Binding Energy and Catalysis: Deoxyfluoro Sugars as Probes of Hydrogen Bonding in Phosphoglucomutase†

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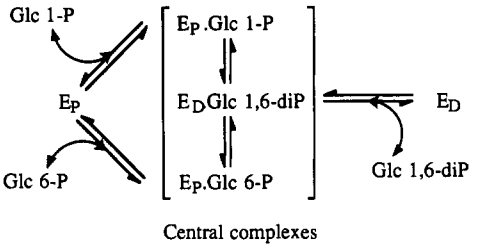
ABSTRACT: Estimates of the contributions of hydrogen-bonding interactions with each of the sugar hydroxyls to the binding of the substrate α-D-glucopyranosyl phosphate both in the ground state and at the transition state for the initial phosphoryl transfer have been obtained by kinetic studies. Michaelis parameters (k_{cat} and K_m) for a complete series of deoxy- and deoxyfluoro-α-D-glucopyranosyl phosphates provide insight into specific interactions with each hydroxyl at the transition state. Inhibition constants (K_i) for a series of deoxygenated and fluorinated analogues of the competitive inhibitor 6-deoxy-6-fluoro-α-D-glucopyranosyl phosphate provide insight into ground-state interactions. Interactions at each hydroxyl are found to strengthen only slightly upon progressing from the ground state to the transition state in contrast to that seen with glycogen phosphorylase [Street et al. (1989) *Biochemistry 28*, 1581] where transition-state interactions became much stronger. This is in accord with the mechanisms for these two enzymes where no distortion of the sugar ring occurs for phosphoglucomutase, whereas considerable distortion is expected for glycogen phosphorylase.

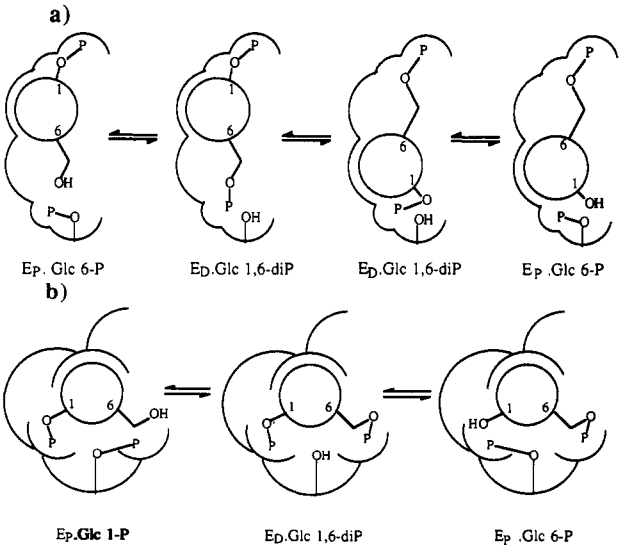
Phosphoglucomutase is a phosphoenzyme which is responsible for the interconversion of glucose-1-P[1] and glucose-6-P. The mechanism is generally considered to proceed via the pathway shown in Scheme I, in which the initial step involves a phosphoryl transfer from the enzyme to the sugar phosphate to form a dephosphoenzyme/glucose-1,6-diP complex (Ray & Peck, 1972). Reorganization of this complex is followed by the transfer back of the other phosphate moiety from the diphosphate to the enzyme to give the alternate glucose monophosphate. While the glucose diphosphate ordinarily dissociates only rarely (approximately 1 in 20 cycles) (Ray & Roscelli, 1964; Britton & Clarke, 1968; Ray et al., 1989), it is required in the reaction mixture to keep the enzyme in its phosphorylated state. Since there is only a single phosphorylation site on the enzyme (serine-116) (Ray et al., 1983), two different mechanisms have been proposed to achieve the reorganization necessary to allow the transfer of either phosphate moiety from the glucose diphosphate (Ray et al., 1973); see Scheme II. The first (Scheme IIa) is the exchange mechanism in which the glucose diphosphate can flip over in the active site without dissociating, thus placing either phosphate adjacent to the serine. The second (Scheme IIb) is the minimal motion mechanism in which the sugar has a single binding mode, and an enzymic conformational change repositions the serine hydroxyl relative to the glucose diphosphate. The minimal motion mechanism has the advantage of requiring only a single sugar binding and recognition mode, while the exchange mechanism requires two such modes. However, the exchange mechanism requires the construction of only a single catalytic site for phosphoryl transfer whereas the alternative requires two such sites. It has been argued (Knowles, 1980) that the construction of a catalytic site is more difficult than construction of a recognition site; thus an exchange mechanism might be expected on this basis. This paper, in part, and the following paper (Percival & Withers, 1992) more fully address this question.

Scheme I: Reaction Scheme for the Interconversion of Glucose-1-P and Glucose-6-P by Phosphoglucomutase[a]



[a] From Ray and Peck (1972).

Scheme II: Schematic Representation of Two Mechanisms of Reorganization of the Dephosphoenzyme/Glucose-1,6-diP Complex in Phosphoglucomutase: (a) Exchange Mechanism; (b) Minimal Motion Mechanism[a]



[a] From Ray et al. (1973).

Binding and specific recognition of the substrate requires that the active site provides appropriately oriented hydro-

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gen-bonding groups to interact with each of the sugar hydroxyls. This is essential for the interaction, since the hydrogen bonds between the sugar hydroxyls and water are lost upon binding and thus *must* be replaced. Any increase in affinity requires improvement of the geometry of each hydrogen bond as well as optimization of hydrogen bond type. Since enzymes catalyze reactions by stabilization of transition states (Pauling, 1946; Jencks, 1975; Fersht, 1985), and since such stabilization is frequently achieved through utilization of binding energy, it might be expected that individual hydrogen bonds would be stronger at the transition state than they were in the ground-state complex. However, this will probably be true to a significant extent only if the portion of the substrate under investigation undergoes a change in conformation in going from the ground state to the transition state, since only then can the enzyme exploit differential binding of the ground state versus the transition state.

The usage of the intrinsic binding energy by phosphoglucomutase has been explored previously by Ray and coworkers (Ray & Long, 1976a,b; Ray et al., 1976), but at a lower "resolution" since they did not measure contributions of individual hydroxyl groups on the sugar, but rather the separate contributions of the phosphate and sugar moieties. This was achieved by a study of the half-reaction involving the transfer of the phosphoryl moiety of the phosphoenzyme to a variety of acceptors ranging from the substrate sugar phosphates (glucose-1-P and glucose-6-P) to water. It was found that the enzyme effects a phosphoryl transfer to the 6-hydroxyl group of glucose-1-P some (3×10^{10})-fold faster than to water, but that the hydrolysis rate (intrinsic hydrolysis rate constant = 3×10^{-8} s^{-1}) could be augmented considerably by the coaddition of sugar or phosphate analogues. The effects of the noncovalent interaction on the hydrolysis rate could be enormous, an enhancement of approximately 10^3-fold being provided by simple phosphate analogues which presumably bind in the other phosphate site, and a factor of (2×10^5)-fold being observed in the presence of xylose-1-P. Similarly, rates of phosphoryl transfer from the phosphoenzyme to free sugars, sugar phosphates, and simple alcohols were measured and also found to be dependent upon structure, hence upon the ability to employ intrinsic binding energy. For example, phosphoryl transfer to the 1-hydroxyl of xylose occurred with a k_{cat} of 2.1×10^{-3} s^{-1}, but when phosphite was also included in the reaction mixture, it occurred at a rate of 60 s^{-1}. This latter rate is approximately 0.2 of that for phosphoryl transfer to the 1-hydroxyl of glucose-6-P. On the basis of a series of such experiments, values for the intrinsic binding energy provided by interaction with the sugar and phosphate moieties have been forwarded, and their sum suggested to account for essentially the entire rate difference of 3×10^{10} between the normal phosphoryl transfer and hydrolysis.

In this paper we present our results on the measurement of hydrogen bond strengths in both ground-state and transition-state complexes of phosphoglucomutase. This is achieved in an equivalent fashion to that employed previously for similar studies on glycogen phosphorylase (Street et al., 1986, 1989), by kinetic studies with deoxygenated and fluorinated substrates and inhibitors. The choice of such substitutions for the sugar hydroxyls is based upon their sterically conservative nature and upon their different capabilities for hydrogen bonding. As discussed in more detail elsewhere (Street et al., 1986, 1989), the hydrogen substituent in a deoxy sugar cannot be involved in any significant hydrogen bonding whereas the fluorine substituent can arguably act as a hydrogen bond (proton) acceptor, but cannot possibly act as a donor. Thus a deoxy substrate in which one particular hydroxyl has been replaced by hydrogen will not be able to engage in any hydrogen bonding at that position. Reductions in rate (V_{max}/K_m) as a consequence of that substitution might therefore be ascribed to the loss of hydrogen bonding at that position in the transition-state complex. In the case of the fluoro sugars, hydrogen bonding may still be possible, so the absence of a rate reduction for the fluoro sugar does not necessarily indicate that there is no important hydrogen bonding. However, should the deoxy substrate be slow and the corresponding fluoro substrate be fast, then it would indicate that the polarity of the hydrogen bond is such that the enzyme is acting as the proton donor.

Experimental Procedures

Syntheses. The syntheses of all the modified α-D-glucopyranosyl phosphates have been described previously (Withers et al., 1986, 1989; Percival & Withers, 1988). The syntheses of 1-deoxy-D-glucose 6-phosphate (1,5-anhydroglucitol 6-phosphate) and α-D-glucopyranosyl fluoride 6-phosphate were achieved from the appropriate nonphosphorylated precursor sugar (Street et al., 1986; Hayashi et al., 1984) by phosphorylation with ATP using hexokinase in the presence of necessary cofactors essentially according to a published method (Drueckhammer & Wong, 1985). Thus, previously dialyzed hexokinase (Sigma H-5500, 1 mg) was added to 1-deoxy-D-glucose (1,5-anhydro-D-glucitol) (75 mg, 50 mM) plus ATP (55 mM) in 0.25 M triethanolamine buffer, pH 7.6, containing 5 mM magnesium chloride, the pH readjusted to pH 7.6, and the solution incubated at room temperature. The pH was occasionally readjusted during incubation. Upon completion of reaction, protein was removed using a Centricon microconcentrator and the solution diluted to 200 mL with water and applied to a DE-52 cellulose column (1.8 × 30 cm) equilibrated with 40 mM ammonium bicarbonate, pH 8.0. Washing with 200 mL of water was followed by elution with a linear gradient of 0–0.2 M ammonium bicarbonate in a volume of 2 L. Buffer was removed from the desired fractions by repeated lyophilization and the isolated product characterized by ^{31}P NMR. The phosphorylation of α-glucosyl fluoride was carried out in a similar manner, but with slightly different reaction conditions. The reaction mixture contained α-glucosyl fluoride (20 mg, 0.2 M), ATP (0.22 M), hexokinase (Sigma H-5875, 0.3 mg), and acarbose (a generous gift of Bayer Chemical Co.) (2 mM). Products had the following NMR parameters.[2] 1-Deoxy-D-glucose 6-phosphate: ^{31}P NMR (121 MHz, D_2O buffer, pH 6.8) δ −6.79 (t, $J_{P,H-6,6'}$ = 6 Hz). α-D-Glucosyl fluoride 6-phosphate: ^{31}P NMR δ −6.83 (t, $J_{P,H-6,6'}$ = 6 Hz), ^{19}F NMR (254 MHz, D_2O buffer, pH 6.8) δ 157 (dd, $J_{F,H-1}$ = 53.4, $J_{F,H-2}$ = 25.9 Hz).

Synthesis of 3-deoxy-3-fluoro-α-D-glucopyranosyl 6-phosphate (tetraammonium phosphate) (3-fluoroglucose-1,6-diP) was achieved by a modification of the method of Hanna and Mendocino (1970). 3-Deoxy-3-fluoro-D-glucose 6-phosphate pyridinium salt (0.52 mmol) (Drueckhammer & Wong, 1985) was thoroughly dried by suspending in and evaporating from dry pyridine (5 mL) three times. It was then suspended in a further 6 mL of dry pyridine, and acetic anhydride (2 mL)

[1] Abbreviations: glucose-1-P, α-D-glucopyranosyl phosphate; deoxyglucose-1-P, deoxy-α-D-glucopyranosyl phosphate; fluoroglucose-1-P, deoxyfluoro-α-D-glucopyranosyl phosphate; glucose-6-P, D-glucose 6-phosphate; glucose-1,6-diP, α-D-glucose 1,6-bisphosphate.

[2] NMR chemical shifts are referenced to the following materials: ^{19}F NMR referenced to $CFCl_3$, δ = 0 ppm; ^{31}P NMR referenced to H_3PO_4, δ = 0 ppm; ^{1}H NMR referenced to DSS, δ = 0 ppm.

was added and stirred rapidly at room temperature until dissolved. After 2 days at 4 °C under anhydrous conditions the solvents were evaporated and the resulting gum dried in vacuo over phosphorus pentoxide for 24 h. The dry gum was dissolved in tetrahydrofuran (10 mL), anhydrous phosphoric acid (0.35 g) was added, and after evaporation of solvent, the resulting gum was heated to 55 °C in vacuo for 2.5 h. Ice-cold 2 M lithium hydroxide solution was added, the pH adjusted to 10.0, and the solution left overnight at room temperature. Lithium phosphate precipitate was removed by filtration, the pH adjusted to 8.0, and the solution applied to a DE-52 cellulose column (1.6 × 30 cm) equilibrated in 50 mM ammonium bicarbonate buffer, pH 8.0 at 4 °C. After washing with water, the product was eluted with a linear gradient (0–0.3 M) of ammonium bicarbonate, the bisphosphate eluting at approximately 0.15 M salt. Rechromatography on the same system, but with a 0.05–0.25 M gradient, followed by lyophilization yielded a white powder containing the desired material and about 16% of its β-anomer. The α-anomer was characterized by NMR as follows: ^{1}H NMR (300 MHz, D_2O) δ 5.25 (dt, 1 H, $J_{1,P}$ = 7.0, $J_{1,2}$ = 3.3, $J_{1,F}$ = 3.3 Hz, H-1), 4.65 (dt, 1 H, $J_{3,F}$ = 51.0, $J_{3,4}$ = 8.9, $J_{3,2}$ = 8.9 Hz, H-3), 4.2–3.8 (m, 5 H, H-2, -4, -5, -6, -6′); ^{31}P NMR (121 MHz, D_2O buffer, pH 6.8, {^{1}H}) δ −7.20 (P-6), −5.04 (P-1): ^{19}F NMR (254 MHz, D_2O buffer, pH 6.8) δ 201.29 (dt, $J_{F,3}$ = 55, $J_{F,2}$ = 13, $J_{F,3}$ = 13 Hz). The β-anomer was characterized as follows: ^{31}P NMR (121 MHz, D_2O buffer, pH 6.8, {^{1}H}) δ −7.20 (P-6), −5.48 (P-1); ^{19}F NMR (254 MHz, D_2O buffer, pH 6.8) δ 196.03 (dt, $J_{F,3}$ = 54, $J_{F,2}$ = 13, $J_{F,3}$ = 13 Hz).

Enzyme Studies. Rabbit muscle phosphoglucomutase was prepared according to a procedure kindly provided by Prof. W. J. Ray, Purdue University. In all cases phosphoglucomutase was preactivated prior to assaying by the method of Peck and Ray (1971) and concentrations of enzyme were determined from their absorbance at 278 nm, a 1% w/v solution having an absorbance of 7.0 (Ray et al., 1983). Demetallated enzyme was prepared according to Rhyu et al. (1984).

Concentrations of glycosyl phosphates were determined by assay of acid-labile phosphate according to Peck and Ray (1971) while inorganic phosphate was determined according to Fiske and Subbarow (1925). Acid-stable phosphate esters were determined according to Bartlett (1959).

Kinetic Studies. (*A*) *Substrates.* All assays of phosphoglucomutase were performed in a buffer containing 25 mM Tris, 1.3 mM EDTA, and 2.5 mM $MgCl_2$ at pH 7.40. The activity of phosphoglucomutase at saturating substrate and cofactor concentrations was determined by the colorimetric assay according to Peck and Ray (1971). Time courses and values of V_{max} and K_m for each modified substrate were determined according to the method of Ray and Roscelli (1964) at 6–10 different concentrations of each substrate. This assay involves the determination of residual acid-labile phosphate after allowing the glycopyranosyl phosphate to be converted to the acid-stable sugar 6-phosphate for known periods of time by phosphoglucomutase. Acid hydrolysis times were extended for the deoxyfluoroglycopyranosyl phosphates to allow for the known (Withers et al., 1986) slower hydrolysis rates, as follows: 2-fluoro, 30 min; 3-fluoro, 20 min; 4-fluoro, 20 min. All deoxyglycopyranosyl phosphates were hydrolyzed for 10 min. Data were analyzed by a weighted Lineweaver–Burk analysis.

(*B*) *Inhibitors.* Rates of conversion of glucose-1-P to glucose-6-P were determined at 5 different substrate concentrations (typically 5–50 μM) at each of 5–6 different inhibitor concentrations. Results were plotted using a weighted Lineweaver–Burk analysis to give $K_{m,app}$ values which were replotted to give the K_i value for the inhibitor. A coupled enzyme assay was used for these studies in which the assay mixture contained the following: 0.4 mL of assay mixture containing 25 mM Tris buffer, pH 7.4, 2.5 mM $MgCl_2$, 1.3 mM EDTA, 30 μM NADP, 0.4 unit/mL glucose-6-phosphate dehydrogenase (Sigma G-5760), and 1.3 μM glucose-1,6-diP; 0.1 mL of phosphoglucomutase (approximately 0.006 unit/mL) in activation buffer; and 0–50 μL of inhibitor in 25 mM Tris, pH 7.4. A total volume of 0.6 mL was employed, the remainder of the volume being made up with 25 mM Tris buffer, pH 7.4. Reactants were incubated in cuvettes for 10 min prior to initiation of the reaction by addition of substrate, and reaction was followed by the change in absorbance at 340 nm. Suitable controls were performed to ensure that no reagents were rate-limiting, that the inhibitor did not affect the coupling enzyme, and that the rate of anomerization of glucose-6-P was not rate-determining. This coupled assay was also used in some of the work to follow time courses.

UV Spectral Studies. Spectra were recorded on a Pye-Unicam PU 8800 double-beam spectrophotometer using a band path width of 0.5 nm and a scanning speed of 0.2 nm/s. Difference spectra were recorded in a split cell at 25 °C in 20 mM Tris buffer, pH 7.5, containing 1 mM EDTA at a final enzyme concentration of approximately 30 μM. After thermal equilibration for 10 min in the cell block, spectra were recorded and the data transferred to an Apple IIe computer. Difference spectra were calculated using the program Vidichart. When necessary, multiple spectra were recorded and summed using this program to improve signal to noise ratio.

Measurement of Equilibrium Constants. The equilibrium constant for the isomerization of each fluorinated glucose-1-P with its 6-phosphate was determined by ^{19}F NMR. Reaction mixtures containing the fluoroglucose-1-P and catalytic amounts of glucose-1,6-diP and phosphoglucomutase were incubated at 30 °C for several hours at pH 7.4 in the assay buffer. Proton-decoupled ^{19}F NMR spectra of each mixture were then measured and relative concentrations of each species determined from both peak heights and integration. Each spectrum was redetermined 1 h later to ensure that equilibrium had been achieved. In addition, a third spectrum was run in each case in which the pulse delay was tripled to ensure that differences in T_1 values did not invalidate the results.

Results

Syntheses. Successful syntheses of all the glycopyranosyl phosphates have been demonstrated previously (Withers et al., 1986, 1989), but syntheses of the glucose 6-phosphate analogues described here deserve comment. In previous studies of the specificity of hexokinase (Bessel & Thomas, 1973; Machado de Domenech & Sols, 1980; Drueckhammer & Wong, 1985) it had been shown that 1,5-anhydro-D-glucitol (1-deoxy-D-glucose) was slow substrate, but that α-D-glucopyranosyl fluoride had no substrate activity. We were, however, successful in utilizing hexokinase to phosphorylase α-glucosyl fluoride by using high (0.22 M) concentrations of substrate. Since commercial hexokinase is contaminated with a small amount of α-glucosidase which could rapidly hydrolyze the glucosyl fluoride prior to phosphorylation, all the hexokinase fractions available from Sigma Chemical Co. were assayed for α-glucosidase activity using *p*-nitrophenyl α-D-glucoside as substrate, and the one with lowest activity (H-5875) was selected. Phosphorylation with hexokinase was then performed in the presence of acarbose, a potent α-glucosidase inhibitor, to minimize any residual hydrolytic activity, allowing synthesis of the desired product in modest yields.

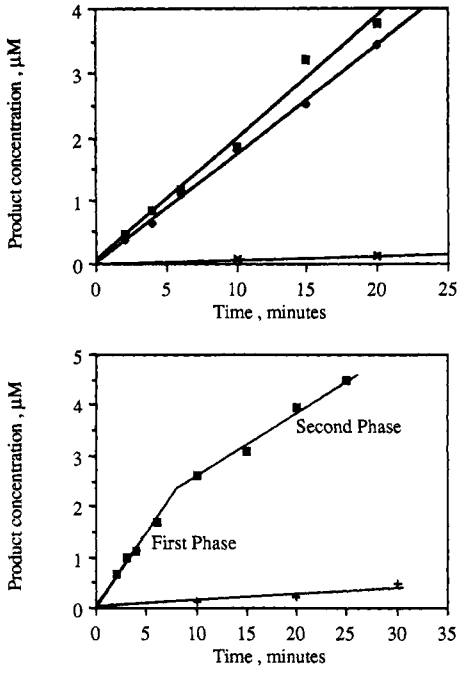


FIGURE 1: Plot of product versus time determined for the reaction of fluoroglucose-1-P analogues with phosphoglucomutase in the presence of different concentrations of glucose-1,6-diP activator. (a) 4-Fluoroglucose-1-P (25 μM) with the following concentrations of glucose-1,6-diP: (×) 0.0 μM; (◆) 0.04 μM; (■) 2.5 μM. (b) 3-Fluoroglucose-1-P (25 μM) with the following concentrations of glucose-1,6-diP: (+) 0.04 μM; (■) 2.5 μM.

Kinetic Studies. (A) Reaction Time Courses. All the glucose-1-P analogues, with the obvious exception of those substituted at the 6-position, were found to be substrates of phosphoglucomutase. In order to perform accurate determinations of V_{max} and K_m, it was necessary to first demonstrate that linear initial rate kinetics could be observed for a reasonable percentage of the reaction course; thus time courses were determined for all substrates. Since the reaction with these modified sugar phosphates must proceed via the corresponding modified glucose-1,6-diP, but since such activators were not available to add to the reaction mixtures, glucose-1,6-diP was added instead. Time courses were determined at two different concentrations of this activator. Linear time courses to at least 15% reaction at both low (0.04 μM) and high (2.5 μM) concentrations of glucose-1,6-diP were seen with all of the 2- and 4-substituted glucose-1-P analogues. Essentially equal rates of reaction were seen at these two activator concentrations, as shown in Figure 1a for 4-fluoroglucose-1-P, while no significant reaction was seen in the absence of activator. In the case of 3-deoxy- and 3-fluoroglucose-1-P biphasic reaction kinetics were observed with a fast initial phase followed by a slower second phase. In addition, the reaction in the presence of the lower concentration of glucose-1,6-diP was much slower than at the higher concentration. Figure 1b shows an example of such time courses for 3-fluoroglucose-1-P; an essentially identical pattern was seen for the 3-deoxy analogue.

In a separate set of experiments to probe the cause of the biphasic plots (data not shown), the release of product as a function of time was monitored at a single concentration (100 μM) of each of the fluoroglucose-1-P analogues at several different concentrations (2–20 μM) of glucose-1,6-diP. The colorimetric assay for acid-labile vs acid-stable sugar phosphates was used for this experiment. Time courses were monitored until 40 μM of the 6-phosphate had been produced, and these were found to be linear for all the 2- and 4-substituted subtrates. However, in the case of 3-fluoroglucose-1-P biphasic plots were obtained, with a large decrease in reaction rate occurring at a product concentration corresponding to the amount of glucose-1,6-diP initially included in the reaction mixture. Further, the rate of this second phase increased with increasing glucose-1,6-diP concentration up to 5 μM, but was not significantly increased at higher diphosphate concentrations than this.

These latter experiments were repeated using the direct coupled assay in place of the colorimetric assay as a means of identifying the sugar phosphate product being released at different times. A large amount of the coupling enzyme, glucose-6-phosphate dehydrogenase, was used to compensate for its low activity with the fluorinated glucose-6-P analogues. Essentially identical results to those just described were obtained for the 3-fluoro- and 4-fluoro substrates. Linear traces throughout the reaction were obtained for the 4-fluoroglucose-1-P and biphasic plates for the 3-fluoroglucose-1-P, with break points at a product concentration equal to the concentration of glucose-1,6-diP added. In the case of 2-fluoroglucose-1-P, nonlinear kinetics were observed, similar to those seen for the 3-fluoro substrate. However, in this case the biphasic behavior is due to the fact that 2-fluoroglucose 6-phosphate is a much poorer substrate for glucose 6-phosphate dehydrogenase than the other fluoroglucose 6-phosphates (Bessel & Thomas, 1973) and thus is not turned over significantly under these conditions. This confirms that the initial product obtained is glucose-6-P, but that once the glucose-1,6-diP has been exhausted, the product becomes 2-fluoroglucose-6-P as will be discussed later.

Redetermination of the time course for 3-fluoroglucose-1-P in the presence of a saturating (>5 μM) concentration of synthetically prepared 3-fluoroglucose-1,6-diP in place of glucose-1,6-diP eliminated the break point, giving a single rate equal to that of the slower phase of the reaction.

(B) V_{max} and K_m Determinations. The values of V_{max} and K_m for these substrate analogues were determined as follows, using the same colorimetric procedure. For the 2- and 4-substituted substrates 1 μM glucose-1,6-diP was added and a complete time course determined for the lowest concentration of substrate to be used in the assay. Since this course was found to be linear to at least 20% reaction, rates at all other concentrations were determined using a single assay interval such that conversion of substrate to product was approximately 20% in all cases. Determination of accurate rates for 2-deoxyglucose-1-P was very difficult due to the extreme lability of this compound (Percival & Withers, 1988). Higher enzyme concentrations and shorter (1–2-min) assay intervals were employed to minimize spontaneous hydrolysis, but results were nonetheless considerably less accurate in this case. The kinetic parameters for the 3-substituted analogues were obtained by measuring full time courses containing both phases of the reaction at each substrate concentration in the presence of a constant (5 μM) concentration of glucose-1,6-diP. The K_m and V_{max} values for 3-fluoroglucose-1-P were also determined in the presence of 5 μM 3-fluoroglucose-1,6-diP. In this case linear plots were obtained allowing simple determination of kinetic parameters. Finally, values of the apparent V_{max} and apparent K_m for 3-fluoroglucose-1,6-diP were determined at a single concentration (503 μM) of 3-fluoroglucose-1-P by the same method. Values of the true V_{max} and K_m were calculated from these according to Ray and Roscelli (1964). All values of V_{max} and K_m determined are presented in Table I.

(C) Inhibition Constants. The K_i values for the 1- or 6-substituted glucose phosphates determined as described earlier

Table I: Kinetic Constants Determined for the Deoxygenated and Fluorinated Glucose 1-Phosphate Analogues with Phosphoglucomutase

substrate	K_m[a] (μM)	V_{max}[a] [μmol/(min·mg)]	V_{max}/K_m [L/(min·mg)]	$\Delta\Delta G^{\circ\dagger}$ (kcal/mol)[b]
glucose-1-P	13 ± 2	875 ± 64	67	NA
2-fluoroglucose-1-P	222 ± 66	24 ± 5	0.11	3.8
3-fluoroglucose-1-P (first phase)	128 ± 22	64 ± 6	0.5	2.9
3-fluoroglucose-1-P (second phase)[c]	113 ± 9	7.6 ± 0.3	0.07	4.1
4-fluoroglucose-1-P	217 ± 44	31 ± 5	0.14	3.6
2-deoxyglucose-1-P	60 ± 15	43 ● 8	0.7	2.7
3-deoxyglucose-1-P (first phase)	153 ± 40	68 ± 12	0.44	3.1
3-deoxyglucose-1-P (second phase)	220 ± 20	9 ● 0.6	0.04	4.5
4-deoxyglucose-1-P	153 ± 26	17 ± 2	0.11	3.8
3-fluoroglucose-1,6-diP	0.9 ± 0.1	4.5 ± 0.4	NA	NA

[a] At pH 7.4 and 30 °C. [b] Values calculated from $RT \ln [(V_{max}/K_m)_1/(V_{max}/K_m)_2]$, where T = 303 K, R = 1.987 cal/(K·mol), 1 refers to the native substrate glucose-1-P, and 2 refers to the modified substrate. [c] This value was determined with 3-fluoroglucose-1,6-diP as the cofactor. A similar value was obtained when glucose-1,6-diP was used instead, this value being obtained from the second phase of the biphasic plots.

Table II: Inhibition Constants Determined for Deoxygenated and Fluorinated Glucose Phosphate Analogues with Phosphoglucomutase

inhibitor	K_i (μM)[a]	$\Delta\Delta G^{\circ}$ (kcal/mol)[b]	DBE[c] (kcal/mol)
6-fluoroglucose-1-P	20		
6-deoxyglucose-1-P	73	0.7	
2,6-difluoroglucose-1-P	2430	2.8	1.0
3,6-difluoroglucose-1-P	1710	2.6	0.3
3-deoxy-6-fluoroglucose-1-P	1350	2.6	0.5
4,6-difluoroglucose-1-P	720	2.1	1.5
4-deoxy-6-fluoroglucose-1-P	740	2.1	1.7
α-glucosyl fluoride-6-P	41	0.5	
1-deoxyglucose-6-P	32	0.3	

[a] At pH 7.4 and 30 °C. [b] Values calculated from $RT \ln (K_1/K_2)$, where T = 303 K, R = 1.987 cal/(K·mol), K_1 is the K_i of the inhibitor, and K_2 is the K_i of 6-fluoroglucose-1-P, 20 μM. [c] DBE = differential binding energy ($\Delta\Delta G^{\circ\dagger} - \Delta\Delta G^{\circ}$), the additional binding energy expressed at the transition state above that expressed at the ground state.

are shown in Table II. All inhibitors were found to be competitive with substrate. Values of $\Delta\Delta G^{\circ}$ were calculated as described in the table footnotes and refer to differences in binding free energy between the modified substrate and its parent sugar phosphate.

(D) Equilibrium Constants. Equilibrium constants determined for the isomerization of each fluoroglucose-1-P with its 6-phosphate are the following, expressed as the ratio of the 6-phosphate to the 1-phosphate: 2-fluoro, 23:1; 3-fluoro, 14:1; 4-fluoro, 9:1. These can be compared with the value of 18.7:1 determined (Post et al., 1989) for the parent glucose phosphates.

UV Spectroscopy. UV difference spectroscopy was used to compare the binding modes of selected inhibitors used in this study with similar data determined previously (Ma & Ray, 1980) for the binding of the two parent sugar phosphates. The 3,6-difluoroglucose-1-P was chosen as representative of the inhibitors in question, and the majority of the studies were performed with this compound. The difference spectrum obtained by subtraction of the spectrum of the phosphoenzyme from that of the phosphoenzyme–3,6-difluoroglucose-1-P complex is shown in Figure 2a. Immediately below, in Figure 2b, is shown the difference spectrum obtained by subtraction of the spectrum of the phosphoenzyme from that of the phosphoenzyme–methylphosphonate complex. The spectrum produced by binding of 3-fluoroglucose-1-P to demetalated phosphoglucomutase (not shown) was identical to that in Figure 2a. Concentrations of enzyme and inhibitor employed are shown in the figure legend.

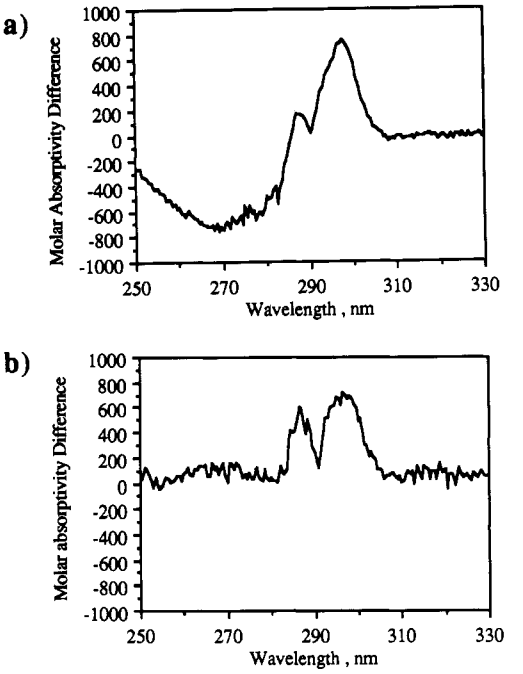


FIGURE 2: Inhibitor-induced UV/vis difference spectra produced by the binding of inhibitors to demetalated phosphoenzyme. (a) 3,6-Difluoroglucose-1-P. The concentration of enzyme was 38.1 μM, and that of inhibitor, 20 mM. (b) Methylphosphonate. The concentration of enzyme was 24.6 μM, and that of inhibitor, 30 mM.

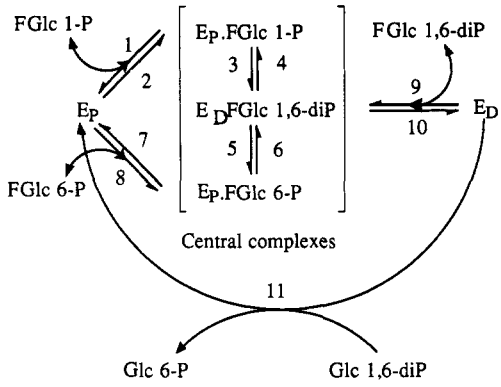
Discussion

All the glucose-1-P analogues, with the obvious exception of those substituted at the 6-position, were found to be substrates of phosphoglucomutase. Both 2- and 3-deoxyglucose-1-P have been shown previously (Egyud & Whelan, 1963) to be substrates, but no kinetic parameters were determined. In other work on the phosphoglucomutase from *Saccharomyces cerevisiae* (Wright et al., 1972) it was found that 3-fluoroglucose-1-P was not a substrate and that 3-fluoroglucose-6-P was a very weak inhibitor of the enzyme. Differences from our findings may be related to the source of the enzyme, or to the coupled assay they used.

Since it was possible that the substitution of fluorine at different positions around the ring might affect the equilibrium constant for the interconversion of the 1- and 6-phosphates, these values were determined by ^{19}F NMR in each case. There was a surprisingly large variation in the numbers, which is not readily understood, but the most important point for this study is that all the equilibria still lie heavily in favor of the 6-phosphate species; thus kinetic analyses can be performed in the same manner as for the parent substrate.

Kinetic Mechanism for Substituted Substrates. The observed kinetic behavior of these substituted glucose-1-P analogues is complicated by the lack of the appropriately substituted glucose-1,6-diP necessitating the use of glucose-1,6-diP instead. The consequence of this is that the reaction pathway

Scheme III: Reaction of Phosphoglucomutase with a Fluoroglucose-1-P Analogue in the Presence of Glucose-1,6-diP



is diverted, at least initially, as shown in Scheme III. Although the following diagrams and discussions refer to fluorinated substrates, *exactly* the same situation applies to the deoxy analogues. In the first step fluoroglucose-1-P binds to, and is phosphorylated by, the phosphoenzyme, giving the dephosphoenzyme/fluoroglucose-1,6-diP complex. Since there is no fluoroglucose-1,6-diP in the reaction mixture, it dissociates from the enzyme and is replaced by glucose-1,6-diP which rephosphorylates the enzyme and then dissociates as glucose-6-P. This latter step is very fast and would probably not be rate-limiting (Lowry & Passonneau, 1969; Ray et al., 1989). Thus the reaction follows steps 1, 3, 9, and 11 in the diagram with the net result shown:

$$\text{FGlc-1-P} + \text{Glc-1,6-diP} \rightarrow \text{FGlc-1,6-diP} + \text{Glc-6-P}$$

This reaction will continue until the glucose-1,6-diP is used up, or until the concentration of the fluoroglucose-1,6-diP reaches a sufficient level [when $(k_{cat}/K_M)_{FG\text{-}1,6\text{-}diP}$[FG-1,6-diP] > $(k_{cat}/K_m)_{G\text{-}1,6\text{-}diP}$[G-1,6-diP]] for it to compete with the glucose-1,6-diP. At this point the reaction will gradually change over to its normal pathway (1, 3, 5, and 7), with the fluoroglucose-1-P as substrate and fluoroglucose-6-P as product as was shown for 2-fluoroglucose-1-P by following time courses using both the colorimetric and the coupled assays. The fact that there is no change in the colorimetrically determined rate for the 2- and 4-substituted substrates upon depletion of the diphosphate suggests that the rate-determining step for these two substrates is the initial phosphoryl transfer since this is the only step common to both pathways. Likewise, the *first* phase of the biphasic kinetics observed for the 3-substituted sugars must also represent the rate of initial phosphorylation of the sugar phosphate by the enzyme. However, in this case once the glucose-1,6-diP has been consumed, a subsequent step becomes rate-limiting: either the reorganization of the dephosphoenzyme/glucose-1,6-diP complex or the second phosphoryl transfer. Distinguishing between these two possibilities would be a nontrivial task, but the finding that 3-fluoroglucose-1,6-diP binds relatively weakly (vide infra) lends support to the notion that it is the reorganization which becomes rate-limiting. In accord with this explanation (though not proving it), monophasic kinetics are observed when 3-fluoroglucose-1,6-diP is used as the activator for turnover of 3-fluoroglucose-1-P, with a rate equal to that of the slower second phase observed previously.

Diphosphate Binding. The kinetic pattern just described requires that the 2- and 4-substituted diphosphate species bind very tightly to the dephosphoenzyme while the 3-substituted species binds quite poorly. In fact, since the maximum concentration of the substituted diphosphate that can be produced in the first phase is equal to the concentration of glucose-1,6-diP initially added, the 2- and 4-substituted diphosphates must bind with a K_d significantly lower than 0.04 μM since the rate of reaction observed at 0.04 μM glucose-1,6-diP is almost the same as that at higher concentrations; thus sufficient diphosphate has been generated at this lower concentration to saturate the enzyme. [Self-generation of glucose-1,6-diP from the phosphoenzyme is quite insignificant at the low (0.001 μM) enzyme concentration employed.] The 3-substituted diphosphate must bind much more weakly since the rate of the second phase for 3-substituted substrates was found to depend upon the concentration of glucose-1,6-diP added, at least up to a concentration of approximately 5 μM. This postulated poor binding of the 3-fluoroglucose-1,6-diP was confirmed by measuring its K_m, the value of 0.9 μM determined being quite consistent with the above findings. The K_m of glucose-1,6-diP has been determined previously (Ray & Long, 1976b) to be 0.01 μM, thus the 2- and 4-substituted diphosphates must bind with a surprisingly similar affinity to that of the parent diphosphate (K_m values less than 0.04 μM), while the 3-fluoroglucose diphosphate (and presumably also the 3-deoxyglucose diphosphate) binds some 90-fold more weakly. This suggests that the interactions between the protein and the diphosphate at the 3-position are more important for the binding (and possibly the reorganization) of the diphosphate than those at the other positions. This may have important implications for the mode of reorganization of the central complex, as is discussed later.

V_{max} and K_m Values: Transition-State Binding. On the basis of the previous discussion it is assumed that the initial phosphoryl transfer is the rate-determining step for the 2- and 4-substituted substrates and for the initial phase with the 3-substituted substrates. This assumption simplifies the interpretation since all data then refer to a single, known step in the mechanism. This initial phosphoryl transfer occurs at a fairly similar rate for all the analogues; some 2–8% of the rate observed for glucose-1-P (for which this step is not necessarily rate-determining) and K_m values are also quite similar, being some 8–20-fold higher than that for glucose-1-P. Values of V_{max}/K_m are perhaps the most readily interpreted since they reflect the activation barrier from free enzyme plus substrate to the transition state for the initial phosphoryl transfer, and *increases* in this activation barrier resulting from substitution at each hydroxyl are shown in Table I. As discussed previously (Street et al., 1989), these numbers provide an estimate of the "cost" of removing specific interactions at each hydroxyl; thus conversely they provide an estimate of the extent to which these interactions stabilize the transition state for the normal substrate. In similar studies on glycogen phosphorylase (Street et al., 1989), these binding effects were masked somewhat by intrinsic electronic effects arising from the considerable differences in electronegativity of the hydrogen, hydroxyl, and fluorine substituents and their effect upon a highly positively charged transition state. Such electronic effects should be relatively insignificant in the case of phosphoglucomutase since the reaction mechanism involves essentially no charge buildup at the anomeric centers; indeed, similar phosphoryl-transfer reactions have been demonstrated previously to be quite insensitive to electronic effects (Bunton et al., 1958; Withers et al., 1986). Values of $\Delta\Delta G^{\dagger}$ shown in Table I therefore represent the *effective* strengths of interactions (most likely hydrogen bonds) between the enzyme and the substrate at each hydroxyl *at the transition state.*

Inhibition Constants: Ground-State Binding. Since K_m values are not reliable indicators of ground-state affinity, a

series of specific ground-state inhibitors of the enzyme which are similarly modified was synthesized to provide more reliable insight into interactions at each hydroxyl. The synthesis and testing of these disubstituted inhibitors were based upon the observation that substitution of the 6-hydroxyl by hydrogen or fluorine did not significantly affect binding (see Table II), allowing the construction of a series of inert substrate analogues which are sequentially substituted at the other hydroxyls, but still bind. The data for these inhibitors are presented in Table II along with values of $\Delta\Delta G°$, the decrease in binding free energy resulting from each substitution. The missing analogue is 2-deoxy-6-fluoroglucose-1-P which was not synthesized due to anticipated lability. Losses of binding energy are similar at each of the secondary hydroxyls and quite modest, with the greatest being one of less than 3 kcal/mol at the 2-hydroxyl. Further, the effects of replacing the hydroxyl by fluorine or hydrogen are essentially identical, suggesting that all these hydrogen bonds involve the sugar hydroxyl acting principally as the H-bond (proton) donor. Any concern that the similarity of these dissociation constants to each other, and to the values for simple phosphate and phosphonate esters (Ray et al., 1973), might indicate that these inhibitors are binding only through their phosphate moieties, with no recognition of the sugar, is allayed by the results of the UV–visible spectral studies which clearly indicate that they bind in the same mode as glucose-1-P. The effects of substitution at the 1- and 6-positions are quite similar as would be expected since these two hydroxyls must have very similar environments in their respective productive complexes, poised for attack upon the enzymic phosphate group. They also indicate very little loss of interaction energy, which is quite reasonable as this hydroxyl would probably not be involved in strong ground-state interactions since these would have to be broken in proceeding to the transition state; an unfavorable situation. It is, however, interesting to note that the relative binding affinities of the 1- and 6-phosphates are inverted upon deoxygenation (but not fluorination) of the nucleophilic center, suggesting small differences in environment.

Use of Binding Energy in Catalysis. The utilization of binding energy in catalysis can be achieved in two general ways [see, for example, Fersht (1985) and references therein]. First, uniform binding of ground states and transition states by provision of a suitable binding environment to accommodate both states equally will effect catalysis by lowering the overall activation energy relative to the free enzyme plus substrate. Second, the enzyme can take advantage of changes in geometry or charge distribution of the substrate on arriving at the transition state by providing a binding environment which better recognizes the transition state, a phenomenon known as differential binding.

It is instructive to compare the results obtained here with similar analyses (Street et al., 1989) performed on glycogen phosphorylase, an enzyme that uses the same substrate, glucose-1-P, but in a very different reaction. Glycogen phosphorylase catalyzes a glycosyl-transfer reaction which involves considerable deformation of the sugar moiety toward a half-chair conformation at the transition state, but very little distortion of the phosphate. Phosphoglucomutase catalyzes a phosphoryl transfer with no deformation of the sugar at the transition state, but significant changes in phosphate geometry. Thus in the case of glycogen phosphorylase differential binding should be associated with changes in the sugar moiety, with little difference at the phosphate, while in the case of phosphoglucomutase the changes in the phosphate moiety will be the focus of the differential binding, with the sugar moiety playing no significant role. The results described here, and previously (Street et al., 1989), illustrate this point graphically. Both enzymes make some modest use of ground-state binding at each sugar hydroxyl, up to 1.8 kcal/mol for phosphorylase and up to 2.8 kcal/mol for phosphoglucomutase. However, the differential binding energies (differences between $\Delta\Delta G°^{\dagger}$ and $\Delta\Delta G°$) at each hydroxyl are *much* greater for glycogen phosphorylase than for phosphoglucomutase, ranging between 0.3 and 1.7 kcal/mol for phosphoglucomutase while energies of 2.3–5.2 kcal/mol are observed for glycogen phosphorylase (Street et al., 1986, 1989). Thus, as anticipated, phosphorylase exploits differences in sugar geometry which are not available for phosphoglucomutase. As indicated previously, the major changes for phosphoglucomutase are at the phosphate moiety, and some indication of the extent to which this change in geometry is exploited is provided by the work of Percival et al. (1990) in studying the binding of the transition-state analogue 6-vanado-α-D-glucose 1-phosphate to phosphoglucomutase. This analogue was found to bind some (2.5×10^4)-fold more tightly than glucose-1,6-diP, indicating a differential binding energy associated with the phosphate moiety of some 6.0 kcal/mol. Other researchers (Ray & Puvathingal, 1990) have reported that the differential binding energy in this same system may be worth as much as 8–9 kcal/mol and suggest that while 6-vanado-α-D-glucose-1-P is a fairly good transition-state analogue by most standards, it is far from perfect and only utilizes half of the true differential binding energy. Clearly, on this basis the sugar moiety does not have to contribute much stabilization.

Implications for the Mechanism of Reorganization of the Central Complexes. While these data do not permit us to distinguish between "exchange" and "minimal motion" mechanisms, they do provide some interesting insights. The removal of the hydroxyl groups at the 2- or 4-positions of the sugar diphosphate apparently has no significant deleterious effect upon its binding whereas removal of the 3-hydroxyl results in a loss of binding affinity of some 90-fold, suggesting a particularly important role for the 3-hydroxyl in the reorganization. This may be consistent with a previously suggested (I. A. Rose, personal communication) mode of exchange in which the sugar diphosphate rotates around the 3-hydroxyl, with the 2- and 4-hydroxyls essentially exchanging subsites, and the 1- and 6-phosphates likewise since this mechanism would require stronger interactions at the 3-position in order to stop the diphosphate diffusing from the site.

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^{19}F NMR Investigations of the Catalytic Mechanism of Phosphoglucomutase Using Fluorinated Substrates and Inhibitors†

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ABSTRACT: The complexes of phosphoglucomutase with a number of fluorinated substrate analogues have been investigated by ^{19}F NMR and the effects of the binding of Li^+ and Cd^{2+} to these complexes determined. Very large downfield chemical shift changes (−14 to −19 ppm) accompanied binding of the inhibitors 6-deoxy-6-fluoro-α-D-glucopyranosyl phosphate and α-glucosyl fluoride 6-phosphate to the phosphoenzyme. Smaller shift changes were observed for ligands substituted with fluorine at other positions. Addition of Li^+ to enzyme/fluorinated ligand complexes caused a 10^2- to 10^3-fold decrease in ligand dissociation constants as witnessed by the change from intermediate to slow-exchange conditions in the NMR spectra. Measurement of the ^{19}F NMR spectra of complexes of the Li^+–enzyme with each of the fluoroglucose 1-phosphates and 6-phosphates has provided some insight into the environment of each of these fluorines (thus also parent hydroxyls) in each of the complexes. Results obtained argue strongly against a single sugar binding mode for the glucose 1- and 6-phosphates. Two enzyme-bound species were detected in the ^{19}F NMR spectra of the complexes formed by reaction of the Cd^{2+}–phosphoenzyme complex with the 2- and 3-fluoroglucose phosphates. These are tentatively assigned as the fluoroglucose 1,6-bisphosphate species bound in two different modes to the dephosphoenzyme. Only one bound species was observed in the case of the 4-fluoroglucose phosphates. The results from this investigation, and those above, are consistent with an exchange type of mechanism [Ray, W. J., Mildvan, A. S., & Long, J. W. (1973) *Biochemistry 12*, 3724] for the enzyme in which there are two distinct glucose ring binding sites.

As described in greater detail in the preceding paper (Percival & Withers, 1992), the general mechanism of phosphoglucomutase is now largely established as involving an initial phosphoryl-transfer step from the serine phosphate of the phosphoenzyme to the bound sugar phosphate, yielding a dephosphoenzyme/glucose-1,6-diP[1] complex. This complex then rearranges in some way to place the nontransferred

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[1] Abbreviations: glucose-1-P, α-D-glucopyranosyl phosphate; deoxyglucose-1-P, deoxy-α-D-glucopyranosyl phosphate; fluoroglucose-1-P, deoxyfluoro-α-D-glucopyranosyl phosphate; glucose-6-P, D-glucose 6-phosphate; α-D-glucosyl fluoride-6-P, α-D-glucosyl fluoride 6-phosphate; glucose-1,6-diP, α-D-glucose 1,6-bisphosphate; PGM, phosphoglucomutase; 4FMeGlc, methyl 4-deoxy-4-fluoro-α-D-glucopyranoside; 6FMeGlc, methyl 6-deoxy-6-fluoro-α-D-glucopyranoside.

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