

OLIGOSACCHARIDE CONFORMATION AND PROTEIN SACCHARIDE INTERACTIONS IN SOLUTION

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Abstract - The interaction between glycogen and the enzyme phosphorylase has been determined by x-ray diffraction of the protein, complexed to a low-molecular weight analogue of an A-chain of glycogen, maltoheptaose. All of the glucans of the saccharide are observed, even though only four of the residues are in direct contact with the enzyme. The saccharide has a helical secondary structure arising from an O2-O3' hydrogen bonding interaction. The saccharide helix binds in a shallow groove between two α -helices at the protein surface in small sub-domain of 56 amino acids. This subdomain has an unusual $\beta\alpha_3\beta_2$ structure. The protein contacts include hydrogen bonding, hydrogen bond network formation, burial of protein salt bridges, and van der Waals interactions.

INTRODUCTION:

A growing body of evidence indicates that biological recognition and attachment processes commonly involve saccharide-protein complexes (Ref. 1). To understand the selectivity, and origin of the association energy, it is important to know the structure of the saccharides and also the structure and nature of the saccharide-protein interactions.

The determination of the primary structure of saccharides has become possible in recent years through improvements in NMR methods (Ref. 2) and chemical methods so that the primary structure of numerous oligosaccharides are now known. The secondary structure of oligosaccharides remains obscure, though NMR has provided some information about the torsional angles in solution (Ref. 3). Oligosaccharides do not form compact globular structures and often fail to crystallize so x-ray crystallography of single crystals is seldom useful in determining secondary structure. With the exception of cyclohexyl-amylose, the largest oligosaccharide to have been crystallized is a trimer (Ref. 4). Even for these, with the possibility that lattice forces determine conformation in crystals, doubts about the significance of these secondary structure determinations to conformation in solutions are well-founded. These observations have led to the general presumption that saccharides do not have extensive regions of definite secondary structure, i.e., definite 3-dimensional orientation from one saccharide unit to the next in long repeats. A corollary to the concept is that secondary structure should have little significance in the recognition of oligosaccharides with other molecules. The present work provides an example in which secondary structure is present and plays a role in a protein recognition process.

Even though the structure of oligosaccharides has been difficult to determine by x-ray crystallography, the structure of specific protein-saccharide interactions may be studied directly by protein crystallography where suitable crystals of the protein complex exist. To date, excepting lysozyme, there has been no high resolution study on a complex of an oligosaccharide and protein. Presently the protein structures available for such studies include the plant lectins wheat germ agglutinin and concanavalin A (Refs. 5,6), binding proteins for arabinose and galactose (Refs. 7,8) lysozyme and phosphorylase (Refs. 9,10) as well as the glycoproteins IgG, human influenza virus hemagglutinin and ribonuclease B (Refs. 11-13), for example.

Phosphorylase is of special interest because its substrate is a simple long-chain oligosaccharide of glucose, glycogen. This substrate binds both at the active site and at a remote surface allosteric site. We have determined the structure of small oligosaccharides as they bind to this allosteric site using difference Fourier techniques. This study presents two unique opportunities. First, the long-chain oligosaccharide is not bound over its entire length at the surface site, so that the secondary structure of the saccharide itself may be visualized, and the extent to which this secondary structure is modulated by the protein interaction may also be evaluated. Second, this surface site

is different from an enzymatic active site, and could possibly be more similar to the class of recognition proteins, where the protein binds to a saccharide on the surface of a larger particle.

We have reported previously on the structure of maltoheptaose as bound to the surface, allosteric or "storage" site of phosphorylase (Ref. 14). This work showed that the oligosaccharide forms an O2-O3' hydrogen bond between the α ,D-(1,4)-linked glucose units. This H-bond promotes a rigid, x-ray observable structure, even where it extends from the protein surface into the solvent filled space between protein molecules in the protein crystal lattice. Here we present structural information for the maltoheptaose-protein interactions derived from partial refinement of the maltoheptaose-phosphorylase complex at 2.5 Å resolution. The interactions observed at this site are compared with other saccharide-protein interactions both at the phosphorylase active site and in the other protein-saccharide complexes which have been studied.

METHODS

The structure of glycogen phosphorylase is available in a partially refined state at a resolution of 2.1 Å (Sprang & Fletterick, unpublished). There are atomic coordinates for most of the 6,800 non-hydrogen atoms of the 97,400 MW subunit. The errors in these coordinates are a few tenths of an angstrom except in rare cases where the protein chain requires significant rebuilding. All main chain and side chains which are interacting with the oligosaccharide both at the storage site and active site, have been checked and adjusted using a computer graphics program FRODO written by Alwyn Jones. The conformation of oligoglucose at the storage site was determined initially by standard difference Fourier synthesis. Crystals of glycogen phosphorylase a containing glucose and caffeine (to stabilize the conformation of the protein in its T state with its active site "closed") (Ref. 15) were soaked in a solution of 0.3 M maltoheptaose for 12 hours. X-ray data were collected to a resolution of 2.5 Å from 14 crystals using an automatic Nicolet Diffractometer. In total after merging, 12,600 of the strongest reflections were measured. The overall scaling and merging of these data gives a scaling R factor of 0.023 and an average difference in scattering amplitudes for the saccharide of 0.082 (Ref. 14).

An electron density difference map revealing the maltoheptaose attached to the protein was calculated using phases determined from the refined atomic coordinates and the coefficients ($F_{\text{malt}} - F_{\text{parent}}$). The electron density map so calculated was interpreted using a computer graphics system which allowed us to build a series of oligo- α ,D(1,4)-glucose helices encompassing a wide range of possible models as defined by the ϕ and ψ angles about the glycosidic link (Ref. 14). The sugar groups were constructed from the composite coordinates of Arnott & Scott (Ref. 16) as well as from the Brown & Levi analysis of α ,D-(1,4)-glucose (Ref. 17).

The structure of the saccharide was then refined using the same procedure as was applied to the parent data set. Constraints were applied to the bond lengths and angles on the saccharide. In early cycles, an added rigid constraint was applied to the O1-O4 distance (set to 4.34 Å) to preserve the sugar pucker. The angle of the glycosidic linkage was assumed to be 117°. The glycosidic torsion angles were allowed to vary freely in the refinement. Solvent accessible surface calculations were done using the Lee & Richards algorithm (Ref. 18) as programmed by T.J. Richmond. Backbone and surface illustrations were made as described previously (Ref. 14).

RESULTS

Figure 1 shows the position of the allosteric glycogen binding site relative to a schematic picture for the phosphorylase structure (Ref. 15). Maltoheptaose binds to the T-state phosphorylase a crystal structure in two segments at this locus in a major and minor site (Ref. 14).

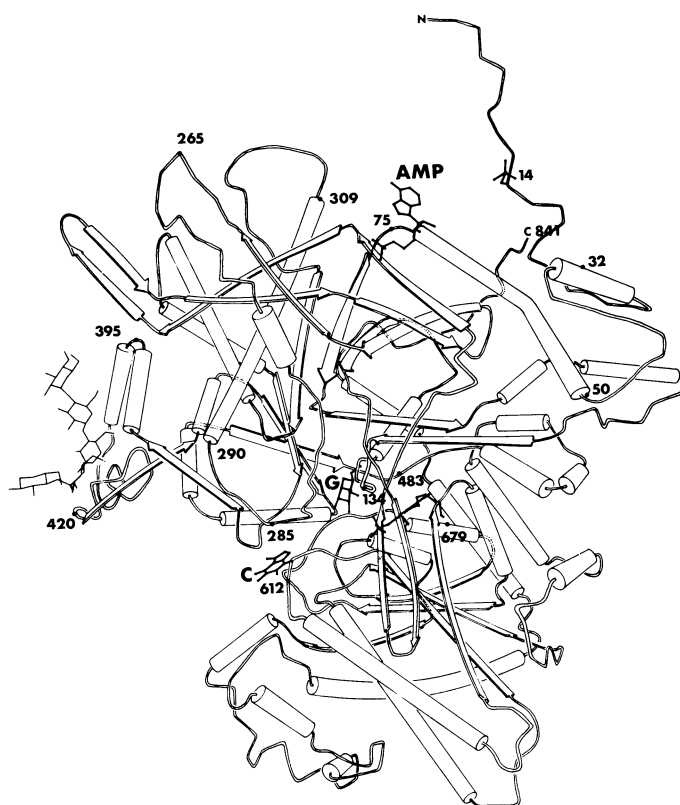


Fig. 1. Phosphorylase α monomer with saccharide binding and active site locations shown.

The Saccharide Structure

Figure 2 shows the original difference map for the major binding site found from the Fourier synthesis as described above. The map shows a continuous band of approximately left-handed helical electron density at the surface of the protein which accommodates seven sugar residues. The phases used in this calculation were based upon a partially refined parent structure with a conventional R of .30 (Ref. 14).



Fig. 2. Helical difference electron density for maltoheptaose bound to phosphorylase (from Ref. 14).

Further refinement of the parent structure and of the maltoheptaose protein complex has led to substantial improvement in the appearance of the electron density. A difference map using particularly refined phases for the complex proves much more revealing (Figure 3). This is an exceptionally clear view of the saccharide. For several of the glucan

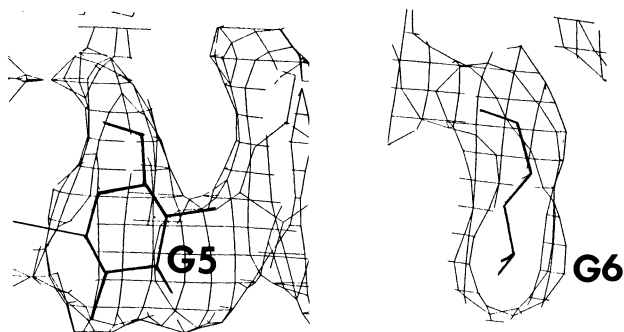


Fig. 3. Improved difference electron density after partial refinement of the phases for some of the residues, the position of each oxygen atom is observed.

units, the position of all the oxygen atoms is clearly indicated in the difference map (Figure 3). Partial refinement and modelling of the saccharide structure shows that the D conformation is maintained along the length of the oligosaccharide.

TABLE 1. Average conformational parameters of α ,D-(1,4) glucan linkages in maltoheptaose

Parameter	Value
ϕ, ψ	$(-15^\circ, -15^\circ)$
O2-O3' H-bond	2.8 Å
Angle C2-O2-O3'	114°
Angle O2-O3'-C3'	107°
Residues per turn	6.6
Rise per residue	-2.3 Å

The accuracy of the original analysis which showed that the structure is helical with a single break is born at the current stage of refinement. The helical parameters for the best average (ϕ, ψ) angles are presented in Table 1. Figure 4 shows a model of the best regular helix of Table 1 and a model of the observed structure.

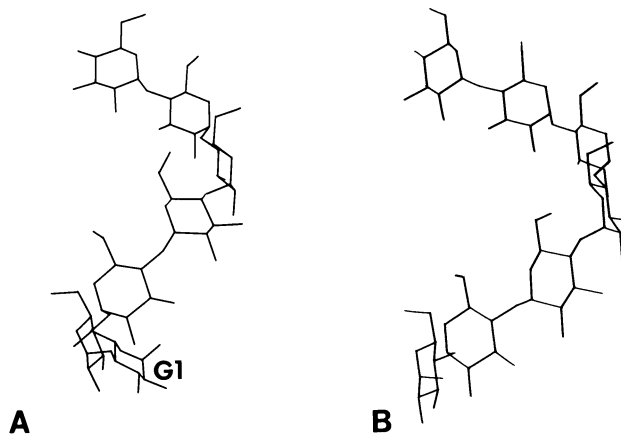


Fig. 4. Best fit to electron density from Ref. 14 (A) and best regular structure (B).

The conformation assumed by the saccharide units in all but one case show the individual glucose units linked by O2-03' hydrogen bonds. Comparison with average hydrogen bond angles in saccharide crystal structures suggests that the glucan pairs take up a conformation that will form a O2-03' hydrogen bond with the best possible geometry (that is, with a C2-02-03' and O2-03'-C3' bond angle of 105° - 120° , Ref. 19). This is consistent with the crystal structures of β -maltose (Ref. 20) and β -methyl maltopyranoside (Ref. 21) which also form this hydrogen-bond.

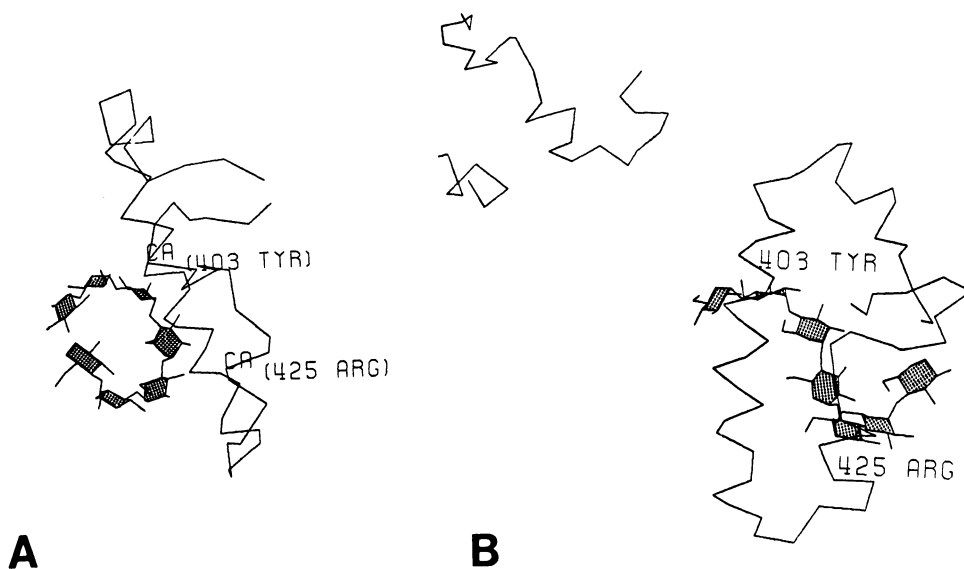


Fig. 5. Position of saccharide on protein α -carbon diagram. A. view down saccharide helix. B. side view of saccharide and protein helices; shows location of nearest protein molecule in lattice.

The Variability in Amount of Protein Contact

Probably the most outstanding and unusual feature of the difference Fourier is that glucan units are observed that are not bound to the protein directly. Figure 5 shows the positioning of the saccharide on the protein. Here the slight changes in the protein structure that occur in the phosphorylase-maltoheptaose complex have been taken into account. The protein backbone, in contact with saccharide, and the location of the

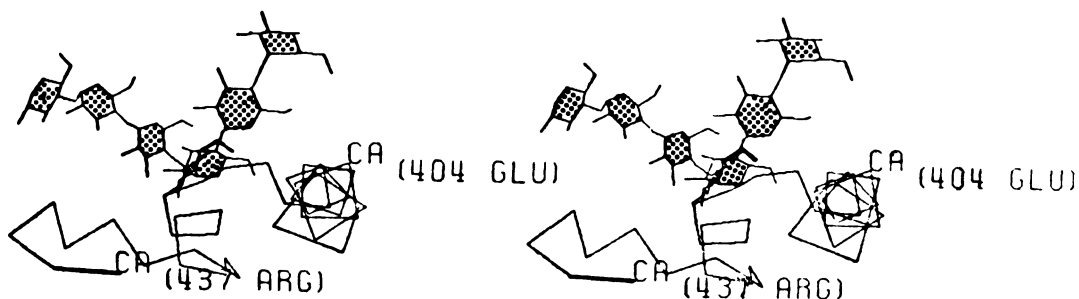


Fig. 6. Stereo view down the two major helices of the binding site shows how saccharide binds between the helices at its center and extends well away from the protein at its ends.

Table 3 summarizes the contacts formed by the saccharide with phosphorylase at the major storage site. Numerous protein-saccharide hydrogen bonds are formed. Various amino acid types capable of hydrogen bonding participate in the interaction: S428, N406, Q407, E432, and R425 and 437. Protein side chains form most of the hydrogen bonds, but the main-chain carbonyl of V430 also interacts. Note that the list of H-bond groups include charged residues as well as neutral groups.

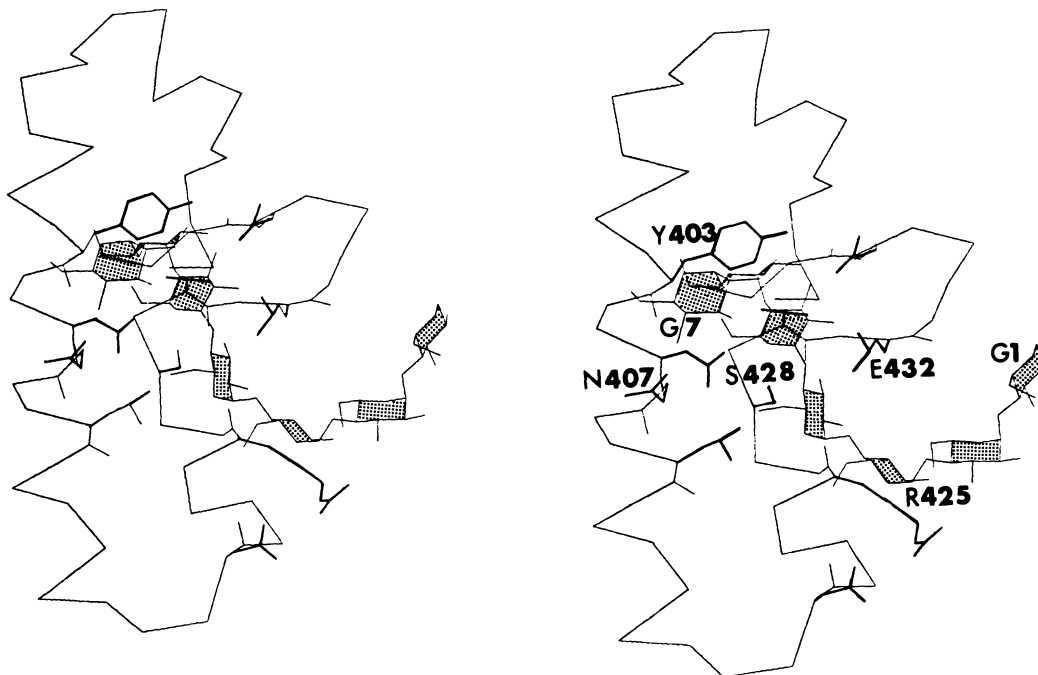


Fig. 7. Stereo view for entire interaction only side-chains in contact with saccharide are shown in heavy lines. Sugar residues are stippled.

Figure 7 shows a stereo view of the interaction. This shows that the hydrogen bonding interactions occur in clusters which are hydrogen bonding networks. The clusters are G2(O3)-R425(NE2)-D420(OG1), G4(O3)-S428(OG)-V430(O), E432(OE1)-G5(O2)-R437(NE1)-G5(O3), and G6(O6)-N406(ND)-Q407(NE). These clusters have in common a central oxygen or nitrogen atom which both donates and accepts a hydrogen bond. One of these, the G2-R425-D420 interaction involves a side-chain not directly in contact with saccharide.

The binding event also changes protein-protein interactions. Two ionic interactions or salt bridge between R425 and D420 and between R437 and E432 form when the saccharide binds. Similar patterns are observed at the minor site, which will be reported elsewhere.

Other Interactions in the Complex

Several hydrophobic interactions with good van der Waals contact occur on the outer surface of the saccharide helix and involve mainly aliphatic side-chains V430, L410, and the aliphatic portion of R425. The aromatic group Y403 participates even more significantly becoming buried by the saccharide on complex formation. Y403 moves by a rotation of 30° , about C α -C β when maltoheptaose binds.

Though these hydrophobic interactions are undoubtedly significant, overall, hydrogen bonding interactions predominate. This can be seen in two ways. First, Table 3 shows that the oxygen atoms form the most contacts. The calculation used to obtain the data on the surface area of the saccharide buried by the interaction (presented in Table 2) also gives the buried surface for each atom. This tabulation shows that the set of

oxygen atoms have the greatest change in solvent accessibility being about twice that of the carbon atoms.

The contacts between individual glucan units and the protein are summarized in Table 2. One of these, G5, is almost entirely buried by the interaction. It also has all of its free hydroxyls involved in hydrogen bonds, and is the only glucose unit to have that feature.

Interaction of Other Oligosaccharides at Storage Site

The interaction of saccharides with the storage site on phosphorylase has been studied in the past using a series of oligoglucose molecules including glucose, maltose, maltotriose, maltopentaose, and maltoheptaose (Ref. 15). Even though glucose does not bind at a concentration of 1 M, the approximate binding affinity, measured kinetically, for maltoheptaose and maltopentaose in solution is of the order of 1 mM. On the basis of occupancy from x-ray diffraction experiments, we would anticipate that the affinity for maltose or maltotriose is also in the range 1-10 mM. Previous work from binding maltose, and maltotriose showed that the preferred sites for these smaller oligosaccharides are G4 and G5 for maltose and G3, G4, G5 and G6 for maltotriose. In this case four sites are seen for the three glucose groups because the trisaccharide binds equally to subsite positions 3-4-5 and 4-5-6.

DISCUSSION

Conformation of Glucose Oligosaccharides in Solution

Obviously, our experiment of the binding of maltoheptaose to phosphorylase is not a direct view of a glucose oligosaccharide free in solution. However, two major features of the conformation which maltoheptaose displays when bound to phosphorylase suggests that glucose oligosaccharides have a longer-range order, and more restricted conformation in solution than previously thought (Ref. 22). The first observation is that glucose units are well-ordered in the structure, even where they cannot be directly stabilized by protein contacts. The second observation is that all the conformations we observe, with a single exception, retain the O2-O3' hydrogen bond whether or not they are to the protein. Therefore, we might expect the hydrogen bond to have some long term stability in solution also. This hydrogen bond is observed in small molecule crystal structures, but with a slightly different geometry (Ref. 14). In these small molecular structures, the O2-O3' hydrogen bond is stronger than one might expect from the structure of glucose (Ref. 15) and the (ϕ, ψ) pairs for those structures (Figure 4 of Ref. 14), because the sugar pucker of the individual glucans adjusts itself slightly to improve the hydrogen-bond geometry. It is of interest also that the observed conformation is very similar to that predicted as a minimum in the rather shallow hard-sphere potential for α, D (1-4) linked glucans (Refs. 22, 23). The presence of the O2-O3' hydrogen bond for glucans not bound to protein might seem surprising in view of the fact that the molecule is completely solvated. However, it may be that the loss in rotational entropy due to the internal hydrogen bond, where only 2 degrees of freedom must be frozen, is less than the loss in rotational and translational entropy of the solvent. This situation is very different from the α -helix in proteins, where six rotational degrees of freedom must be lost in forming the first hydrogen bond, and the helix formation is cooperative, i.e., forms more easily for longer helices (Ref. 24).

The Exoanomeric Contribution

The exoanomeric affect has been found to be the major determinant of conformation (Ref. 25) in cases where other orienting factors are not available, in particular for a methyl group at the O1 position on a sugar. The ϕ angle is always $\sim 65^\circ$. We note that the (ϕ, ψ) pairs observed here do not (for 4 of 5 cases) coincide with predictions of the exoanomeric effect ($\psi = -65, -40$) for α, D -(1,4)-linked glucans, rather are 40° rotated away in the direction that shortens the O2-O3' distance (the transformation from the helical convention used in Table 1 to the ϕ convention of Ref. 25 is $+132^\circ$). Our results here show that the intramolecular hydrogen-bond is a dominating factor, both for the solution structure of the oligosaccharide and in its interaction with protein. However, where this hydrogen-bond is broken at G4-G5, the resulting conformation, even in the presence of other orienting interactions from protein side chains is closer to the predictions of the anomeric effect, with $\phi = -40, \psi = -35$, which gives a ϕ (Ref. 25) = 80° .

Protein Recognition of Secondary Structure

The question then arises whether this secondary structure is sufficiently energetically favorable that the protein is designed to recognize it. Of the 3 (ϕ, ψ) pairs, we observe (for the 4 glucans bound to protein) 2 (ϕ, ψ) pairs are the same as observed where at least one of glucans has no protein contacts. Thus the protein is organized to 'recognize' this unperturbed conformation to some extent. For the most strongly interacting

pair (G4-G5), however, the conformation is very different and the hydrogen bond is broken. Here the protein provides a set of alternative interactions in the form of a salt-bridge triad of bonds for one glucan OH and a simple hydrogen bonding network for the other OH (see Fig. 7). The collection of all the saccharide conformations we observe have an analogy in (ϕ, ψ) plots for proteins: some conformations are favored, but a much broader spectrum of conformation is available to accommodate other influences in the environment.

One feature of the design of this allosteric site is that it binds oligomers of glucose, including maltose, but not glucose itself. This is a physiologically necessary feature of the enzyme, because it must shut down when glucose is in great supply. Structurally, the lack of glucose binding stems from the fact that only one glucan binding site (G5) saturates the hydrogen bonding capacity of the sugar. At this site, conformational changes occur on the protein (Y403). The necessity of these conformational changes could prohibit the interaction with glucose alone. This situation is very different from that at the active site where five of the six oxygen atoms on glucose are hydrogen bonded.

Other Protein-Saccharide Interactions

Figure 8 is a schematic of the interactions of glucose in the active site of phosphorylase (Ref. 26). A number of features similar to the storage site can be noted. First, there is a predominance of hydrogen bonding interactions. Second, the hydrogen bonding

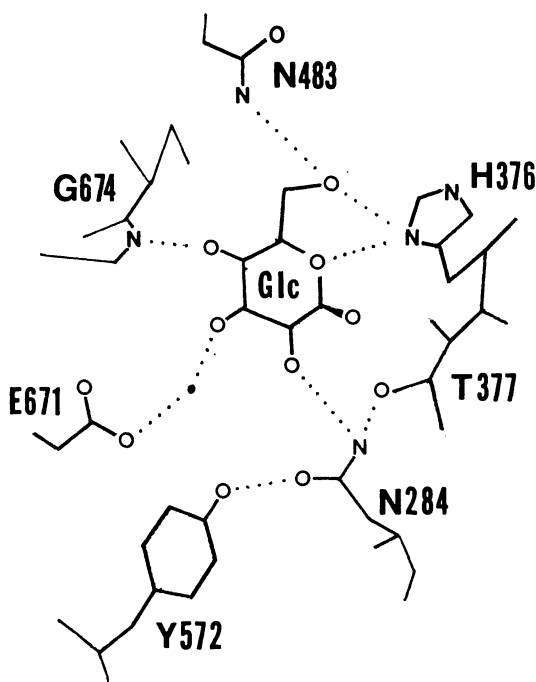


Fig. 8. Glucose interactions at active site (Ref. 26). Hydrogen bonds are marked by dashed lines, and a water molecule is represented by a dot.

patterns involve network formation, and in one case, involve a charged residue. In this case however, no salt bridges are present. The present structure has been thoroughly refined in this region of the structure. One important conclusion is that the protein-saccharide hydrogen bonds all have excellent geometry by the criterion discussed above.

TABLE 4. Summary of protein-saccharide binding sites

Protein	Ligand	Solvation	Locus	Back- bone	Polar	Charged	Non- Polar	Kd M
ABP	L-arabinose	Buried	Inter- domain		N423 N405	K10 E14 D30	W16	3×10^{-7}
GBP	D-galactose	Buried	Inter- domain		N91 N211	D14 K11	F233	3×10^{-7}
HXX	α ,D-glucose	Partially buried	Inter- domain		T277 N188 N245 N188 N215	D189		1×10^{-3}
WGA	diNAG	Partially buried	Inter- subunit	64NH 110NH	Y71	E111	A60 Y71	1×10^{-5}
<u>Pa</u>	α ,D-glucose	Buried	Inter- domain	674NH	N284 N483	H376 E571	A672 V454 L139	3×10^{-3}
<u>Pa</u>	Maltoheptose	Slightly buried	Surface	430CO	S428 N407 Q408	R425 R437 E432	L410 V430 Y403	1×10^{-3}
LYZ	NAM-NAG-NAM	Partially buried	Cleft	107CO 57CO 109NH	N46 N59 N103 W63	D52 D101 E35	W108 V109	3×10^{-3}

Table 4 lists the protein-saccharide sites that have been studied to date. Despite the fact that the overall shape of these sites, and the topology of the protein making up the sites is different, certain definite patterns emerge on how saccharide binding sites are made. The binding site includes van der Waals contacts with an aromatic side-chain, hydrogen bonding with polar side-chains, especially asparagine, and hydrogen bonding to charged side-chains. The group of aromatic saccharide interactions is interesting because the contact always involves only one or at most two saccharide carbon atoms. The group of interactions with charged side-chains shows a greater variation. Wheat germ agglutinin (Ref. 5), lysozyme (Ref. 9), and phosphorylase active site use aspartic or glutamic acids with non-compensated charges to form an interaction, whereas ABD (Ref. 7), GBP (Ref. 8), and the phosphorylase storage sites bury salt bridges in forming the interaction.

Hydrogen Bonding Contributions

One of the main conclusions from analysis of the saccharide binding sites on phosphorylase is that hydrogen bonding is a predominant type of interaction. Ross & Subramanian (Refs. 27,28) have pointed out that localized hydrogen bonds, as in a protein, or in a protein ligand interaction can be enthalpically more favorable than a protein-solvent or ligand solvent H-bond by about 1 kcal. Further, Jeffrey (Ref. 18) has shown that hydrogen bonding networks can have shorter hydrogen bonds for central oxygen atom by about 0.1 Å. This suggests that hydrogen bonds in networks are stronger bonds. We noted in the study of glucose binding to phosphorylase that the protein-glucose interaction involves networks of hydrogen bonds. This is undoubtedly a feature of saccharide recognition, and may also be a means of modulating the binding energy.

In the storage site, several charged side chains are prominent in the interaction. Two at this site form complimentary pairs (Ref. 24) showed that formation at salt bridges on a protein helix can give a negative free energy, and Ferscht (Ref. 29) estimated $\Delta G = -2$ to -3 kcal/mole for formation of salt bridges in chymotrypsin. The significance of buried salt bridges in recognition and binding strength is not yet clear but it is an interesting possibility that part of the ligand binding energy can arise from the formation of new intraprotein interactions.

Relative Importance of Hydrophobic, Van der Waals and Hydrogen Bonding

What interactions dominate the energetics of protein-saccharide interactions? The proposal of Janin & Chothia (Refs. 30,31) that the hydrophobic effect, or solvent disordering effect, plays a dominant role in protein-protein interactions and protein-ligand interactions has gained wide acceptance. However, in a few cases, the necessary thermodynamic measurements to distinguish a hydrophobic-dominated interaction ($\Delta S > 0$, $\Delta H \approx 0$) from an attraction-dominated process ($\Delta S < 0$, $\Delta H < 0$) now have been carried out (Refs. 27,28). Although the hydrophobic effect does dominate at some temperatures for a few protein-protein associations, it has not yet been shown to be significant in a protein ligand interaction. In a study on the phosphorylase binding site for caffeine, where a hydrophobic effect certainly might be expected, the thermodynamic measurements showed $\Delta H < 0$, $\Delta S < 0$, indicating that even in this completely hydrophobic binding site, the attractive forces of van der Waals interactions by stacking, and dipole-dipole interactions are the most significant (Ref. 32).

For the saccharide binding, the necessary thermodynamic measurements have been carried out for lysozyme (Ref. 33), where attraction-dominated ($\Delta S < 0$, $\Delta H < 0$) process is observed. We do not have measurements for the phosphorylase-maltoheptaose interaction. However, we note that the storage site is predominantly a hydrophilic site and that the majority of protein-saccharide interactions are hydrogen-bonding interactions (Table 3). These interactions also involve the favorable process of network formation. Further, we have found from studies with small oligosaccharides, maltose and maltotriose, that the G4, G5 subsites are strongest binding sites. These sites form the most hydrogen bonds (Table 3) and have the greatest proportion of hydrophilic atoms buried in the interaction.

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