Hydrogen Bonding and Specificity. Fluorodeoxy Sugars as Probes of Hydrogen Bonding in the Glycogen Phosphorylase–Glucose Complex†

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ABSTRACT: The affinities of a large number of deoxy and fluorodeoxy sugars for the glucose binding site in glycogen phosphorylase have been measured, and polarities and relative strengths of the hydrogen bonds at each position have been predicted on the basis of these data. Comparison with the recently refined X-ray crystal structure of the phosphorylase–glucose complex shows a generally good correlation between predicted and observed bond strengths, vindicating this approach to the evaluation of hydrogen bonding. Estimates of the net contributions of hydrogen bonds of different types (neutral–neutral and neutral–charged) are essentially identical with those obtained by a complementary approach on the tyrosyl tRNA synthetase–substrate complex [Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Cortes, P., Waye, M. M. Y., & Winter, G. (1985) *Nature (London) 314*, 235–238]. The carbohydrate binding site structure determined is compared with that recently determined for the arabinose binding protein.

The interactions between a ligand and its protein receptor form the basis of biological specificity and are responsible for, amongst others, the remarkable selectivities exhibited by most enzymes for their substrates and by antibodies for their antigens. The nature of the forces responsible for such interactions are fairly well documented, but the relative contributions of each to both the overall binding interaction and to the specificity thereof are very poorly understood. The present dogma on this subject is that hydrophobic interactions contribute the major driving force for association and that hydrogen bonding interactions and salt-bridge formation primarily provide the specificity (Fersht, 1984), with relatively little direct contribution to the interaction energy. However, relatively few studies have provided clear information on this topic, though a recent elegant study (Fersht et al., 1985) has permitted a quantification of the contribution of specific hydrogen bonds to a ligand/protein interaction. This study, on a protein–ligand complex of known three-dimensional structure, involved the systematic removal of side chains involved in hydrogen-bonding interactions with the substrate through site-specific mutagenesis and measurement of the affinity of the ligand for each mutant so generated. Contributions of individual hydrogen bonds ranged from 0.5 to 4.5 kcal/mol.

Carbohydrate/protein interactions have not attracted such detailed attention to data but are no less important in light of their central role in cell–cell recognition, for example. In considering their large number of hydroxyl groups, it might be anticipated that hydrogen-bonding interactions would be relatively more important in these cases, particularly for completely buried monosaccharides. The most useful information on ligand–protein interactions has usually derived from X-ray crystallographic studies of ligand–protein complexes. This is no less true for carbohydrate/protein interactions, but unfortunately relatively few such structures have been determined at the necessary resolution. Structures currently available, at differing degrees of resolution, include concanavalin A (Becker et al., 1975), wheat germ agglutinin (Wright, 1980), the enzymes lysozyme (Kelly et al., 1979) and glycogen phosphorylase (Sprang et al., 1982), and binding proteins for galactose (Vyas et al., 1983; Mowbray & Petsko, 1983) and arabinose (Quiocho & Vyas, 1985). The last of these, a 1.7-Å refined structure is undoubtedly the best in terms of providing insight into specific hydrogen-bond interactions. Indeed, ten significant hydrogen-bonding interactions between the protein and arabinose are observed, an average of two per oxygen atom.

The complementary approach to that applied by Fersht in probing hydrogen-bond involvement would involve specific modifications of the ligand rather than the protein. Binding studies with carefully selected analogues should provide essentially identical information, and such studies can be and have been applied previously to carbohydrate–protein systems. The deoxy and fluorodeoxy analogues have proved the most useful in such studies primarily due to their sterically conservative nature. Substitution by fluorine is particularly interesting in light of both its electronegativity and the similarity in size of the substituent to that of the hydroxyl, precluding any binding of water in the empty space present but not necessarily filled, for deoxy sugars. In addition fluorine cannot possibly act as a hydrogen-bond donor but can indeed act as an acceptor, albeit weakly. A variety of such studies has been conducted, involving hexokinase (Bessell et al., 1972; Machado de Domenech & Sols, 1980), galactokinase (Thomas et al., 1974), antibody receptor fragments (Ittah & Glaudemans, 1981), a sugar transport protein (Rees & Holman, 1981) and several other systems (Penglis, 1981). However, none of these studies has involved the necessary combination of systematic measurements of affinity of deoxy and fluorodeoxy analogues compared with refined X-ray crystal structure data on the carbohydrate–protein complex. We have taken this combined approach, using glycogen phosphorylase as our system.

Glycogen phosphorylase catalyzes the reversible phosphorolysis of glycogen to produce glucose 1-phosphate. It is an allosteric enzyme whose inactive (T-state) conformation is stabilized by glucose and whose active (R-state) conformation by substrates and activators. Detailed kinetic studies have been performed on both phosphorylase *a* and phosphorylase *b*

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(Graves & Wang, 1972; Madsen & Withers, 1986) delineating their kinetic mechanisms and inhibition patterns. In addition the X-ray crystal structures of both phosphorylase *a* (Sprang & Fletterick, 1979; Sprang et al., 1982; Withers et al., 1982) and *b* (Johnson et al., 1980) have been determined to reasonable resolution (2.1 and 2.0 Å, respectively), the former being that of the T-state phosphorylase *a*–glucose complex. This therefore provides an ideal system for such a study since not only are good structural data available and kinetic studies relatively simple but also the information obtained in both these ways refers to interactions occurring within a ground-state (noncatalytic) enzyme–inhibitor complex. It does not rely upon comparisons of ground-state structural data with transition-state kinetic data. In addition it provides the opportunity to explore oligosaccharide/protein interactions at the glycogen storage site, and some studies on this have already been reported (Goldsmith et al., 1982; Goldsmith & Fletterick, 1983).

This paper provides binding data for a large series of deoxy and fluorodeoxy sugars and an interpretation of these data in terms of hydrogen-bonding interactions observed in the recently refined X-ray crystal structure. It therefore provides an evaluation of the validity of use of such analogues to probe the role of hydrogen bonding in ligand/protein interactions as well as an evaluation of the importance of hydrogen bonding in the generation of biological specificity.

Materials and Methods

Syntheses. Syntheses of 3-deoxy-3-fluoro-, 4-deoxy-4-fluoro-, and 6-deoxy-6-fluoro-D-glucose have been described elsewhere (Withers et al., 1986b). Other syntheses were performed according to the literature procedures wherever possible, and these compounds and their appropriate references are listed below. All compounds had melting points and NMR[1] data consistent with the literature values where available: β-D-glucopyranosyl fluoride, (Helferich & Gootz, 1929); α-D-mannopyranosyl fluoride (Hyashi et al., 1984); 2-deoxy-2-fluoro-D-glucose, 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride, 2-deoxy-2-fluoro-β-D-mannosyl fluoride, and 2-deoxy-2-fluoro-D-mannose (Adamson et al., 1970); 2-deoxy-2-fluoro-β-D-glucosyl fluoride and 2-deoxy-2-fluoro-α-D-mannosyl fluoride (Hall et al., 1971); β-D-glucopyranosylamine (Cusack et al., 1973); 2-deoxy-1,5-anhydroglucitol (1,2-dideoxyglucose) (Fisher, 1914). The remaining deoxy sugars were prepared as follows.

1,5-Anhydroglucitol (1-Deoxyglucose). A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (1.42 g, 3.4 mmol) and 12.7 mg of α,α′-azobisisobutyronitrile were dissolved in 10 mL of dry toluene and allowed to stir under an atmosphere of dry nitrogen. To this solution was added tributyltin hydride (1.7 g, 5.8 mmol) and the reaction temperature maintained at 80 °C for 2 h. After the solution cooled to room temperature the solvent was removed in vacuo and the remaining gum dissolved in acetonitrile, any remaining tributyltin removed by washing with *n*-pentane and evaporated again to produce 1.05 g of a white crystalline solid. The solid was redissolved in 10 mL of 0.1 M sodium methoxide and left to stir at room temperature for 30 min. The reaction was neutralized by addition of Dowex 50 W-X8(H^+) resin and filtered and the solvent removed in vacuo. The final product was recrystallized from ethanol: yield, 0.372 g (68%); mp 140–141 °C [lit. mp 142–143 °C (Ness et al., 1950)].

Methyl 2,3,6-Tri-O-benzoyl-4-deoxy-4-chloro-α-D-glucopyranoside. Methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside (10 g, 20 mmol) was dissolved in 50 mL of anhydrous pyridine and cooled to 0 °C. Sulfuryl chloride (3.5 g, 26 mmol) was added in a dropwise fashion to the reaction and then the mixture allowed to stir at room temperature for 18 h. The reaction was quenched by addition to a large volume of iced water and the product extracted into methylene chloride. The organic phase was washed with 1 M hydrochloric acid, followed by water, and dried with sodium sulfate. The product crystallized on removal of the solvent and recrystallized from hot ethanol: yield, 8.72 g (85%); mp 136–138 °C; ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 8.12–7.31 (m, 15 H), 6.08 (t, 1 H, J = 8.0 Hz), 5.24–5.20 (m, 2 H), 4.82–4.69 (m, 2 H), 4.35 (m, 1 H), 4.22 (t, 1 H).

Methyl 2,3,6-Tri-O-benzoyl-4-deoxy-α-D-glucopyranoside. Methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-4-chloro-α-D-glucopyranoside (7.0 g, 13.3 mmol) and α,α′-azobisisobutyronitrile were dissolved in dry toluene (100 mL) and placed under an atmosphere of dry nitrogen. Tributyltin hydride (4.7 g, 16 mmol) was then added and the reaction temperature maintained at 80 °C for 24 h. After the solution cooled and the solvent was removed in vacuo, the resulting gum crystallized on trituration with *n*-pentane. Recrystallization from ether/*n*-pentane gave the title compound: yield, 5.48 g (84%); mp 119–120 °C; ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 8.12–7.31 (m, 15 H), 5.80 (dt, 1 H, J = 5.3, 11.2 Hz), 5.33 (dt, 1 H, J = 3.5, 10.1 Hz), 5.19 (d, 1 H, J = 3.8 Hz), 4.52–4.37 (m, 3 H), 3.47 (s, 3 H), 2.50 (m, 1 H), 1.93 (m, 1 H).

Methyl 4-Deoxy-α-D-glucopyranoside. Methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-α-D-glucopyranoside (5.0 g, 10.2 mmol) was suspended in 35 mL of 0.1 M sodium methoxide in methanol and left to stir at ambient temperature overnight. The reaction was neutralized by addition of Dowex 50W-X8(H^+) ion-exchange resin and filtered and the solvent removed in vacuo. The oily product was purified by flash chromatography using 90% ethyl acetate/10% methanol. The pure product crystallized from ethanol/ether mixtures: yield, 1.27 g (70%); mp 90–92 °C [lit. mp = 90–91 °C (Lopes et al., 1979)]; ^1H NMR (D_2O) δ 4.81 (d, 1 H, J = 4.0 Hz), 3.88 (m, 2 H), 3.64 (dd, 1 H, J = 4.0, 11.6 Hz), 3.54 (dd, 1 H, J = 6.0, 12.0 Hz), 3.46 (dd, 1 H, J = 4.0, 9.0 Hz), 3.30 (s, 3 H), 1.94 (ddd, 1 H, J = 2.0, 3.5, 12.0 Hz), 1.40 (q, 1 H, J = 12.0 Hz).

4-Deoxy-D-glucopyranose. Methyl 4-deoxy-α-D-glucopyranoside (1.2 g, 6.7 mmol) was dissolved in 100 mL of water, Dowex 50W-X8(H^+) added and the mixture heated to a gentle reflux. After 24 h the reaction mixture was cooled and filtered and the solvent removed in vacuo to give a colorless gum: yield, 1.0 g (91%); ^1H NMR (D_2O) for an anomeric ratio of 35% α, δ 5.24 (d, 1 H(α), J = 3.8 Hz), 4.55 (d, 1 H(β), J = 8.0 Hz), 4.05 (m, 1 H(α)), 3.94 (m, 1 H(α)), 3.76–3.55 (m, 5 H(β) + 2 H(α)), 3.44 (dd, 1 H (α), J = 4.0, 9.5 Hz), 3.13 (t, 1 H(β), J = 8.5 Hz), 1.96 (m, 1 H(α) + 1 H (β)), 1.42 (m, 1 H(α) + 1 H(β)).

1,2:5,6-Di-O-isopropylidene-3-deoxy-3-chloro-α-D-glucofuranose. 1,2:5,6-Di-*O*-isopropylidene-α-D-allofuranose (10 g, 38 mmol) was dissolved in 60 mL of anhydrous pyridine cooled in an ice bath and sulfuryl chloride (6.7 g, 50 mmol) added over a period of 15 min. The reaction mixture was then allowed to warm to room temperature and left to stir for 18 h. The reaction was again cooled to 0 °C and quenched by careful addition of 2 mL of water. The pyridine was removed in vacuo and the remaining oil dissolved in methylene chloride,

[1] Abbreviations: DAST, (diethylamino)sulfur trifluoride; DMF, dimethylformamide; DTT, dithiothreitol; NMR, nuclear magnetic resonance.

washed several times with water, and dried with sodium sulfate. After removal of the solvent the product mixture was purified by flash chromatography using a solvent system of 90% methylene chloride/10% ethyl acetate. Fractions containing the major product were pooled, and the solvent was removed to give the desired product as a pale yellow oil: yield, 5.8 g (55%); ^{1}H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 5.93 (d, 1 H, J = 3.5 Hz), 4.71 (d, 1 H, J = 3.5 Hz), 4.40 (d, 1 H, J = 2.0 Hz), 4.36–4.30 (m, 1 H), 4.21–4.10 (m, 2 H), 4.05 (m, 1 H), 1.50 (s, 3 H), 1.41 (s, 3 H), 1.35 (s, 3 H), 1.31 (s, 3 H).

1,2,4,6-Tetra-O-acetyl-3-deoxy-3-chloro-D-glucopyranose. 1,2:5,6-Di-*O*-isopropylidene-3-deoxy-3-chloro-α-D-glucofuranose (2.5 g, 9.1 mmol) was dissolved in 100 mL of 30% v/v aqueous ethanol, Dowex 50W-X8(H^+) ion-exchange resin added and the mixture left stirring at room temperature for 7 days. After this time the reaction mixture was filtered, the solvent removed in vacuo, and the resultant gum dried over phosphorus pentoxide for 24 h. This gum was dissolved in 17 mL of anhydrous pyridine and cooled in an ice bath and 10 mL of acetic anhydride added slowly. After a further 72 h at room temperature, pyridine was removed in vacuo and the remaining oil added to a stirred solution of saturated sodium bicarbonate. The product was extracted into methylene chloride and the organic phase washed with both 1 M HCl and water and dried with sodium sulfate. After removal of the solvent, the product was obtained as a colorless gum: yield, 3.07 g (92%); ^{1}H NMR [$CDCl_3$, $(CH_3)_4Si$] for an anomeric ratio of 50% α/50% β, δ 6.30 (d, 1 H, J = 3.5 Hz), 6.15 (d, 1 H, J = 8.0 Hz), 5.3–5.1 (m, 4 H), 4.27 (m, 1 H), 4.23–4.20 (m, 2 H), 4.12 (m, 1 H), 4.09–3.98 (m, 3 H), 3.78 (m, 1 H), 2.16–2.09 (6 × s, 24 H).

1,2,4,6-Tetra-O-acetyl-3-deoxy-D-glucopyranose. 1,2,4,6-Tetra-*O*-acetyl-3-deoxy-3-chloro-D-glucopyranose (2.0 g, 5.5 mmol) and 32 mg of α,α'-azobisisobutyronitrile were dissolved in 30 mL of dry toluene. The solution was placed under an atmosphere of nitrogen, tributyltin hydride (1.9 g, 6.5 mmol) added, and the reaction maintained at 80 °C for 20 h. After the reaction was complete the solvent was removed in vacuo and the product dissolved in acetonitrile. This solution was extracted four times with *n*-pentane to remove any remaining tributyltin. After the acetonitrile had been removed in vacuo, the product was recovered as a colorless gum: yield, 1.83 g (100%); ^{1}H NMR [$CDCl_3$, $(CH_3)_4Si$] for an anomeric mixture containing 50% α/50% β, δ 6.21 (d, 1 H, J = 3.2 Hz), 5.64 (d, 1 H, J = 8.0 Hz), 5.00 (m, 2 H), 4.88 (m, 3 H), 4.20 (m, 4 H), 3.89 (m, 1 H), 3.81 (m, 1 H), 2.64 (m, 1 H), 2.35 (m, 1 H), 2.20–1.98 (5 × s, 24 H).

3-Deoxy-D-glucose. 1,2,4,6-Tetra-*O*-acetyl-3-deoxy-D-glucopyranose (500 mg, 1.5 mmol) was dissolved in 5 mL of 0.1 M sodium methoxide in methanol and left to stir at room temperature for 30 min. After neutralization with Dowex 50-W-X8(H^+) the mixture was filtered and the solvent removed in vacuo to give a colorless gum: yield, 237 mg (96%).

Enzyme Studies. Rabbit muscle phosphorylase *b* was prepared by the method of Fischer and Krebs (1962) using DTT instead of cysteine and recrystallized at least three times before use. Rabbit liver glycogen (type III) purchased from Sigma Chemical Co. was purified on a Dowex 1-Cl column and assayed by the method of Dishe (Ashwell, 1957). All other buffer chemicals and substrates were obtained from Sigma Chemical Co. except for DTT, which was obtained from Bio-Rad Laboratories.

Initial reaction rates were determined by the Fiske–Subbarow phosphate analysis in the direction of saccharide synthesis as described by Engers et al. (1970). The buffer employed for all kinetic studies contained 50 mM triethanolamine hydrochloride, 100 mM KCl, 1 mM EDTA, and 1 mM DTT, pH 6.8. Reaction mixtures were 0.2 or 0.5 mL containing 1 mM AMP and 1% glycogen, and studies were performed at pH 6.8, 30 °C. Inhibitors were first tested in a "range-finding" experiment wherein a range of inhibitor concentrations (typically 0 → 250 mM) was tested at a fixed (4 mM) glucose-1-P concentration. An estimate of K_i could be obtained from the slope ($K_m/K_i([S] + K_m)$) of a plot of $v_{uninhibited}/v_{inhibited}$ against [I]. Those inhibitors showing a K_i value greater than 100 mM were not tested further and are quoted at approximate ($\approx K_i$) values since inhibition at these concentrations could result from nonspecific binding. Such numbers therefore represent an approximate minimum estimate of K_i. Inhibitors of $K_i < 100$ mM were subjected to a full kinetic analysis at five different inhibitor and five different substrate concentrations and data analyzed by means of a Hill plot, K_i values being determined from a replot of the apparent K_m values. Inhibition by α- and β-D-glucose was quantitated by using solutions of α- and β-D-glucose made immediately before each experiment to minimize anomerization of each sample. Inhibition was assayed at a series of concentrations of each inhibitor but at a fixed (4 mM) concentration of glucose 1-phosphate, by using 2-min reaction times, such that all assays were completed within 6 min of dissolution of the glucose. Resultant data were plotted according to Dixon.

Table I

no.	name	K_i (mM)	anomeric (% α)[c] composition
1[a]	D-glucose	2.0	36
2	1-deoxy-D-glucose	10.7	
3[a]	α-D-glucopyranosyl fluoride	0.6	100
4	β-D-glucopyranosyl fluoride	3.8	0
5	β-D-glucosylamine	10	0
6[a]	2-deoxy-D-glucose	27	47
7[a]	2-fluoro-2-deoxy-D-glucose	1.9	45
8	2-fluoro-α-D-glucosyl fluoride	0.2	100
9	2-fluoro-β-D-glucosyl fluoride	1.6	0
10	2-fluoro-α-D-mannosyl fluoride	75	100
11	2-fluoro-β-D-mannosyl fluoride	$\gg$100	0
12	D-mannose	$\gg$100	67
13	α-D-mannosyl fluoride	$\approx$225	100
14	2-fluoro-2-deoxy-D-mannose	$\approx$90	68
15	1,2-dideoxy-D-glucose	$\approx$600	
16	3-fluoro-3-deoxy-D-glucose	$\approx$200	45
17	4-fluoro-4-deoxy-D-glucose	25	41
18	6-fluoro-6-deoxy-D-glucose	$\approx$90	44
19[b]	3-deoxy-D-glucose	$\gg$100	25
20	4-deoxy-D-glucose	$\gg$100	34
21[a]	6-deoxy-D-glucose	$\gg$100	45

[a] Data taken from Sprang et al. (1982). [b] This compound is also present in its furanose forms (23%) (Pfeffer et al., 1980), and was therefore tested for inhibition at much higher concentrations. [c] Data from Phillips and Wray (1971) or this paper.

Results and Discussion

All analogues were tested as inhibitors of glycogen phosphorylase *b* at constant glycogen and variable glucose 1-phosphate concentrations. No such measurements were made with phosphorylase *a* since it had been shown previously (Sprang et al., 1982) that T-state inhibitors have similar affinities for both phosphorylase *a* and *b*. Inhibition constants determined for the deoxy and fluorodeoxy analogues are presented in Table I, along with their anomeric composition. All analogues that were found to be inhibitory exhibited nonlinear competitive kinetics in the same manner as glucose itself. In order to interpret these data an understanding of hydrogen-bonding energetics in aqueous solution and of the

hydrogen-bonding capabilities of fluorine is required, as follows.

Hydrogen-Bonding Energetics in Water. Hydrogen bonding between ligands and proteins in aqueous solution is a complex process due to the competition of water for the hydrogen-bonding sites on each species (Klotz & Franzen, 1962; Jencks, 1964). In order to understand the role of hydrogen bonding in the energetics of such interactions a full hydrogen-bond inventory must be performed (Fersht, 1984; Fersht et al., 1985), as formulated previously (Jencks, 1969; Hines, 1972). Thus the hydrogen bonding present at a single site in a ligand–protein association in an aqueous medium can be expressed as in eq 1, where the enzyme E has a hydrogen-bond

$$E\text{–}H\cdots OH_2 + HOH\cdots A\text{–}L \leftrightarrow [E\text{–}H\cdots A\text{–}L] + HOH\cdots OH_2 \quad (1)$$

donor H which pairs with the acceptor A of the ligand L in the complex. Hydrogen-bonding roles could, of course, be reversed. Thus since there are two hydrogen bonds on each side, both numbers and types of hydrogen bonds are conserved in the process, though hydrogen-bond geometries may be quite different. The binding energy expressed therefore results partially from enthalpic differences due to different hydrogen-bond geometries but mainly from the increase in entropy associated with water release from the active site and the ligand into bulk water.

Removal of a hydrogen-bonding group from the ligand L need not lead to the loss of the full energy of the hydrogen bond since in that case no hydrogen bond would exist between the substrate analogue and water before binding, as shown in eq 2. Thus any energetic difference between two systems

$$E\text{–}H\cdots OH_2 + HOH + L \leftrightarrow [EH\ L] + HOH\cdots OH_2 \quad (2)$$

described by eq 1 and 2 will not be equal to the full hydrogen-bond energy but to the difference in strengths of hydrogen bonds formed with the protein and with water. This could, however, be quite significant when comparing a hydrogen bond in an evolved enzyme–ligand complex with that formed between a relatively mobile water molecule and a protein residue.

The two cases to be considered in this paper involve deoxygenation (OH to H) and fluorination (OH to F). The former is equivalent to the types of mutations performed by Fersht et al. (1985) and will result in complete removal of the hydrogen bond, leaving a space in the complex that might accommodate a water molecule but is probably too small. The latter substitution (OH to F) results in very little size difference, certainly not permitting coincident binding of water, but is more complex since the fluorine could be involved in a hydrogen bond but only as a hydrogen bond acceptor (vide infra).

Thus two situations could obtain for the fluoro sugar as shown below, where in one case (eq 3) the enzyme donates a hydrogen bond at that position, which the fluorine can (weakly) accept. In the other case (eq 4) the enzyme accepts

$$E\text{–}H\cdots OH_2 + HOH\cdots F\text{–}L \leftrightarrow [E\text{–}H\cdots F\text{–}L] + H\text{–}OH\cdots OH_2 \quad (3)$$

$$E\text{–}A\cdots H\text{–}O\text{–}H + HOH\cdots F\text{–}L \leftrightarrow [E\text{–}A\ F\text{–}L] + HOH\cdots OH_2 \quad (4)$$

a hydrogen bond at that position and thus cannot interact favorably with the fluorine. Since the fluorine can still hydrogen bond as acceptor with the water, there is net loss of a hydrogen bond in this case, and considerable affinity decrease should result. In such a situation the deoxy sugar might have greater affinity than the fluoro sugar.

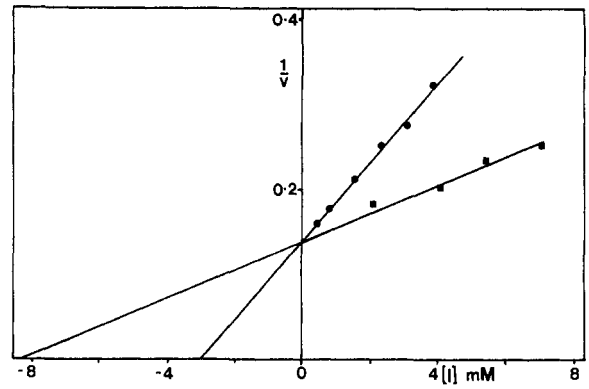

FIGURE 1: Dixon plot of inhibition of glycogen phosphorylase *b* by α-D-glucose (●) and β-D-glucose (■), determined at saturating (1%) glycogen and 4 mM glucose 1-phosphate concentrations by using 2-min reactions. All kinetic studies were completed within 6 min of dissolution of the inhibitor. The slope of each plot = $K_m/V_{max}\cdot K_i\cdot[S]$. Therefore, since K_m, V_{max}, and [S] are identical in each case, the slopes are inversely proportional to the K_i values.

Hydrogen Bonding to Fluorine. The involvement of fluorine in hydrogen bonding is well documented and in fact some of the strongest known hydrogen bonds occur in fluorine-containing systems (Sheppard et al., 1969; Pauling, 1980). However, the involvement of fluorine attached to carbon is a matter of debate. Its high electronegativity favors bonding of this type, but since the electrons to be shared are held tightly to the fluorine nucleus, resultant hydrogen bonds will be weak. Documentation of the process in solution is somewhat sparse but is available as in, for example, the relatively high boiling point of difluoromethane, which has been attributed (Sheppard et al., 1969) to intermolecular hydrogen-bonding interactions involving fluorine and also in the dimerization of 2,2,2-trifluoroethanol in the gas phase (Curtiss et al., 1978). Probably the best evidence arises from X-ray crystallographic data. In a review (Murray-Rust et al., 1983) of over 260 structures containing the C–F fragment, nine such interactions were found, the majority of which were C–F···H–N hydrogen bonds. Other more recent structures have provided further evidence for discrete C–F···H–O bonds in a fluorinated carboxylic acid (Karipides & Miller, 1984) and in a difluorinated sugar (Withers et al., 1986a). In the latter example both the fluorines were involved in weak, but significant, interactions as well as both the protons of the CHF fragments at C-1 and C-2.

Thus sufficient evidence exists to suggest that the C–F fragment can act as a weak proton acceptor and that when a suitable hydrogen bond donor is appropriately positioned as in a crystal lattice or an enzyme–ligand complex, hydrogen bonds may be formed.

Interactions at C-1 and C-2 of Glucose. It has been suggested previously (Cori & Cori, 1940) and effectively confirmed by X-ray crystallography (Sprang et al., 1982) that glycogen phosphorylase binds α-D-glucose in preference to or to the exclusion of its β-anomer. However, since this finding has been disputed and equal affinities claimed for α- and β-D-glucose (Ariki & Fukui, 1977), further studies were performed by us on relatively short time scales in order to minimize anomerization. The slopes of the respective Dixon plots (Figure 1) revealed a 3-fold lower K_i value for α-D-glucose than for β-D-glucose, and this will represent a minimum estimate of their affinity ratios since any anomerization occurring during the experiment will reduce this value. Approximate K_i values of 1 and 3 mM for α-D-glucose and β-D-glucose, respectively, obtained from this plot are completely consistent with the K_i value of 2 mM determined for the anomeric mixture (36% α; 64% β) of D-glucose. Binding

constants for analogues modified at C-1 should therefore be compared with this (1 mM) value. However, since the anomeric compositions of most other analogues are similar to that of glucose (Table I), their binding data will be compared with that of the equilibrium mixture of glucose anomers for simplicity.

The energetic consequences of substitution at C-1 and C-2 as expressed in their binding affinities are quite similar in many respects but different in others. In each case (**1** → **2**; **1** → **6**), substitution by H results in a loss of ≈1.5 kcal/mol in binding energy,[2] whereas replacement by F (**1** → **3**; **1** → **7**) has no significant overall energetic consequences. This would suggest the presence of a hydrogen bond at each position in which the fluorine can apparently replace the hydroxyl completely, suggesting that the fluorine, thus the original hydroxyl, acts as the hydrogen-bond acceptor. A more extensive interpretation of these numbers is unwarranted given the uncertainties inherent in such an analysis, such as the unknown relative hydrogen-bond strengths of fluorine to water and its donor in the active site, the slightly smaller overall size of the fluorine (2.74 Å) relative to the oxygen (2.83 Å) and the possible role of the fluorine in promoting other hydrogen-bonding interactions involving nearby C–H and C–OH protons as seen (Withers et al., 1986a) and proposed (Bessell et al., 1972) previously in related systems. However, had the original hydrogen bond been of the opposite polarity, requiring the fluorine to act as a donor, a considerable loss in affinity should have resulted (eq 4).

Differences between the 1 and 2 positions are apparent upon comparing the energetic consequences of inversion of configuration at each position. Inversion at C-1 (**3** → **4**; **8** → **9**) results in a 6- to 8-fold loss in affinity of the fluoro sugars, all of which (1.1 kcal mol^{-1}) can be accounted for by the loss of the positive interaction associated with the axial anomeric substituent. This suggests that no significant repulsive interactions result from introducing a relatively bulky equatorial substituent at C-1 and would appear to be confirmed by the data on 1-deoxyglucose and β-glucosylamine (**2** → **5**). Data are thus consistent with a preferred, but not exclusive, binding of α-glucose over its β-anomer. Inversion of configuration at C-2, however (**1** → **12**; **3** → **13**; **7** → **14**; **8** → **10**), results in a considerable loss in affinity, greater than that expected simply on the basis of loss of the hydrogen-bonding interaction with the equatorial substituent. These differences are best quantitated in the difluorinated series, where inversion of configuration at C-2 in the 2-fluoro α-fluoride series (**8** → **10**) results in at least a 375-fold (≈3.6 kcal/mol) loss in affinity. Loss of the positive interaction with the equatorial fluorine at C-2 accounts for 1.5 kcal mol^{-1} (from **1** → **6**); thus the remaining ≈2 kcal mol^{-1} must arise from repulsive interactions associated with the bulky axial substituent. A considerable energetic price is therefore paid for a relatively small (F vs. H = 0.45 Å) increase in substituent size, indicating a rather precise fit between ligand and protein at this point. No significant inhibition could be measured for 2-fluoro-β-D-mannosyl fluoride (**11**) in which both fluorines occupy "incorrect" orientations. This is unsurprising since the K_i value would be predicted to be 600 mM on the basis of data from **8**–**10**.

Introduction of two fluorines generally leads to increased affinity over that predicted. Thus a K_i value of 0.6 mM would be predicted for 2-fluoro-α-D-glucosyl fluoride (**8**) on the basis of values for **3** and **7**, but the measured value is 0.2 mM. Similarly a value of 3.8 mM would be predicted for 2-fluoro-β-D-glucosyl fluoride (**9**) on the basis of values for **4** and **7**. These small increases in affinity are probably related to the secondary effects of fluorine substitution in promoting other hydrogen bonding, noted earlier. The exact converse is observed for 1,2-dideoxy-D-glucose (**15**), where a K_i value of 135 mM is estimated on the basis of values for **1**, **2**, and **6** but a value of ≈600 mM determined. Deactivation of these secondary effects on hydrogen bonding by the less electronegative substituent could be responsible for this.

Since the introduction of fluorine substituents into sugar rings has been shown previously (Hall & Manville, 1969) to result in distortion of the native conformation in some cases, which could invalidate our procedure, a conformational study was performed on that fluoro sugar in our study most likely to be distorted on the basis of dipolar repulsions. As reported elsewhere (Withers et al., 1986a), 2-fluoro-β-D-mannopyranosyl fluoride (**11**) was found to adopt a completely normal $^4C_1$ conformation both in the solid state (by X-ray crystallography) and in solution (by two-dimensional *J*-resolved NMR). Since this sugar is not distorted it is reasonable to assume that all other fluoro sugars studied adopt a normal chair conformation.

Interactions at C-3, C-4, and C-6. The energetic consequences of modifications at these positions are considerably different from those at C-1 and C-2 since deoxygenation at each position (**19**–**21**) results in a large loss in affinity (>3 kcal mol^{-1}), which can only partially be reinstated by fluorination, if at all. Thus 3-fluoro-3-deoxy-D-glucose (**16**) and 6-fluoro-6-deoxy-D-glucose (**18**) bind very poorly with a loss of approximately 2.7–3.2 kcal mol^{-1} of binding energy, while 4-fluoro-4-deoxy-D-glucose (**17**) binds a little better but still 1.5 kcal mol^{-1} more weakly than glucose. The simplest interpretation is that strong hydrogen bonds exist at the 3 and 6 positions in which the sugar hydroxyl ordinarily acts as the hydrogen-bond donor. Thus the fluorine cannot participate. In addition, since 6-fluoroglucose binds slightly better than the 6-deoxy derivative an additional hydrogen bond of opposite polarity is suggested. Similarly the results at the 4-position suggest a relatively weak hydrogen bond (1.5 kcal mol^{-1}) in which the sugar hydroxyl acts as the donor, plus another hydrogen bond of opposite polarity.

The predicted order of hydrogen bond strength and the role [donor (D) or acceptor (A)] of the sugar hydroxyl at each position can thus be summarized as below.

$$3(D) \approx 6(D + A) > 4(D + A) > 2(A) \approx 1(A)$$

This analysis cannot reliably predict the presence of multiple hydrogen bonding since it cannot detect additional weak hydrogen bonds in the presence of a strong one.

Comparison with Recent X-ray Crystallographic Data. Details of the interactions between glucose and phosphorylase in the recently refined (*R* factor = 18.5%) X-ray crystal structure of the phosphorylase *a*–glucose complex have been very kindly supplied prior to publication by Dr. S. Sprang and Dr. R. J. Fletterick and are presented in Table II in the form of close contacts observed between atoms of each species. Although the uncertainties in the hydrogen bond lengths quoted discourage detailed interpretations, it is apparent that, for example, interactions at 1-OH are relatively weak, whereas elsewhere, and particularly at 6-OH, quite short strong interactions are observed. Moreover, the polarity of the hydrogen bonds observed is consistent with that predicted. The principal hydrogen bonds at 1-OH and 2-OH both involve the hydroxyl as a proton acceptor; in the first case from a backbone

[2] Free energy differences $\Delta\Delta G°$ are calculated from the expression $\Delta\Delta G° = RT \ln (K_{i2}/K_{i1})$, where K_{i2} and K_{i1} are the inhibition constants for the two analogues under comparison.

Table II: Hydrogen-Bonding Interactions at the Active Site of Phosphorylase *b*

sugar hydroxyl	protein residue, atom involved, and its role (donor or acceptor)	distance (Å) (±0.2 Å)	angle about oxygen (deg)
1-OH	Leu 136 N(D)	3.4	121
2-OH	Asn 284 ND2 (D)	2.9	106
	Tyr 573 OH (D/A)	3.3	146
3-OH	Glu 672 OE2 (A)	3.1	119
	Ser 674 N (D)	3.1	126
4-OH	Asn 284 OD1(A)	3.3	160
	Gly 675 N (D)	2.9	100
6-OH	His 377 ND1 (D)	2.7	147
	Asn 484 OD1 (A)	2.8	131

NH and in the second case from the nitrogen of an asparagine residue. Principal hydrogen bonds at 3-OH involve it as a donor to a glutamate side chain and as acceptor of a hydrogen bond from the backbone NH of Ser 674. Donor and acceptor roles are required at 4-OH and probably also at 6-OH. More reliable, perhaps, is the information on the types of interacting groups since all but two hydrogen bonds involve neutral amino acid side chains or main chain atoms. One exception is Glu 672, which interacts with 3-OH and which is certainly ionized since it also interacts quite strongly with Lys 574. The other is His 377, which interacts with 6-OH and which is probably charged since Asp 339 is quite close to it. Thus both these interactions (Glu 672–3-OH and His 377–6-OH) are charged–neutral interactions, which is particularly interesting since the kinetic studies predicted that the strongest hydrogen bonds would be at 3-OH and 6-OH.

An indication of the source of the high specificity against epimeric sugars was obtained upon attempting to superimpose the coordinates for 2-fluoro-β-D-mannosyl fluoride onto those for glucose at its binding site, by using computer graphics. A severe steric clash (F → O = 2.1 Å) is observed between the axial fluorine at C-2 and the main chain carbonyl of His 377. Such a clash does not occur with a proton at that position (H → O ≈ 2.55 Å) but rather a close fit. This therefore explains the kinetic data obtained with the mannosyl derivatives.

Comparison with Results of Fersht et al. (1985). Comparison of these results with those of Fersht et al. (1985) on the affinities of mutants of tyrosyl tRNA synthetase for its substrates is quite instructive since the two complementary studies provide essentially identical results. The overall energetic consequence (based upon k_{cat}/K_m) of removal of a hydrogen bond between uncharged donor/acceptor pairs in the tyrosyl tRNA synthetase–substrate complex was a loss of 0.5–1.5 kcal mol^{-1}. However, deletion of a hydrogen bond within a neutral–charged pair reduces the affinity by approximately 4 kcal mol^{-1}, leading to the conclusion that specificity is indeed caused to some extent by hydrogen bonding but that it is best mediated by charged residues.

A similar situation appears to obtain in the phosphorylase–glucose complex. The only charged (or potentially charged) residues involved in hydrogen bonding are Glu 672 and His 377, which are bonded to 3-OH and 6-OH, respectively. Deletion of all hydrogen-bonding capabilities at these positions (deoxy analogues) results in an affinity decrease of greater than 100-fold (>3 kcal mol^{-1}). Introduction of fluorine, limiting the hydrogen bonding, has similar consequences at the 3 position (≥3.2 kcal mol^{-1}) and a slightly lesser effect at the 6 position (2.7 kcal mol^{-1}). Unfortunately, due to the limited "range" of our procedure, occasioned by the relatively high K_i for glucose and uncertainties surrounding the nature of inhibition by glucose analogues at high (>100 mM) concentrations, we are unable to reliably assess affinity losses greater than ≈3 kcal mol^{-1}. Thus the strongest hydrogen bonds appear to involve charged–neutral interactions. The hydrogen bonds involved at 1-OH and 2-OH-2 are of the neutral–neutral type, and their deletion results in a loss of 1.5 kcal mol^{-1}, as also observed by Fersht. Relatively large affinity loss at 4-OH for the deoxy analogue may be the result of loss of two weaker H bonds, whose presence is suggested by the 4-fluoroglucose data. Neither of the interactions observed at 4-OH involves charged residues.

The source of the stronger hydrogen bonding associated with charged–neutral pairs is uncertain. However, its origin may lie in the fact that hydration of (and therefore hydrogen bonding to) ions is much greater than that of neutral species. Thus since the measured hydrogen-bond energies reflect differences in relative hydrogen-bond strengths to water and to its hydrogen-bonding partner (which will also be suitably polarized) it is not unreasonable to expect that these *differences* will be greater when one partner is charged than when both are neutral. Whatever the cause of the effect, it is extremely gratifying that two very different systems investigated by complementary methods arrive at such similar results.

Comparison with the Arabinose Binding Protein System. A brief comparison of the carbohydrate binding site in phosphorylase with that recently published (Quicho & Vyas, 1985) for the arabinose binding protein is instructive in terms of structure/function relationships. The sole function of this protein is the efficient transport of arabinose across the cell membrane, and it has evolved to optimize this capability through generating very high affinities for both α- and β-L-arabinose ($K_D \approx 2 \times 10^{-7}$ M) (Clark et al., 1982; Newcomer et al., 1981). Through studies of the temperature dependence of binding (Clark et al., 1982) the interaction was shown to be an enthalpy-driven process with a small negative entropy ($\Delta H = -9.5$ kcal mol^{-1}, $\Delta S \approx -2.5$ eu), indicating the importance of attractive forces between arabinose and the protein. This was borne out in the X-ray crystallographic study of the complex where a total of 10 hydrogen bonds were observed between arabinose and the protein, of average length 2.82 Å. Only two of these were greater than 3.0 Å in length. By comparison, the average hydrogen-bond length in the relatively low affinity ($K_D = 2 \times 10^{-3}$ M) phosphorylase–glucose complex is 3.05 Å with only four of the nine observed hydrogen bonds being shorter than 3.0 Å. Thus the greater affinity of L-arabinose for its protein would appear to derive from its more numerous shorter and hence stronger hydrogen bonds. The tightness and degree of hydrogen bonding is the more noteworthy in consideration of the fact that arabinose has one less hydroxyl available for such interactions than does glucose.

The other remarkable feature of the arabinose binding protein site is the large number of charged residues involved in interactions with arabinose. The results described in this paper along with those of Fersht et al. now clarify the role of these charged residues, since it would appear that the best way of improving the affinity of a hydrophilic ligand for its binding site is to provide a large number of charged residues as hydrogen-bonding partners since they can compete very effectively with water as hydrogen-bonding partners, giving rise to shorter and stronger hydrogen bonds, as observed. Indeed, as more structures for carbohydrate–protein complexes become available it will be interesting to see whether a good correlation does indeed exist between the association constant and the number of charged residues in the binding site.

Thus it would appear that phosphorylase has overcome the seemingly difficult task of providing a binding site for glucose

of high specificity but of relatively low affinity by incorporating steric constraints to prohibit the binding of unwanted epimers, as observed for the manno derivatives, and by construction of a network of hydrogen bonds, most of which are relatively weak, by utilizing predominantly neutral hydrogen-bonding partners.

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Registry No. **1**, 50-99-7; **2**, 154-58-5; **4**, 7617-95-0; **5**, 7284-37-9; **8**, 75414-45-8; **9**, 103960-04-9; **10**, 103960-05-0; **11**, 98808-81-2; **12**, 3458-28-4; **13**, 2713-54-4; **14**, 38440-79-8; **15**, 13035-11-5; **16**, 14049-03-7; **17**, 29218-07-3; **18**, 4536-08-7; **19**, 2490-91-7; **20**, 7286-46-6; 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, 572-09-8; α,α′-azobis(isobutyronitrile), 78-67-1; methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-4-chloro-α-D-glucopyranoside, 41881-07-6; methyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside, 3601-36-3; methyl 2,3,6-tri-*O*-benzoyl-4-deoxy-α-D-glucopyranoside, 19488-41-6; methyl 4-deoxy-α-D-glucopyranoside, 13241-00-4; 1,2:5,6-di-*O*-isopropylidene-3-deoxy-3-chloro-α-D-glucofuranose, 32785-94-7; 1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose, 2595-05-3; 1,2,4,6-tetra-*O*-acetyl-3-deoxy-3-chloro-D-glucopyranose, 104013-04-9; 1,2,4,6-tetra-*O*-acetyl-3-deoxy-D-glucopyranose, 5040-09-5; α-D-glucopyranose, 492-62-6; β-D-glucopyranose, 492-61-5; phosphorylase *b*, 9012-69-5.

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