme. It is established that recognition of glucose 1-phosphate is highly specific. Here, we show by crystallographic analysis of the α-D-glucose–phosphorylase *a* complex and by analysis of inhibition by a variety of glucose analogues the nature and specificity of the recognition of the glucosyl group by the T-state enzyme. The catalytic site at which glucose is bound is located at the confluence of the N- and C-terminal domains. Each is an α/β structure consisting of a β-sheet core surrounded by a double tier of α helices. The active-site residues are located on flexible loops of polypeptide chain emanating from the domain boundaries. Glucose participates in at least five well-defined hydrogen bonds with these residues and presents a complementary molecular surface to the active site at the hydrogen-bonded positions of the ligand. Inhibition and model-building studies show that changes in chirality or substitution at any of the glucose hydroxyl groups can abolish or drastically reduce the binding affinity of the ligand. Absence or low activity in glucose analogues can be rationalized as a reduction in hydrogen bonding capacity and/or the introduction of steric conflicts with the enzyme. Although there are substantial differences between the T- and R-state enzymes with respect to active-site conformation, both conformers exhibit specific binding of the glucosyl moiety of α-D-glucose on the one hand (T) and glucose 1-phosphate or half-chair glycosyl analogues (which mimic the proposed carbonium ion intermediates or transition state) on the other (R). A structural interpretation of these observations is presented. By means of inhibition studies with several glucose 1-phosphate analogues and also by inspection of the crystal structure, it is demonstrated that the substrate binding site, in the R-state enzyme, comprises adjacent phosphate and glycosyl subsites. Analogues of the substrate which differ substantially in their carbohydrate moiety demonstrate competitive inhibition by occupation of the phosphate subsite alone.

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the reversible transfer of the glucosyl moiety from nonreducing ends of the α-1,4 linked glucose chains in glycogen (Graves & Wang, 1972; Fletterick & Madsen, 1980) to P_i.[1] The structures of both the phosphorylase *a* (Sprang & Fletterick, 1979) and phosphorylase *b* (Weber et al., 1978; Johnson et al., 1980) enzymes are known from X-ray crystallographic analysis at moderately high resolution. These studies reveal two subtly different conformations of the phosphorylase molecule even though the two forms crystallize in isomorphous unit cells. The phosphorylase *b* structure has at least some characteristics of the catalytically active R-state conformation by virtue of its ability to bind the substrate glucose-1-P without sustaining lattice disorder (Johnson et al., 1980). Phosphorylase *a*, in contrast, undergoes substantial conformation changes, interpreted as a shift toward the R state, when either substrate glucose-1-P or substrate P_i is introduced into the crystals (Madsen et al., 1978). Phosphorylase *a* can be stabilized in an inactive, T-state, conformation by incorporating α-D-glucose into the crystals (Sprang & Fletterick, 1979).

The effects of glucose on rabbit muscle phosphorylases *a* and *b* have been studied by a variety of techniques. Kinetic studies have shown it to be an allosteric inhibitor (Helmreich et al., 1967) competitive with glucose-1-P and acting synergistically with purine nucleosides or derivatives such as caffeine (Kasvinsky et al., 1978) to stabilize the incative T conformation of the enzyme. The allosteric effects of glucose are manifested in its ability to induce disorder at the N terminus of phosphorylase, the recognition site of the modifying enzymes phosphorylase phosphatase and phosphorylase kinase. This is evident from the results of chemical cross-linking and protease degradation studies (Dombradi et al., 1980, 1982) as well as the acceleration by glucose of the phosphatase reaction itself (Dombradi et al., 1982; Stalmans et al., 1970). Physical techniques have been employed to study the effect of glucose on the tertiary and quaternary structure of the enzyme. Ultracentrifugal studies showed it to stabilize the dimeric form of the enzyme (Wang et al., 1965; Withers et al., 1979), and ^{31}P NMR techniques indicate that it stabilizes a local environment of the phosphate moiety of the pyridoxal 5′-phosphate coenzyme similar to that observed in the presence of nucleosides or in nonactivated phosphorylase *b* (Withers et al., 1979). Both glucose and the substrates, glucose-1-P and P_i, render the coenzyme inaccessible to solvent, as evidenced by their ability to prevent the quenching of the PLP fluorescence by iodide (Honikel & Madsen, 1973). Glucose was found to bind at the phosphorylase active site (Sprang & Fletterick, 1979) in approximately the same orientation as the substrate glucose-1-P (Weber et al., 1978; Johnson et al., 1980).

It is perhaps surprising that the two similar compounds should promote significantly different catalytic site conformations by interacting at the same locus. It has been suggested that the phosphate group acts as a conformational trigger, promoting the T → R transition (Madsen et al., 1978). The active site exhibits high specificity for glucose-1-P (Graves & Wang, 1972) as substrate since the glucosyl moiety is exclusively bound by the R conformation of the enzyme. Earlier studies have suggested that the T conformation is equally

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[1] Abbreviations: glucose-1-P, α-D-glucose 1-phosphate; P_i, orthophosphate; DTT, dithiothreitol; PLP, pyridoxal phosphate; Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-5364$01.25/0

specific in its recognition of α-D-glucose as an inhibitor. β-D-Glucose, for example, was shown to be noninhibitory (Cori & Cori, 1940), although more recent research casts doubt on this finding (Ariki & Fukui, 1977).

In this report, we examine in some detail the structure of the catalytic site of glucose-inhibited phosphorylase *a*. The earlier analysis of the structure at 2.5-Å resolution (Sprang & Fletterick, 1979) has been extended with X-ray diffraction data to 2.1 Å and partially refined atomic coordinates. Difference Fourier analysis directly reveals the binding mode of α-D-glucose within the parent enzyme, the high specificity of the interaction, and its contribution to the stability of the T conformation. The inhibition constants of several glucose analogues have been determined, and the relative efficacies of the inhibitors are rationalized in terms of specific interactions within the active site. These studies provide a structural basis for the observed specificity of T-state phosphorylase for α-D-glucose and also suggest how specificity is appropriately modified for recognition of glucose-1-P and a possible catalytic intermediate, in the R-state enzyme. A detailed analysis of the structural changes that occur upon activation of the enzyme with a good substrate analogue is described in the following paper (Withers et al., 1982).

Materials and Methods

Crystallographic Analysis. Parent crystals of rabbit muscle phosphorylase *a* were grown in the presence of 50 mM glucose as previously described (Fletterick et al., 1976). "Native" phosphorylase *a* crystals are prepared by allowing glucose to diffuse out of the parent crystals by twice soaking the crystals for 3 h at 22 °C in the standard buffer (10 mM Bes, 10 mM magnesium acetate, 1 mM DTT, and 1 mM EDTA, pH 6.8). The native data were measured with a Syntex $P2_1$ diffractometer by using methods previously described (Fletterick et al., 1976). X-ray data for 14000 unique reflections (of about 26000 observable to 2.5-Å resolution) were measured from 14 crystals. These represent the most intense 60% of the observable reflections. The merging *R* factor

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

is 3.8%, where I_{hkl} is the mean intensity for the reflection 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 previously collected data set. The latter are measured at the onset of data collection for one crystal and the conclusion for the succeeding crystal. The *R* factor is a measure of the errors from the usual instrumental and experimental sources including radiation damage to the crystals, which is more severe in the absence of glucose. The correction for radiation damage never exceeded 30%.

The model for glucose was fit into a difference electron density map prepared by using Fourier coefficients $F_{parent} - F_{native}$. Crystallographic phases were calculated from the current atomic coordinates for phosphorylase *a*. These were originally derived from a 2.5-Å resolution X-ray diffraction analysis of the glucose-inhibited (T-state) structure (Sprang & Fletterick, 1979) and have subsequently been refined with 2.1-Å resolution data to an *R* factor of 0.30. The atomic coordinates for glucose were taken from the neutron diffraction analysis by Brown & Levi (1979). Active-site coordinates for glucose were derived from the model structure by a rigid body rotation–translation that gave the best fit to the appropriate difference electron density.

The binding mode of several glucose analogues with phosphorylase *a* was approximated by a model-building procedure. The atomic coordinates for each analogue were translated and rotated as a rigid group so as to minimize the least-squares deviation from the corresponding atoms of α-D-glucose bound at active site. Only the fragment of the analogue known to be conformationally and chirally equivalent to α-D-glucose was included in the comparison. Atomic coordinates for the following analogues were derived from crystallographic determinations: α-D-galactose (Sheldrick, 1976), α-D-mannose (Longchambon et al., 1976), glucuronic acid (DeLucas et al., 1975), β-D-glucose (Arnott & Scott, 1972), and D-glucono-(1,5)lactone (Hackert & Jacobson, 1971). It was assumed that the conformations exhibited in the crystal structures would obtain in the putative complexes. Where steric conflicts were observed between the parent phosphorylase coordinates and the analogue model, no attempt was made either to adjust the conformation of ligand or protein or to move the position of the ligand from that determined from the least-squares procedure.

The electron density fitting procedure was aided by a computer graphics program written by O. Jones, and Figures 1–3 were generated with the graphics program MIDS implemented at the UCSF Computer Graphics Laboratory.

Molecular Surface Calculation. The three-dimensional envelope described by integrating over all possible paths of a water molecule as it "rolls" over the van der Waals surface of another molecule is referred to as a solvent-accessible "molecular surface". For a macromolecule in which constituent atoms are not closely packed, molecular surfaces can be defined by interior cavities as well as the external boundary. This concept has been defined algorithmically by Lee & Richards (1971), and a program to compute and display the image of such a surface on an Evans & Sutherland Picture System II computer graphics terminal has been designed and written by M. Connolly (unpublished results). The molecular surface for the catalytic site of phosphorylase *a* has been computed from the coordinates described above. A similar calculation was carried out for the coordinates of α-D-glucose bound at the catalytic site. The superposition of the two surfaces (Figure 5) approximates the juxtaposition of van der Waals surfaces of protein and ligand within the complex.

Kinetic Studies. Buffer chemicals and most glucose analogues were obtained from Sigma Chemical Co. 2-Fluoroglucose was obtained from Calbiochem and DTT from Bio-Rad Laboratories, and D-glucal was a kind gift from Dr. R. U. Lemieux. All glucose analogues were examined for the presence of contaminating glucose by paper chromatographic analysis using the system described previously (Withers et al., 1981). Less than 1% glucose contaminant was observed in each case.

A Radiometer PHM 62 pH meter was used for all pH measurements. Rabbit muscle phosphorylase *b* was prepared by the method of Fischer & Krebs (1962), using DTT instead of cysteine, and was 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.

Initial reaction rates were determined by the Fiske–Subbarow phosphate analysis in the direction of saccharide synthesis as described by Engers et al. (1970). Reaction mixtures

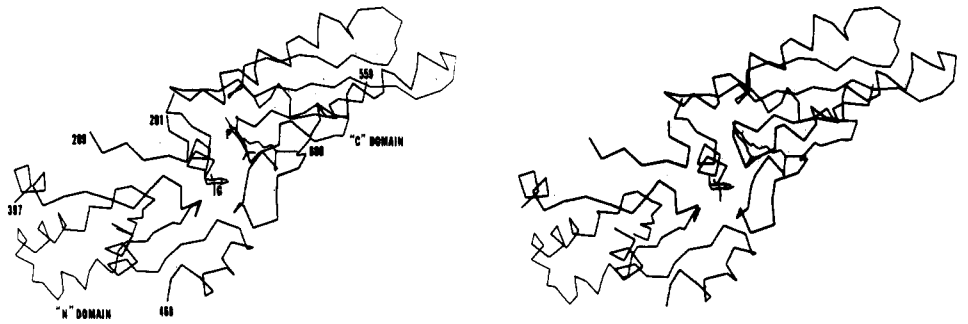

FIGURE 1: Trace of the α-carbon backbone of the two fragments of the N- and C-terminal domains of phosphorylase *a* which converge upon the catalytic site. The positions of the covalently bound pyridoxal phosphate coenzyme (P) and the molecule of α-D-glucose (G) bound to crystals of the parent enzyme are shown.

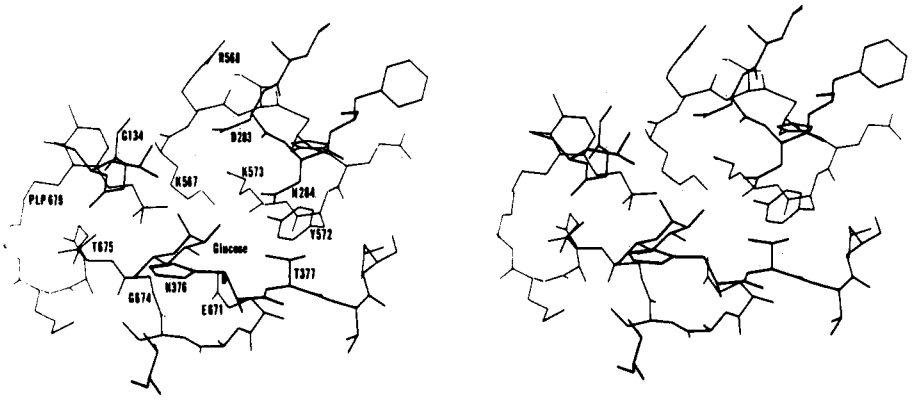

FIGURE 2: Residues surrounding the active-site tunnel of the glucose-inhibited enzyme. The view is approximately orthogonal to that shown in Figure 1.

were 0.2 or 0.5 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. An initial estimate of the K_i value for each compound was obtained by measuring the rates at a fixed concentration of glucose 1-phosphate (4 mM with phosphorylase *b* and 3 mM with phosphorylase *a*) and concentrations of analogue from 0 to 100 mM. Those analogues showing no inhibition at a concentration of 100 mM were not studied further and were entered in the results as $\gg$100 mM. Those showing an apparent inhibition constant less than 100 mM were subjected to a full kinetic analysis to determine the K_i value. K_i values for phosphorylase *b* were determined with 1 mM AMP, 1.0–20 mM glucose-1-P, 1% glycogen, and 3–7 μg of enzyme per reaction mix by using concentrations of inhibitor ranging from approximately 0.2 to 5 mM. Phosphorylase *a* was assayed similarly, in the absence of AMP, by using 1–3 μg of enzyme and including 0.1 mg of BSA/reaction.

Results

Description of the Glucose Binding (Catalytic) Site. The catalytic site of phosphorylase is nested between the cores of the two domains that form the enzyme (Figure 1). Each domain is constructed around a central sheet of parallel β strands, typical of the class described by Richardson as parallel doubly wound β sheet (Richardson, 1980). The two domains meet such that the C termini of the β sheets are in direct opposition. Individual residues lining the catalytic site are attached to loops of polypeptide chain that extend from one of the β strands and retreat into the domain structure, in most cases forming α helices. Constituent polypeptide chains of the active site, especially those from the C-terminal domain, arise from interior β strands.

The catalytic site is distinguished by the high density of ionizable and polar residues. There are 14 ionizable groups within 10 Å of the glucose molecule. These are R138, D283, D338, H340, H376, E381, H458, K567, R568, H570, K573, E663, E671, and the pyridoxal phosphate. With the caveat that the protonation states of the active-site histidine residues are not known and that detailed calculations of the electrostatic potential within the cavity have not been carried out, we note that in the immediate environment of the glucose there is a slight excess of negatively charged to positively charged side chains. Five of the charged residues may be involved in salt linkages, viz., R769/E381 and R568/D283 or K573/D283 as suggested by their mutual proximity. It is possible that the latter three residues are involved in a hydrogen-bonded or salt-linked triad; however, their relative disposition in the present atomic model does not offer a favorable geometry for such an interaction. The R769/E381 pair is exposed at the surface of the molecule, while the other three residues are found within the active-site cavity (Figure 2). Table I lists the intra- and interdomain nonbonded contacts involving ionizable or polar residues in the immediate vicinity of the glucose binding site. The majority of these occur within domains, the only significant exception being the charge interactions involving D283 and E381. Nearly all of the catalytic site residues are inaccessible to solvent; this is true without exception for main-chain atoms. Side groups with significant solvent accessible surface either are exposed near the exterior of the molecule (D283, E381, E571, and R769) or lie adjacent to a cavity in the neighborhood of the active site (H340, E671, and the phosphate group of the pyridoxal phosphate coenzyme).

The environment of the pyridoxal phosphate comprises both hydrophobic and hydrophilic residues (Figure 3). The phosphate moiety is buried deep within the cavity but surrounded by polar or ionizable side chains. K573, E663, E671, and T675 are less than 5 Å away. The pyridoxal ring is in a fairly hydrophobic neighborhood, in the proximity of Y90,

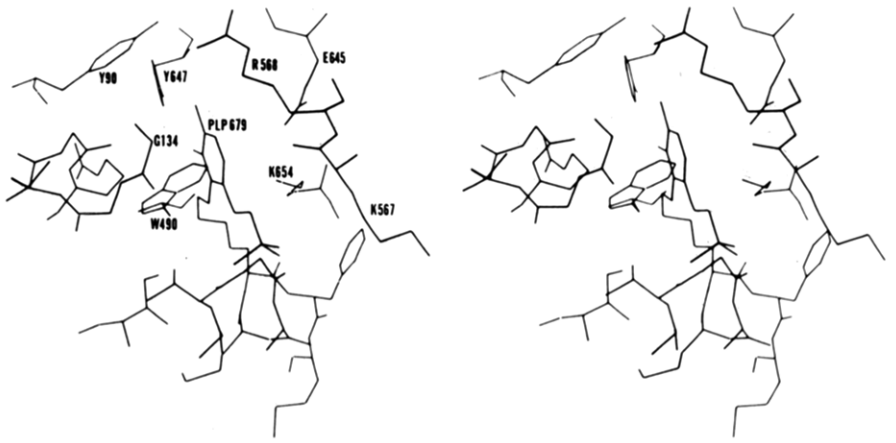

FIGURE 3: Environment of the covalently bound pyridoxal phosphate coenzyme. The view taken is similar to that of Figure 1 at the α-D-glucose binding site.

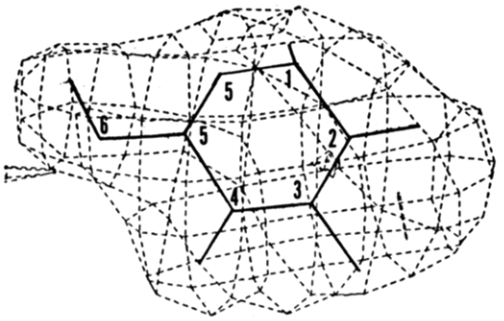

FIGURE 4: Molecule of glucose fitted to the positive difference electron density contour at two standard deviations above background is shown for the difference map calculated with coefficients $F_{parent} - F_{native}$ and phases calculated from the atomic model of the parent structure.

G134, W490, and Y647, although the E645 Oε2 and K654 Nζ atoms are within 5 Å of the pyridoxal ring nitrogen, N1, and the enolic O3 substituent, respectively. The phosphate substituent of the pyridoxal group does not appear to be tightly bound in the T-state enzyme. There are two possible hydrogen bonds from the backbone amide nitrogens of T675 and G676 to two of the phosphate oxygen atoms. A possible charge pair interaction may exist with K573, although the contact distance is greater than would be expected (Table I).

The electron density for the pyridoxal group in the $2F_{obsd} - F_{calcd}$ map based on model phases and 2.1-Å resolution data (Sprang & Fletterick, unpublished) is most consistent with a planar trans conformation about the C–N bond linking the pyridoxal group to K679. This observation confirms the well-established spectroscopic data (Shaltiel & Cortijo, 1970; P. J. Kasvinsky, unpublished results), indicating a Schiff base linkage of the coenzyme to the protein.

Table I: Nonbonded Interactions between Polar and Ionizable Amino Acid Residues within the Active Site[a]

residue	atom	residue	atom	distance (Å)
		Intradomain Contacts[b]		
N284	Oδ1	T377	Oγ	3.1
H376	Nε2	D338	Oδ2	3.8
K567	Nζ	K573	O	2.5
R568	Nη2	A608	O	3.6
H570	Nδ1	Y612	Oζ	3.9
K573	Nζ	PLP679	OP2	4.5
E645	Oε2	PLP679	N1	4.3
K654	Nζ	PLP679	O3′	4.9
T675	N	PLP679	OP1	2.8
G676	N	PLP679	OP1	3.0
		Interdomain Contacts		
N133	Nδ2	R568	Nη1	3.3
G134	O	PLP679	N1	3.6
D283	Oδ1	R568	Nη1	4.0
D283	Oδ2	K573	Nζ	3.2
D284	Nδ2	Y572	Oη	2.8

[a] With exception of contacts involving the PLP, only contacts less than 4.0 Å are listed. [b] With exception of contacts involving the PLP, contacts between residues separated by fewer than four residues in sequence are ignored.

Interaction of Glucose with Phosphorylase. A model of α-D-glucose in the $^4C_1$ chair conformation (Brown & Levi, 1979) can be fit into the electron density without ambiguity (Figure 4). The glucose molecule is clearly observed to adopt a *gt* conformation about the C5–C6 bond, consistent with the hexose conformers observed in a variety of crystal structure

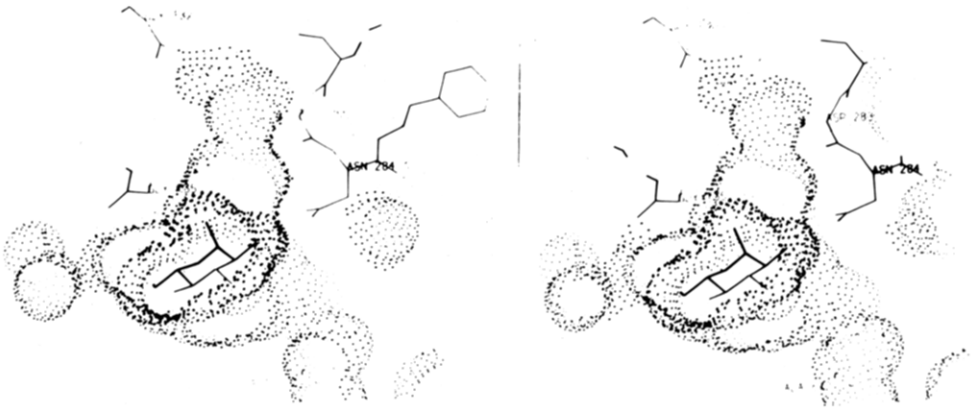

FIGURE 5: van der Waals molecular surface of glucose-inhibited phosphorylase *a* (with glucose coordinates excluded) superimposed on that of α-D-glucose.

Table II: Interactions of α-D-Glucose with T-State Phosphorylase

Hydrogen Bond Interactions			
glucose atom	protein atom	distance (Å)	angle about glucose oxygen (deg)
O2	N284 Nδ	3.0	130
O3	solvent		
O4	G674 N	2.5	180
O5	H376 Nε1	2.6	
O6	H376 Nδ1	2.8	107
	N483 Nδ	3.0	120

van der Waals Contacts[a]			
glucose atom	protein[b] residue(s)	glucose atom	protein[b] residue(s)
O1	G135	O4	T675
C1	H376, T377	C4	G674, A672
O2	T377	C5	H376
C2	T377	O6	L139, V454
O3	G674	C6	L139, H376, V454

[a] Less than 3.5 Å. [b] Residues already involved in hydrogen bond with the glucose atom not included.

analyses (Sundaralingam, 1968).

Glucose is totally enclosed within its binding site. A calculation of the solvent-accessible surface area of the bound glucose molecule reveals it to be completely buried by the enzyme (Figure 5). Removal of glucose from the phosphorylase *a* coordinate set results in an increase in the accessible surface area of binding site residues by 140 Å^2, equivalent to about five solvent water molecules. It is noteworthy that in nearly all of the residues for which there are significant accessibility changes, the only atoms substantially buried by the interaction are the potential hydrogen bond donors or acceptors. The only exceptions are the Cα atom of G135 and the Cβ atom of L136. These residues are contained in a strand of polypeptide that forms hydrophobic van der Waals contacts with the glucose molecule and leads to a short α-helical segment.

Glucose participates in a network of hydrogen bond interactions involving several amino acid residues within the loops of polypeptide chains surrounding the active site. These contacts appear to stabilize the T-state geometry within the catalytic "tunnel" (Figure 5) and strengthen the interdomain contact. The hydrogen bond assignments listed in Tables I and II are based on the observed distances between the putative donor and acceptor atoms as well as the angles formed by donor, acceptor, and an adjacent, covalently bonded atom. Likely hydrogen bonds involve donor–acceptor distances of 2.5–3.0 Å and C–O···O angles within the range of 100–130°. These observations of self-consistent group interactions and their attendant geometries serve to indicate the quality, as well as the limitations of the atomic coordinates derived from the electron density interpretation.

Two hydrogen bond networks are observed (Figure 6). The Nδ atom of N284 interacts with the 2-hydroxyl of glucose and, by inference from the kinetic data with analogue compounds (see below), appears to act as a donor. The former is linked, presumably as a hydrogen acceptor, to Oγ of T377. An interdomain contact appears as a hydrogen bond between Oδ of N284 and the η-hydroxyl group of Y572. A second network involves the 6-hydroxymethyl glucose oxygen and the adjacent ring oxygen at the 5 position. These form a donor/acceptor tetrad with Nδ1 on H376 which is donor to the glucose O5 atom and acceptor from the glucose O6. The latter receives a hydrogen bond from the Nδ atom of N483 (Figure 6). Although the 3-hydroxyl of glucose is too distant from the

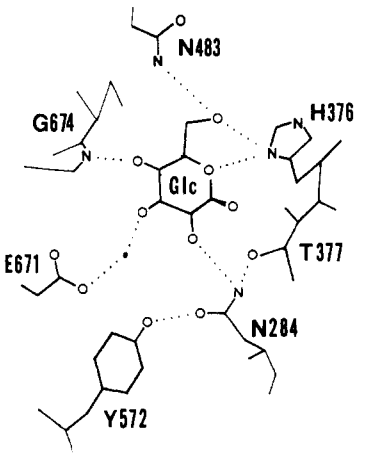

FIGURE 6: Schematic diagram of the hydrogen bond network involving α-D-glucose and the phosphorylase *a* amino acid residues.

carboxylate of E671 to form a hydrogen bond, there is crystallographic evidence that the two groups are linked by an intervening water molecule. While the solvent is not clearly visible in the parent electron density map, its movement upon introduction of a substrate analogue into the catalytic site is clearly evident in the difference electron density map of the binding of a substrate analogue (Withers et al., 1982). The anomeric oxygen, O1, does not appear to hydrogen bond with the enzyme.

Structural Changes on Glucose Binding to Native Phosphorylase a. There are few changes in the protein structure on removing glucose from the crystals. Paired peaks of positive and negative electron density near H376 may be interpreted as a movement of that residue toward the glucose position. A positive peak is observed near the position taken by the glucose 4-hydroxyl group which might result from reordering of solvent. The general absence of structural change is consistent with the 2.5% change in scattering amplitude we observe on removing glucose from these crystals. There is no change in lattice constants. The allosteric effects manifested by glucose in vitro are not observed in the crystal structure.

Inhibition Studies. A series of analogues of glucose have been tested as inhibitors of both phosphorylase *a* and phosphorylase *b* (Table III). Similar studies have been carried out in the past (Ariki & Fukui, 1977), but the present work extends the list considerably. The analogues differ from α-D-glucose in chirality (viz., mannose and galactose), substitution at certain ring carbon atoms (viz., 2-fluoroglucose, 3-aminoglucose, and glucuronic acid), unsaturation at a C–C bond (D-glucal), or number of carbon atoms (D-xylose). All the glucose analogues that were inhibitory exhibited nonlinear competitive inhibition for both phosphorylase *a* and phosphorylase *b* in the same manner as glucose itself. Each inhibitor was tested at several concentrations for each substrate concentration, the data placed on a Hill plot, and the apparent K_i values determined from a replot of the apparent K_m's. We did not determine K_i precisely for compounds that showed no significant inhibition above 100 mM since inhibition at such concentrations or greater could result from nonspecific effects on the enzyme. In general, the analogues showed similar K_i's for both phosphorylase *a* and phosphorylase *b*, as does glucose itself (K_i = 2 mM).

The results of these studies are summarized in Table III. With the exception of 3-aminoglucose and D-glucal, analogues substituted at any but the 2 position had no significant inhibitory activity ($K_i \gg 100$ mM). On the other hand, modification at the 2 position yielded a range in K_i values over 2

Table III: Inhibition Constants and Model-Building Results for Glucose Analogues

compound[a]	modification	K_i (mM) Phos[b] *b* + AMP	K_i (mM) Phos *a*	model building: H-bond loss	model building: steric conflict
glucose		2	2		
mannose	O2 axial	⩾100	⩾100	N284 Nδ	T377
2-deoxyglucose		27	25	N284 Nδ	
2-fluoroglucose		1.9	1.3		
2-aminoglucose		10	5		
D-glucal	1,2 unsat	80		N284 Nδ	H376
3-aminoglucose		90			
3-*O*-methylglucose		⩾100		H_2O	E671
galactose	O4 axial	⩾100		G674 N	A672
xylose	6-H	⩾100			
6-deoxyglucose	6-CH_3	⩾100		N483 Nδ, H376	
glucuronic acid	6-carboxylate	⩾100			V454
fucose	4 and 6 posn	⩾100			
phosphorylated analogues					
mannose-1-P		35	6.6		
1-P-glucuronate			15		
phenyl-P		19	10		

[a] All analogues are α-D. [b] Phos, phosphorylase.

orders of magnitude (in order of inhibitory activity, glucose = 2-fluoroglucose > 2-aminoglucose > 2-deoxyglucose ≫ mannose). D-Glucal, unsaturated at the 1–2 bond, exhibits measurable, though low, inhibition as does 3-aminoglucose.

The possible binding modes of several glucose analogues to T-state phosphorylase *a* were investigated by computer graphics assisted molecular model building. The results of this study (Table III) show that it is possible to rationalize in stereochemical terms the reduction in inhibitory activity of the analogues relative to α-D-glucose. Bound at the glucose site in a position and orientation similar to that of glucose itself, the analogues exhibit either steric conflicts or loss of hydrogen bonding with the enzyme. Analogues that differ only in chiralty from α-D-glucose, mannose (O2 axial) and galactose (O4 axial), exhibit both effects. The equatorial configuration of O1 in β-D-glucose leads to a steric clash as does methylation of the 3-hydroxyl. Replacement of the methylene hydroxyl at the 6 position by a carboxyl group necessitates a *gg* conformation about the 5–6 bond (DeLucas et al., 1975) and consequently a steric conflict with the binding site. None of the above compounds exhibit significant inhibitory properties. Reduction in inhibitory power appears to parallel loss in hydrogen-bonding capability. Whereas 2-deoxyglucose (one hydrogen bond lost; Table III) is partially effective as an inhibitor, 6-deoxyglucose (two lost) is inactive.

We have also investigated the inhibitory activity of several phosphorylated compounds as competitive inhibitors of glucose-1-P, the physiological substrate of phosphorylase (Graves & Wang, 1972). All of the analogues (Table III) proved to be inhibitory with similar values of K_i. We find that the inhibitory activity of the phosphorylated analogues does not reflect the inhibition constants of their dephosphorylated counterparts, none of which are effective inhibitors in the manner of α-D-glucose. The phosphorylated analogues all exhibit lower inhibition constants for phosphorylase *a* than for phosphorylase *b*, indicating tighter binding to the activated enzyme form of the enzyme. We have been unable to demonstrate crystallographically the binding of either xylose 1-phosphate or phenyl phosphate to crystalline phosphorylase *a* even at high concentrations (100 mM) or in the presence of substrate activator maltopentaose.

Discussion

Active-Site Environment. The active site of phosphorylase repeats a structural motif found in other enzymes of the glycolytic pathway (Branden, 1980; Richardson, 1980), namely, the confluence of two parallel β-sheet domains, edge to edge with the C termini of the respective sheets opposing. The structural and functional ramifications of such protein construction have been discussed by Branden (1980), and the structure of phosphorylase *a* illustrates two of these: a balance between stability and flexibility in active-site construction and the contribution of the domain structure itself to the binding of a negatively charged substrate. The former is achieved by attachment of active-site chain residues to loops of polypeptide chain which emanate from strands in the interior of the β sheet. Flexibility in the interdomain contact obtains from the inherent conformational freedom of the polypeptide loops which form the internal boundary of the active site. The stability of the supporting β strands arises from the network of hydrogen bonds which tie them into the interior of the parallel β sheet. The majority of polypeptide loops reenter their respective domains as α helices which, it has been suggested (Hol et al., 1978), provide oriented dipoles directed toward the active (interdomain) site. These may contribute to the favorable binding of negatively charged substrates; in the case of phosphorylase *a*, inorganic phosphate as well as the covalently bound coenzyme pyridoxal phosphate.

Nonbonded contacts between the domains across the catalytic site cavity are few, the outstanding example being a possible ion pair interaction involving D283 with R568 or K573. The role of these residues in the conformation changes that accompany binding of activators is discussed in the following paper (Withers et al., 1982). Glucose participates in hydrogen-bonded networks involving residues from both domains, thus stabilizing the interdomain contact of the T-state phosphorylase molecule, but it resides primarily in the N-terminal domain. Jeffrey & Takagi (1978) have pointed out the cooperative effects of hydrogen-bonded donor–acceptor chains in strengthening the individual interactions. Evidence for the stabilizing effects of glucose is also provided by the ability of glucose to protect the enzyme from iodoacetamide modification of a cysteine residue (C142) located near the domain interface and the active site (Withers et al., 1979). The same study shows, by ^{31}P NMR, that glucose promotes the phosphorus resonance (form 1) characteristic of T-state phosphorylase.

Active-Site Specificity. The active site of T-state phosphorylase is as specific in its recognition of the inhibitor glucose as is the R-state (catalytically active) enzyme in binding the

substrate glucose-1-P. Specificity requires overall complementarity between inhibitor and protein (Figure 5) and is achieved through a network of five or more precisely oriented hydrogen bonds (Figure 6). Several analogues differing from glucose in chirality or by substitution at one of the hydroxyl groups have been tested kinetically (Table III) and are shown to be inactive ($K_i \gg 100$ mM). These results can be rationalized stereochemically as a loss of complementarity which could result in steric conflicts between inhibitor and binding site residues (Table III).

Glucose is recognized by its ability to engage in a specific set of hydrogen bond interactions within the binding site. The loss of such capability can render an analogue partially or completely unable to bind. 6-Deoxyglucose makes two fewer hydrogen bonds than does glucose and accordingly has no significant inhibitory activity (Table III). 2-Deoxyglucose loses only one potential interaction and exhibits a 10-fold increase in K_i, equivalent to a 1–2 kcal/mol loss in binding energy. Two other ligands, 2-fluoroglucose and 2-aminoglucose, potentially offer to the binding site a molecular surface similar to that of α-D-glucose itself but differ in their hydrogen bonding and, consequently, their inhibitory properties. The fluoro substituent is potentially a strong hydrogen bond acceptor and is as inhibitory as glucose itself (Table III), suggesting that the hydroxyl group of the latter is also an acceptor (Figure 6). 2-Aminoglucose is 5-fold less active than glucose and is likely to be a better donor than acceptor. In that capacity it may act as donor to Oγ of T377 but in so doing would disrupt the hydrogen bond network interlacing Y572, N284, and T377 (Figure 6). Such subtle effects could explain the modest difference in inhibitory power with respect to glucose.

The sensitivity of the enzyme to the hexose ring conformation is demonstrated by the weak binding of D-glucal. This analogue is unsaturated at the 1–2 bond and, like 2-deoxyglucose, cannot participate in a hydrogen bond at the 2 position. However, while the latter is modestly inhibitory, D-glucal is relatively ineffective. Absence of hydrogen-bonding potential at the 1 position should not be important since glucose itself makes no hydrogen bonds at this site. The low activity is probably related to the half-chair ring conformation resulting from the trigonal geometry about the 1 and 2 positions. A model building study with a conformationally related analogue, 1,5-gluconolactone (Hackert & Jacobson, 1971), suggests that the C1 substituent could be in steric conflict with H376. This finding is significant in that two different laboratories have suggested that a possible carbonium ion intermediate of the phosphorylase-catalyzed reaction would be expected to adopt a half-chair conformation (Gold et al., 1971; Tu et al., 1971). The latter should be readily accommodated by the binding site of the R-state enzyme. The suggestion for the stabilized carbonium ion transition intermediate arose from the two laboratories' interpretations of the complex inhibition patterns observed with 1,5-gluconolactone.

P. J. Kasvinsky (unpublished results) has demonstrated weak noncompetitive inhibition of phosphate binding in the presence of AMP for D-glucal, indicating that while it probably binds to R-state phosphorylase, D-glucal is a poorer transition state analogue than the lactone. Klein et al. (1981) have recently demonstrated that potato phosphorylase is able to convert D-glucal to 2-deoxyglucose in the presence of oligosaccharides. The reaction is known to proceed through a covalent intermediate, although the protein residues involved are not known.

These inhibition studies have shown that the glucosyl binding site has evolved a high degree of specificity for α-D-glucose. It is significant that the T-state enzyme exhibits the same selectivity for inhibitor analogues of glucose as does the R state for analogues of glucose-1-P. The two ligands bind at the catalytic site in nearly the same position and orientation. However, glucosyl recognition in the R-state enzyme differs in some significant respects from that in the T state. The most obvious is the substitution of the hydrogen bond between the 2-hydroxyl group of glucose and N284 of the T-state enzyme for an interaction, as yet poorly defined, with the 1-phosphate group of glucose-1-P in the R state [see the following paper (Withers et al., 1982)]. In addition, the latter can accommodate a half-chair conformation, represented by 1,5-gluconolactone and characteristic of a proposed (Graves & Wang, 1972; Gold et al., 1971; Tu et al., 1971) carbonium ion intermediate, while the T state cannot. This flexible, yet specific recognition of the glucosyl moiety by the catalytic site is achieved by the mode of molecular surface complementarity and the disposition of hydrogen bonds between ligand and protein. Although glucose-1-P binds with a slight rotation and translation with respect to glucose, crystallographic evidence (Withers et al., 1982) demonstrates that the tight complementarity at the 4–6 positions is maintained. Consequently, both active and inactive conformers recognize that portion of the glucosyl moiety that is least affected by phosphorylation at the 1 position or a possible chair/half-chair transition.

The phosphorylase active site actually contains two adjacent subsites which bind, respectively, the glucose moiety and orthophosphate (or the 1-phosphate group). Only a virtual phosphate binding site exists in the T-state enzyme because it is partially occupied by a residue of the protein itself, the negatively charged carboxyl group of D283. In the R-state enzyme, this residue is presumed to be disordered, and the binding site is accessible (Withers et al., 1982). We have studied several phosphorylated inhibitors that appear to be competitive with glucose-1-P. All of these are moderately inhibitory (Table III) with similar inhibition constants (K_i) for glucose-1-P. They differ greatly from one another and from glucose-1-P in the character of the moiety replacing the glucosyl group, the most extreme being phenyl phosphate. However, none of the corresponding glucose analogues alone are able to occupy the glucose subsite of the T-state enzyme. The pattern of inhibition we observe is thus not consistent with binding of the phosphorylated analogues into both subsites of the glucose-1-P pocket. Rather, it appears that the phosphate group of the analogue is bound at the phosphate subsite while the glucose subsite remains empty. These analogues thus act as inhibitors by competing with glucose-1-P for the phosphate subsite. The fact that these inhibitors bind more tightly to the phosphorylase *a* than to the phosphorylase *b* enzyme simply reflects the higher ratio of R over T conformers in the phosphorylated enzyme. Less highly modified analogues of glucose-1-P such as 5-thioglucose 1-phosphate (Madsen et al., 1978) and α-D-glucose cyclic 1,2-phosphate (Withers et al., 1982) bind with full occupation of both glucose and phosphate subsites, as reflected in their smaller dissociation constants.

Acknowledgments

We thank Shirley Schechosky and Laura Glew for expert assistance with the kinetic analysis and for growing the crystals.

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