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Fluorinated and Deoxygenated Substrates as Probes of Transition-State Structure in Glycogen Phosphorylase†

Ian P. Street, Karen Rupitz, and Stephen G. Withers*
Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Y6
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ABSTRACT: A series of deoxyfluoro- and deoxy-α-D-glucopyranosyl phosphates have been tested as substrates of rabbit muscle glycogen phosphorylase *b*. All are found to be utilized by the enzyme, but at substantially reduced rates. Values of V_m/K_m for these analogues range from 10^2 to 10^5 times lower than that for the parent substrate. The large rate reductions are suggested to arise from a combination of intrinsic electronic effects and poorer binding of these substrates at the transition state. The data provide substantial evidence for an oxocarbonium-ion-like transition state. They also provide estimates of the strengths of hydrogen bonds to individual sugar hydroxyls at the transition state of the reaction. Further, comparison of such data with those obtained for glucose analogues binding as inhibitors to T-state phosphorylase suggests that these two glucose subsites are essentially identical; thus, the glucose pocket remains intact during the conformational transition associated with activation of the enzyme.

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the reversible phosphorolysis of glycogen, producing glucose-1-P[1] (Graves & Wang, 1972; Fletterick & Madsen, 1980). The activity of this enzyme is modulated by, among many other effectors, glucose. The binding of glucose stabilizes an inactive T-state conformation of the enzyme that has been shown by a variety of techniques (Helmreich et al., 1967; Wang et al., 1965; Withers et al., 1979) to be different from the active R-state conformation induced by substrates and activators. The structure of the enzyme in this T-state conformation and its interactions with glucose are well understood, as the three-dimensional structure of the phosphorylase *a*–glucose complex has been determined by X-ray crystallography (Sprang & Fletterick, 1979; Sprang et al., 1982). The

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[1] Abbrevations: glucose-1-P, α-D-glucopyranosyl phosphate; deoxyglucose-1-P, deoxy-α-D-glucopyranosyl phosphate; fluoroglucose-1-P, deoxyfluoro-α-D-glucopyranosyl phosphate; DTT, dithiothreitol.

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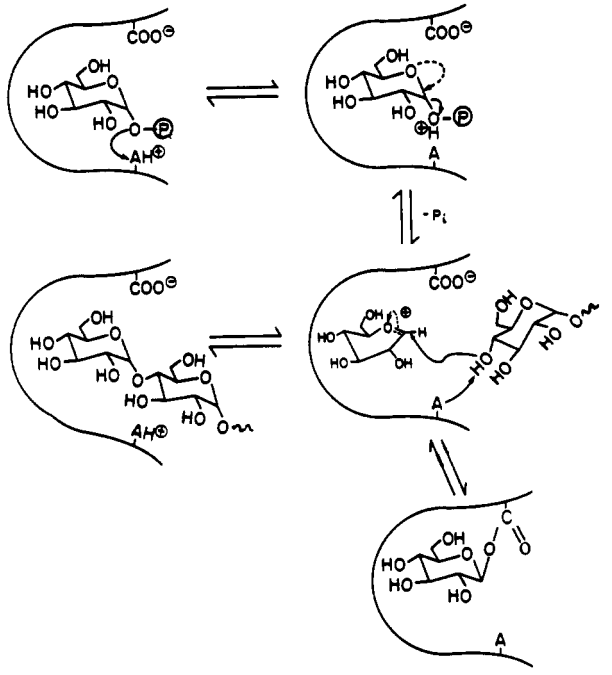
Scheme I



three-dimensional structure of an essentially T-state phosphorylase in the presence of the weak activator IMP has also been described (Weber et al., 1979; Sansom et al., 1985). Further insight has been provided through studies of the binding of glucose analogues (Street et al., 1986) in which hydrogen-bond polarities and strengths at each hydroxyl have been estimated by using a series of deoxy- and deoxyfluoroglucose analogues. Unfortunately, relatively little information is available concerning the structure of the R-state enzyme and its interactions with substrate since neither phosphorylase *a* nor phosphorylase *b* has been successfully crystallized in this conformation, although crystals of a modified R-state phosphorylase are currently under investigation. Some structural information has been obtained by diffusion of substrates or substrate analogues into the crystal and subsequent collection of diffraction data (Withers et al., 1982; McLaughlin et al., 1984; Oikonomakos et al., 1987). However, such data are invarably of relatively poor quality, and any conformational changes observed are certainly attenuated by crystal packing forces.

Structural information on the R-state enzyme is important for the development of an understanding of the catalytic mechanism. Until recently (Klein et al., 1986) it was generally considered [see, for example, Klein et al. (1982) and Withers et al. (1982a)] that the mechanism involved a double displacement, as shown in Scheme I, similar to that of "retaining" glycosidases such as lysozyme or β-galactosidase (Blake et al., 1967; Sinnott, 1984). Such ideas were based on the chemical similarities, namely, a glycosyl transfer with retention of anomeric configuration, on the similarity of reactions catalyzed with a variety of unnatural substrates such as D-glucal (Klein et al., 1982), heptenitol (Klein et al., 1984; McLaughlin et al., 1984), and α-D-glucosyl fluoride (Palm et al., 1983), and on relatively strong inhibition (K_i = 0.025 mM in the presence of phosphate) by D-gluconolactone, a possible transition-state analogue (Gold et al., 1971). The main argument against such a mechanism has been the lack of conclusive evidence for a glucosyl–enzyme intermediate. However, supportive evidence for this intermediate does exist and comes from two different studies on potato phosphorylase. This enzyme is generally considered to operate by the same mechanism as that from muscle, and data from the two enzymes are frequently considered together. First, exchange was observed between the bridging and nonbridging oxygen atoms of glucose-1-P in the ternary complex with cyclodextrin, a nonreactive oligosaccharide analogue. Such evidence has not been obtained for rabbit muscle glycogen phosphorylase since no suitable glycogen analogue is available. Second, some kind of glucosyl–enzyme intermediate was shown (Klein et al., 1981, 1982) to be formed in the reaction between potato phosphorylase and D-glucal, and this glucose residue was subsequently transferred to an oligosaccharide acceptor.

The difficulty of obtaining more conclusive evidence for such an intermediate resides in the rapid equilibrium random kinetic mechanism for phosphorylase wherein the rate-limiting step is the interconversion of the ternary complexes with substrate and product. The enzyme does not adopt the active conformation *until* the full ternary complex is formed, a mechanism that minimizes the likelihood of hydrolysis of any intermediate. A further problem with the proposal of a glucosyl–enzyme intermediate has been the absence of an obvious candidate for the nucleophile in any of the crystal structures obtained [see, for example, Hajdu et al. (1987) and Withers et al. (1982a)]. However, it must be remembered that all the structures obtained are of essentially inactive T-state enzyme and that addition of saturating concentrations of both substrates (oligosaccharide and the phosphorylated substrate) results in extensive cracking of the crystal. Data acquisition has only been successful on crystals that have not undergone the full conformational transition, only limited quantities of the substrates or analogues having been added. In one case where reaction is observed and monitored, the phosphorylation of heptenitol (McLaughlin et al., 1984; Hajdu et al., 1987), reaction occurs in the absence of oligosaccharide. Thus, conformational changes that are known to occur with, and are essential for, the normal substrates do not occur with this substrate. This oligosaccharide-induced conformational change may well be responsible for the placement of the nucleophile in the normal mechanism. Thus, the phosphorylation of the heptenitol may occur by an alternate mechanism, indeed, likely that suggested (Klein et al., 1986) involving a concerted cis addition of phosphate across the double bond not requiring the "backside" stabilization. This might also explain the puzzling lack of reactivity of the heptulose 2-phosphate product since its β-methyl group may serve to block the approach of this nucleophile, hence the "one-way" reaction of this substrate.

Clearly, further information is required before the involvement of a glucosyl–enzyme intermediate of some kind is discounted, as has been suggested recently (Klein et al., 1986). The alternative mechanism proposed, a concerted front-side nucleophilic displacement (S_Ni) of phosphate by oligosaccharide, had been considered previously (Withers et al., 1982a) but discounted as unlikely on the basis of the severe steric requirements imposed by such a mechanism on a transition state involving a bulky nucleophile and leaving group at the active site of an enzyme. The only previous evidence for such mechanistic behavior at the anomeric center of a sugar involves small nucleophiles (water and simple alcohols) and an even smaller leaving group (fluoride) in free solution (Sinnott & Jencks, 1980).

In the absence of structural information on the active enzyme, mechanistic questions are best pursued through kinetic studies and other physical techniques. A commonly employed approach in physical organic chemistry that provides direct information on transition-state structure is the kinetic study of modified substrates in a search for interpretable struc-

Table I: Kinetic Parameters for Glucose-1-P Analogues with Phosphorylase b^a

	muscle phosphorylase b				
compound	V_m (μmol min^{-1} mg^{-1})	K_m (mM)	10^{-4} V_m/K_m (L min^{-1} mg^{-1})	$\Delta\Delta G^\ddagger$ (kcal mol^{-1})	K_i (mM)
G1P	90	4.8	187 500		
2-fluoro-G1P	2.8×10^{-4}	2.6	1.08	7.3	2.0
3-fluoro-G1P	5.6×10^{-3}	25	2.2	6.8	39
4-fluoro-G1P	6.7×10^{-3}	1.8	37	5.1	30
6-fluoro-G1P	2.5×10^{-3}	97	0.26	8.1	25
3-deoxy-G1P	1.8×10^{-2}	31	5.8	6.3	ND^b
4-deoxy-G1P	1.8	45	400	3.7	ND^b
6-deoxy-G1P	7.8×10^{-2}	70	11.1	5.9	ND^b

^{a} Parameters were determined as described under Experimental Procedures by using the following enzyme concentrations and reaction times: glucose-1-P, 4.3 μg mL^{-1}, 5 min; 2-fluoroglucose-1-P, 993 μg mL^{-1}, 1680 min; 3-fluoroglucose-1-P, 615 μg mL^{-1}, 650 min; 4-fluoroglucose-1-P, 615 μg mL^{-1}, 254 min; 6-fluoroglucose-1-P, 1042 μg mL^{-1}, 965 min; 3-deoxyglucose-1-P, 269 μg mL^{-1}, 215 min; 4-deoxyglucose-1-P, 50.4 μg mL^{-1}, 17 min; 6-deoxyglucose-1-P, 504 μg mL^{-1}, 60 min. ^{b} ND, not determined.

ture/activity correlations. Unfortunately, as noted earlier, apart from the unsaturated sugars and glucosyl fluoride, nothing other than glucose-1-P has been shown to act as a substrate for the muscle enzyme. Useful information may, however, be obtained through more conservative modifications of the substrate, producing analogues that differ in their electronic properties and hydrogen-bonding capability but which may still be accepted by the enzyme. We have followed up our recent studies on the binding of glucose to the T-state enzyme (Street et al., 1986) by synthesis and testing as substrates of a series of deoxy- and deoxyfluoro-D-glucose-1-P derivatives. Comparison of the data obtained in this way with our previous data on a model system, the acid-catalyzed hydrolysis of such substituted glucose-1-P derivatives (Withers et al., 1986; 1988), has provided insight into the electronic structure of the enzymic transition state. It has also permitted insight into the hydrogen-bonding interactions with individual sugar hydroxyls both in the ground-state Michaelis complex and at the transition state. This paper does not, however, address the question of the role of the cofactor pyridoxal phosphate in the catalytic mechanism.

Experimental Procedures

Syntheses of bis(cyclohexylammonium) 6-fluoro-, 4-fluoro-, 3-fluoro-, and 2-fluoroglucose-1-P have been described elsewhere (Withers et al., 1986), as have syntheses of their deoxy equivalents (Withers et al., 1988).

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. Phosphorylase *a* was prepared from phosphorylase *b* by the action of phosphorylase kinase (EC 2.7.1.38) (Krebs et al., 1964). Phosphorylase *b* freed from AMP was prepared as described by Withers et al. (1979).

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.

All values of K_m and V_{max} were determined from the initial reaction rates of saccharide synthesis (Engers et al., 1970). All reactions were conducted in a buffer containing 20 mM sodium glycerophosphate, 4 mM DTT, and 1 mM EDTA. Phosphate released was measured according to the procedure of Baginski (Baginski et al., 1967) but using 1.5 times the normal level of assay reagents to ensure full color development at high concentrations of phosphate.

All substrates were employed directly in the assay as the bis(cyclohexylammonium) salts since K_m and V_{max} values for the dipotassium and bis(cyclohexylammonium) salts were shown to be essentially identical. The reactivity of glycogen phosphorylase toward the different substrates varied greatly, and it was found necessary to conduct initial range-finding experiments to determine optimum enzyme concentration and reaction time. During these experiments a single concentration of the substrate (between 20 and 50 mM) was incubated with glycogen phosphorylase, and at various times aliquots from the reaction were removed and assayed for phosphate released. The optimum reaction times and enzyme concentrations determined in this manner are given in Table I. Control experiments were performed in which phosphorylase *b* was incubated under standard assay conditions in the absence of substrate. These showed a negligible loss in activity, even over the longest reaction time given in Table I. Accurate values for K_m and V_{max} were determined from initial rate data at seven or eight substrate concentrations by use of a Lineweaver–Burk analysis.

K_i values were determined by measurement of reaction rates at a series of glucose-1-P concentrations at each of a series of inhibitor concentrations using a buffer system containing 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1 mM EDTA, and 1 mM DTT, pH 6.8.

^{19}F NMR Studies. Spectra were recorded on a Bruker HXS-270 spectrometer fitted with a Nicolet 1180 computer and operating at 254 MHz with a 5-mm fluorine probe. Chemical shifts are quoted relative to $CFCl_3$ and were measured with reference to external hexafluorobenzene. All experiments were conducted with broad-band proton decoupling.

Reaction mixtures (0.7 mL) contained 5.0 mM substrate, 0.5% glycogen, 1 mM AMP, 12.6 mg of glycogen phosphorylase *b*, and 0.2 mL of D_2O. All reactions were conducted at pH 6.8 in a buffer containing 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1 mM EDTA, and 1 mM DTT. For 2-fluoro-glucose-1-P the reaction was run under similar conditions except that 4.5 mg of phosphorylase *a* and 36 mM maltopentaose were used in place of phosphorylase *b* and glycogen. Reaction mixtures were incubated for 12–48 h at 30 °C prior to data acquisition.

Results

Our initial studies on these substrate analogues appeared to show that none of them acted as substrates. Consequently, the fluorinated analogues were tested as inhibitors of phosphorylase *b* to determine whether they could even bind at the active site. All were indeed found to be inhibitors that were essentially competitive with glucose-1-P, though some indication of noncompetitive inhibition was obtained in some cases. K_i values determined are listed in Table I. This prompted a more vigorous search for turnover of these substrates. Experiments were therefore initially performed in NMR tubes at very high enzyme concentrations using ^{19}F NMR to monitor the dissappearance of the resonance due to fluorinated substrate and the appearance of a resonance due to fluorinated

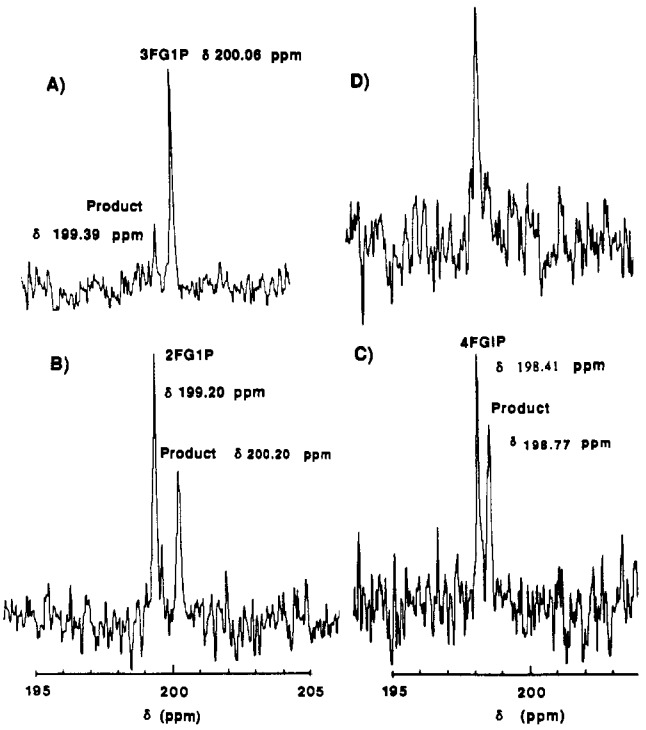


FIGURE 1: ^{19}F NMR spectra of reaction mixtures containing fluoro sugar phosphates and glycogen phosphorylase as described under Experimental Procedures. (A) 3-Fluoro-α-glucose-1-P; (B) 2-fluoro-α-glucose-1-P; (C) 4-fluoro-α-glucose-1-P; (D) as in (C) with omission of AMP and glycogen.

product. Figure 1 shows some examples of such spectra and clearly demonstrates that reaction is occurring to produce a fluorosugar product that is not simply the corresponding deoxyfluoroglucose on the basis of both chemical shifts observed and the absence of a pair of resonances due to the α- and β-anomers expected for the free deoxyfluoroglucose. As is shown in Figure 1, omission of the required activator AMP or the cosubstrate glycogen, or both, from such samples resulted in no product appearance.

Since ^{19}F NMR spectroscopy could not be conveniently used for kinetic studies, an alternative assay procedure, based on phosphate release, was sought, and the ascorbate/molybdate assay (Baginski et al., 1967) was found to be sufficiently sensitive to detect low levels of phosphate in the presence of relatively high enzyme concentrations. However, since this assay is not compatible with the triethanolamine buffer used in our previous studies, all assays were performed in the sodium glycerophosphate system described under Experimental Procedures. This assay has the additional important advantage of being well suited to measurement of inorganic phosphate in the presence of acid-sensitive phosphate esters. This is essential since the deoxy-D-glucopyranosyl phosphates are considerably more acid sensitive than the parent sugar phosphate and hydrolyze under the conditions of the Fiske–Subbarow assay. An alternative colorimetric phosphate assay based upon a coupled enzyme system (Fossati, 1985) was also evaluated. It is more sensitive and does not require acid conditions, thus avoiding problems associated with protein precipitation and with acid-sensitive phosphate esters. However, largely due to high background readings under these conditions, it was found to be unreliable and was abandoned. Substrate analogues were therefore assayed at high (up to 1.0 mg mL^{-1}) enzyme concentrations for long (up to 28 h) time periods in the presence of 1% glycogen and 1 mM AMP. Control experiments with similar samples of enzyme assayed over this time period showed negligible activity loss. Michaelis–Menten parameters determined in this way are shown in Table I. Once again, omission of either AMP or glycogen from such reaction mixtures resulted in no phosphate release. This experiment, in conjunction with the similar ^{19}F NMR experiment discussed earlier, essentially proves that the phosphate-producing reaction being assayed is catalyzed by glycogen phosphorylase. Unfortunately, reliable data could not be obtained for 2-deoxyglucose-1-P since it could not be synthesized at sufficiently high purity for assay with the enzyme and since it is very hydrolytically labile. However, experiments involving D-glucal as a substrate for phosphorylase that produces 2-deoxy oligosaccharides and 2-deoxy-α-D-glucose-1-P as products (Klein et al., 1982) clearly demonstrate that 2-deoxyglucose-1-P should be a reasonable substrate. Indeed, the V_{max} value determined for D-glucal was about 20% of that for glucose-1 P.

Table I also contains the values of the increase in overall activation free energy ($\Delta\Delta G^{\ddagger}$) consequent upon substitution of each hydroxyl by hydrogen or fluorine. These values are readily calculated from the relative values of V_m/K_m for the analogue in question and for the parent substrate by using the expression below, where $(V_m/K_m)_1$ and $(V_m/K_m)_2$ are the kinetic constants for the analogue and the parent substrate, respectively.

$$\Delta\Delta G^{\ddagger} = RT \ln \frac{(V_m/K_m)_2}{(V_m/K_m)_1}$$

DISCUSSION

It is clear from the data in Table I that the substitution of individual hydroxyl groups around the ring by hydrogen and fluorine has enormous consequences on their turnover rates. Indeed, 6-deoxy- and 6-fluoroglucose-1-P had been tested previously as substrates (Graves & Wang, 1972) and were thought to be inactive, whereas the present work shows them to be substrates, but with very low turnover rates. Interpretation of these numbers, however, requires some consideration of what each parameter represents in this case. Since the enzyme follows a rapid equilibrium random kinetic mechanism, values of K_m should represent true dissociation constants and be equal to the values of K_i determined, unless the substitution results in significant accumulation of any bound intermediate species. Values of V_m reflect the activation energy for the rate-determing step (the maximum energetic difference between the transition state in question and its previous bound species). Values of V_m/K_m reflect the overall activation free energy (i.e., energetic difference between free enzyme plus free substrate and the highest transition-state energy, strictly that of the first irreversible step). In the absence of a consensus on the mechanism itself, it is not currently possible to assign measured activation energy differences to specific steps.

The large rate reductions observed, as expressed in decreased V_m and V_m/K_m values, likely arise from some combination of intrinsic electronic effects, as measured previously in the acid-catalyzed hydrolysis of these same glucose-1-P analogues (Withers et al., 1986, 1988) and differences in binding of these analogues, both in their ground state and particularly at the transition state. Evaluation of the exact individual contributions of these two effects to the observed rate reductions is not entirely possible, but can be achieved to a limited extent, as discussed below.

Electronic Effects. The double-displacement mechanism discussed earlier involves transition states with substantial oxocarbonium ion character. Replacement of hydroxyl groups in the sugar ring by the more electronegative fluorine should result in destabilization of such charged transition states and

thus a decrease in turnover rate. The converse should hold true for the deoxy sugars. These electronic effects have been probed previously (Withers et al., 1986) in some detail for the acid-catalyzed hydrolysis of the same series of deoxyfluoro-D-glucopyranosyl phosphates, where reaction is known to proceed via an oxocarbonium-ion-like transition state. Fluorine substitution in the ring resulted in significant rate decreases, with the greatest effect (60-fold) being upon substitution of the 2-hydroxyl. Corresponding rate increases have since been observed as expected (Withers et al., 1988) for the deoxy-D-glucopyranosyl phosphates. The rate decreases observed for the fluoro sugars in the enzymic reaction are considerably greater than those in the acid-catalyzed reaction (10^3–10^5-fold versus 4–60-fold), indicating that some other factor which does not affect the nonenzymic reaction also contributes to the rate reductions. This is likely related to the loss of specific binding interactions that normally contribute to stabilization of the transition state. Such binding, which clearly does not contribute to the nonenzymic reaction, will be discussed later.

Evidence for the involvement of charged transition states in enzymic reactions can be obtained, in favorable circumstances, by comparing substituent effects on enzymic reaction rates with those on a model nonenzymic reaction as was achieved by Poulter et al. (1981) in a study of the rate of farnesylpyrophosphate synthetase catalyzed reaction of a series of fluorine-substituted substrates. A plot of the logarithm of the relative rate constants of the enzymic reaction versus the logarithm of the relative rate constants for acid-catalyzed hydrolysis of each substrate produced an excellent linear free energy relationship with a correlation coefficient (ρ) of 0.993. Since the substituent effects on the acid-catalyzed reaction are primarily electronic in origin and since the linear free energy relationship shows there is a high correlation of substituent effects between the enzymic and nonenzymic reaction, this demonstrates the involvement of a carbonium-ion-like transition state. The high correlation coefficient also showed that binding effects are relatively insignificant in this particular case. A similar analysis applied to the data in this paper is most informative. Figure 2a shows a plot of log V_m for the phosphorylase-catalyzed reaction versus the logarithm of the rate constant for acid-catalyzed hydrolysis. A reasonable correlation (ρ = 0.90) is observed, indicating considerable similarities in the electronic nature of these two transition states. Any contributions to transition-state stabilization arising from additional binding interactions at the transition state that are not present in the previous bound ground state will give rise to scatter in such a plot as will be discussed later. The plot of log V_m/K_m, shown in Figure 2b, is essentially a scatter plot. This is quite reasonable since, as discussed earlier, values of V_m/K_m reflect overall activation free energies (free enzyme and free substrate to transition state); thus, these values contain much greater contributions from binding interactions. These binding differences overwhelm the electronic effects buried therein.

These data therefore provide evidence that is strongly supportive of a mechanism involving transition states with substantial oxocarbonium ion character. This is consistent with the proposed double-displacement mechanism and in line with the previously observed tight binding of the transition-state analogue D-gluconolactone to the enzyme–AMP–phosphate complex (Gold et al., 1971). As discussed earlier (Withers et al., 1982), the absence of a significant secondary deuterium kinetic isotope effect (Firsov & Bogacheva, 1977) does not rule out a mechanism involving oxonium-ion-like transition states since a different step (e.g., a conformational change)

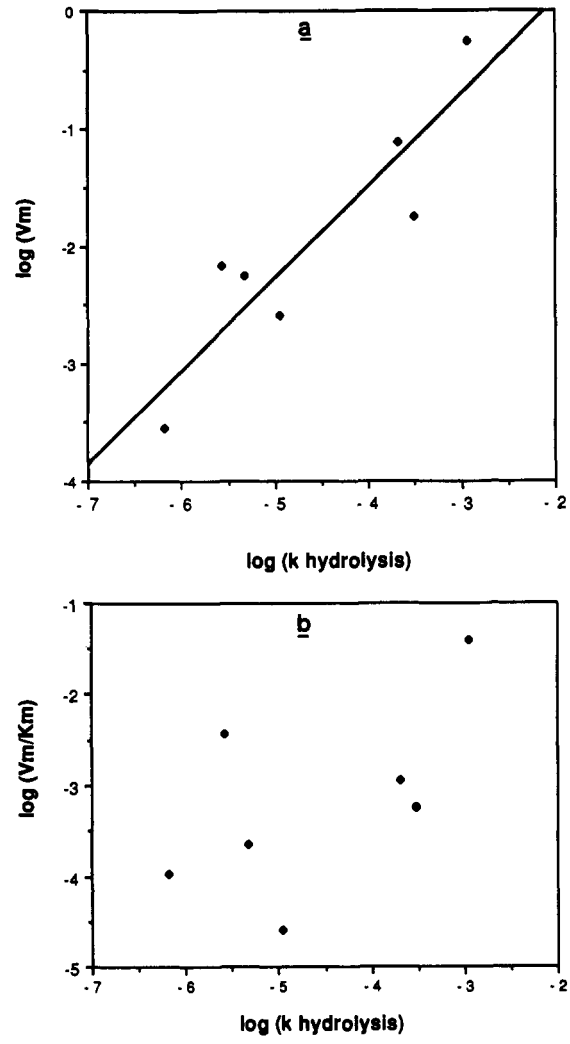


FIGURE 2: Linear free energy correlations between kinetic parameters for the phosphorylase-catalyzed reaction and acid-catalyzed hydrolysis of a series of deoxy- and deoxyfluoro-α-D-glucopyranosyl phosphates: (a) Plot of log V_m for muscle glycogen phosphorylase *b* versus the logarithm of the acid-catalyzed hydrolysis rate constant; (b) plot of log V_m/K_m for muscle glycogen phosphorylase *b* versus the logarithm of the acid-catalyzed hydrolysis rate constant.

may be rate limiting for the natural substrate.

This is, however, inconsistent with *any* truly concerted mechanism, such as that recently proposed (Klein et al., 1986). A less concerted mechanism involving substantial departure of the leaving group prior to attack of the nucleophiles would be compatible. However, it is reasonable to suppose that any such charged transition state would be stabilized electronically by the enzyme.

Binding Effects. As has been discussed many times previously [see, for example, Pauling (1946), Fersht (1985), and Fersht et al. (1986)], enzymes catalyze reactions by binding very specifically to the transition state of the reaction they catalyze, thus stabilizing it and lowering the overall activation free energy. Some very elegant studies involving protein mutagenesis have been performed to measure the strengths of individual hydrogen bonds involved in stabilizing the reaction transition state for the enzyme tyrosyl-tRNA synthetase (Fersht et al., 1986; Fersht, 1987). Certain interactions between enzyme and substrates were found to contribute much more to the binding of the transition state than to the binding of the ground state. This suggests that, during the approach to the transition state, the enzyme takes advantage of the geometrical changes in its substrate to bind it more tightly,

thus facilitating the reaction. A similar situation may obtain with glycogen phosphorylase since the conformation of the sugar ring can be expected to change from its normal chair conformation toward the half-chair conformation of the oxocarbonium ion species at the transition state. Individual hydrogen bonds to sugar hydroxyls might therefore be very important in promoting such a transition. Replacement of a hydroxyl in the sugar substrate, which can both donate and accept hydrogen bonds, by a hydrogen, as in a deoxy sugar, will result in loss of all significant hydrogen bonds at that position. A fluorine substituent, however, can arguably accept hydrogen bonds, but cannot possibly donate them, as demonstrated in recent work on glucose analogues binding to phosphorylase (Street et al., 1986). Therefore, on the basis of binding effects alone, both series (deoxy and deoxyfluoro) should generally be worse substrates than the parent compound, but the deoxy substrates should be poorer than the deoxyfluoro.

The importance of the binding effects is best illustrated by considering initially only the data for the deoxy sugars. On the basis of electronic effects only, the deoxy sugars should be turned over more rapidly than the parent substrate, yet in fact, they are turned over more slowly. This can only be explained if it is assumed that important transition-state interactions at these positions are missing in these cases. The values of $\Delta\Delta G^{\ddagger}$ for the deoxy sugars listed in Table I therefore represent *minimum* estimates of the loss of apparent binding energy at each position at the transition state resulting from removal of the hydroxyl in question, since such substitution has resulted in the observed destabilization of the transition state. The values measured in essence represent the difference in hydrogen-bond strengths between that formed at the transition state between the enzyme and substrate and that formed between the unbound species and water and are therefore referred to as apparent hydrogen-bond strengths. [For a detailed discussion see Fersht (1988).] The values reported here, which will represent the sum of all binding interactions for the hydroxyl in question, are quite large. This is just as might be expected for binding energies *at the transition state*, since such binding should be much tighter than that at the ground state. Apparent binding energies observed are $\approx$6 kcal mol^{-1} for the 3- and 6-positions and 3.7 kcal mol^{-1} for the 4-position.

Previous studies of the apparent strengths of hydrogen bonds between enzymes and ligands have suggested that hydrogen bonds between two neutral partners contribute up to about 1.5 kcal mol^{-1} to overall binding free energy, while those between one neutral and one charged species are worth 3 kcal mol^{-1} or more (Fersht et al., 1985; Street et al., 1986). On that basis, and assuming that lost hydrogen bonding is responsible for the majority of the lost binding energy, this probably suggests that there are several quite strong hydrogen bonds, probably involving charged partners, at each of the 3- and 6-positions and one strong, or several weak, hydrogen bonds at the 4-position. These apparent hydrogen bond strengths are those present at the transition state.

Data on the strength of hydrogen bonds in a ground-state complex are more equivocal. If relative values of K_m and/or K_i are used to calculate differences in interaction energies in the ground-state Michaelis complex, a similar picture emerges. The strongest interactions are those present at the 3- and 6-positions, respectively, while interactions at the 2- and 4-positions are less crucial. However, the presence of any nonproductive binding modes or accumulated intermediates could invalidate these numbers. Indeed, it is important to note that values for K_m and K_i do not all agree well. This is particularly true for 4-fluoroglucose-1-P; thus, these K_i and K_m values were redetermined several times but with essentially the same results. It is possible that this disagreement might be related to the fact that the product of the 4-fluoroglucose-1-P reaction (the 4-fluoroglycogen) will no longer serve as a substrate and thus must dissociate at the end of the catalytic cyclic. This is a complication that would not affect the inhibition constants. This could result in a change of rate-determining step and accumulation of previous intermediates. A further possible cause of differences could be binding of the analogues as inhibitors at the AMP site; indeed, some indications of noncompetitive inhibition were obtained, as mentioned earlier. With these cautions in mind it is interesting that the order of hydrogen-bond strengths found here is similar to that observed in the binding of glucose to the T-state enzyme (Street et al., 1986). In that case, also, the strongest hydrogen bonds measured were those at the 3- and 6-hydroxyls (3.2 and 2.6 kcal mol^{-1}, respectively), with the remainder of the hydrogen bonds being in the 0.5–1.5 kcal mol^{-1} range. Thus, it seems likely that a similar set of hydrogen-bonding partners exists in both the ground-state (glucose-1-P or glucose) and the transition-state (glucose-1-P) complexes but that hydrogen bonds in the latter are stronger. This is quite consistent with expectations that the enzyme binds the transition state preferentially, as suggested by the gluconolactone binding, and provides additional insight into the way in which phosphorylase effects catalysis. The similarity between the ground-state complexes with glucose (T state) and glucose-1-P (R state) suggests that the glucose binding pocket in the T-state enzyme remains essentially intact during the T $\rightarrow$ R conformational transition and becomes the glucose subsite of the glucose-1-P binding site, and this appears to be backed up by the X-ray crystallographic results described below.

Comparison of the hydrogen-bonding pattern predicted in this work with that recently described for the binding of heptulose 2-phosphate, a possible transition-state analogue, to phosphorylase is of interest (McLaughlin et al., 1984; Hajdu et al., 1987; L. N. Johnson, personal communication). Once again, hydrogen bonds involving charged residues (His 377 and Glu 672) are seen at the 6- and 3-positions, respectively, consistent with the strong hydrogen bonds predicted at those positions, and similar to the T-state glucose complex. However, at the current state of refinement Glu 672 appears to be a little further away and interacting also with the 2-hydroxyl. The importance of these interactions is also presumably indicated by the fact that His 377 and Glu 672 (to a lesser extent) both shift upon binding of substrates, the former also affecting the positions of other nearby residues. A second residue at the 2-position, Tyr 573, is also observed to be hydrogen bonded to the phosphate residue of heptulose 2-phosphate. This is quite consistent with the role postulated previously (Madsen & Withers, 1986) for this tyrosine as a possible acid/base catalyst, much as has been proposed for β-galactosidase (Loeffler et al., 1977). This proposal finds further support in the recent findings on base-catalyzed reactivation of pyridoxal(5′)diphospho(1)-α-D-glucose reconstituted phosphorylase (Horinishi et al., 1988). Unfortunately, in the absence of data on 2-deoxyglucose-1-P, there is limited kinetic insight into hydrogen bonding at the 2-position, though the results with 2-fluoroglucose-1-P would suggest that any hydrogen bonding present must be accommodated by the fluorine, thus suggesting the hydroxyl is normally a hydrogen-bond acceptor, consistent with the involvement of tyrosine. Other interactions around the sugar involve neutral hydrogen-bond partners,

consistent with the weaker hydrogen bonds detected at, for example, the 4-position.

In summary, these data provide substantial supportive evidence for a mechanism involving oxocarbonium-ion-like transition states. They also provide some measure of the importance of hydrogen-bonding interactions in the stabilization of the ground-state and transition-state complexes. This permits some quantitation of the contribution of interactions at each individual hydroxyl group to catalysis by the enzyme and provides support for the notion that the glucopyranose site remains intact during the T → R transition.

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Registry No. G1P, 59-56-3; 2-fluoro-G1P, 109959-18-4; 3-fluoro-G1P, 46725-92-2; 4-fluoro-G1P, 109923-28-6; 6-fluoro-G1P, 109923-26-4; 3-deoxy-G1P, 28079-06-3; 4-deoxy-G1P, 28434-41-5; 6-deoxy-G1P, 24809-76-5; glycogen phosphorylase *b*, 9012-69-5.

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