$C_{21}H_{40}O_4Si$: C, 65.57; H, 10.48. Found: C, 65.73; H, 10.64. (B) By reaction with *m*-CPBA: Reaction conditions as in the peracid epoxidations described above. Reaction time: 3 h. Yield: 92%.

Oxidation of 23 to Silyl β-Epoxide Lactone 21. PCC (475 mg, 2.2 mmol) was added to a solution of lactone **23** (423 mg, 1.1 mmol) in dry CH_2Cl_2 (30 mL). The reaction mixture was stirred at rt for 30 min. More PCC (475 mg) was then added, and stirring was continued for 3 h. Quenching and usual workup (8 → 7), followed by column chromatography of the residue on silica gel (30% ethyl acetate in hexane), yielded 205 mg (49%) of **21** as a white solid. Recrystallization from hexane–diethyl ether gave colorless needles: mp 119–120 °C; IR 1767, 1279, 1231, 1153, 1105, 1086, 956, 920, 757 cm^{-1}; ^1H NMR δ 4.19 (d, J = 4 Hz, H-6), 3.70 (dd, J = 11.2, 4.3 Hz, H-9), 2.77 (dq, J = 7, 7 Hz, H-11), 2.39 (dddd, J = 10.5, 7, 6.5, 4 Hz, H-7), 1.90 (m, H-3), 1.70–1.40 (m, H-1, H-2, H-8), 1.40 (s, H-15), 1.19 (d, J = 7 Hz, H-13), 1.02 (s, H-14), 0.94 (t, J = 7.5, $SiCCH_3$), 0.58 (q, J = 7.5, $SiCH_2$); ^{13}C NMR, see Table I. Anal. Calcd for $C_{21}H_{36}O_4Si$: C, 66.27; H, 9.53. Found: C, 66.39; H, 9.69.

Desilylation of 21 to Hydroxy β-Epoxide Lactone 19. A solution of silylated lactone **21** (190 mg, 0.5 mmol) in HOAc/THF/H_2O, 6:1:3 (20 mL), was stirred at 45 °C for 3 h. The reaction mixture was then diluted with water and extracted twice with CH_2Cl_2. The combined organic layers were then washed once with 5% aqueous NaOH and worked up as usual. Column chromatography of the residue on silica gel (75% ethyl acetate in hexane) yielded 94 mg (71%) of **19** as a white solid. Recrystallization from ethyl acetate gave colorless platelets: mp 226–227 °C; IR 3300, 1766, 1188, 1157, 1021, 953, 906 cm^{-1}; ^1H NMR δ 4.18 (d, J = 4.2 Hz, H-6), 3.62 (dd, J = 11.5, 3.8 Hz, H-9), 2.75 (dq, J = 7, 7 Hz, H-11), 2.45 (dddd, J = 11, 7, 6.5, 4.2 Hz, H-7), 1.90 (m, H-3), 1.70–1.40 (m, H-1, H-2), 1.70 (ddd, J = 13.5, 6.5, 3.8 Hz, H-8$_\alpha$), 1.42 (ddd, J = 13.5, 11.5, 11 Hz, H-8$_\beta$), 1.35 (s, H-15), 1.13 (d, J = 7 Hz, H-13), 0.99 (s, H-14); ^{13}C NMR, see Table I; MS m/z (relative intensity) 266 (M^+, 40), 251 (M^+ – Me, 8), 248 (M^+ – H_2O, 10), 238 (9), 233 (7), 223 (98), 209 (53), 205 (80), 193 (45), 175 (66), 153 (82), 135 (92), 131 (100), 123 (83), 109 (96), 95 (95), 55 (99); HRMS m/z calcd for $C_{15}H_{22}O_4$ 266.1518, found 266.1519.

Oxidation of 19 to Keto β-Epoxide 18. Reaction conditions were virtually as described above for 8 → 7. Reaction time: 5 h. Column chromatography on silica gel (10% MeOH in diethyl ether) afforded **18** in 70% as a white solid. Recrystallization from hexane–diethyl ether gave colorless platelets: mp 175–176 °C; IR 1771, 1704, 1250, 1214, 1168, 1115, 1096, 1010, 965, 726 cm^{-1}; ^1H NMR δ 4.49 (d, J = 5.7 Hz, H-6), 3.04 (br ddd, J = 9.5, 7, 5.7 Hz, H-7), 2.86 (dq, J = 7, 7 Hz, H-11), 2.75 (dd, J = 13.6, 9.5 Hz, H-8$_\alpha$), 2.33 (br d, J = 13.6 Hz, H-8$_\beta$), 2.05 (m, H-3), 1.80–1.40 (m, H-1, H-2), 1.47 (s, H-15), 1.30 (s, H-14), 1.23 (d, J = 7 Hz, H-13); ^{13}C NMR, see Table I; MS m/z (relative intensity) 264 (M^+, 6), 249 (M^+ – Me, 6), 246 (M^+ – H_2O, 5), 236 (8), 231 (3), 221 (28), 206 (91), 203 (68), 191 (70), 147 (55), 133 (100), 109 (51), 55 (92); HRMS m/z calcd for $C_{15}H_{20}O_4$ 264.1362, found 264.1361.

Acetylation of 19 to 20 was performed under the standard conditions (Ac_2O–pyridine–DMAP, rt, 12 h). Quenching with water and usual workup yielded an oily residue which was chromatographed on silica gel (50% ethyl acetate in hexane). This gave **20** in 78% yield as a white solid. Recrystallization from hexane–diethyl ether gave colorless cubes: mp 202–203 °C; IR 1753, 1718, 1278, 1238, 1158, 1147, 1096, 1021, 975, 950, 914, 742 cm^{-1}; ^1H NMR δ 4.92 (dd, J = 11, 3.8 Hz, H-9), 4.26 (d, J = 4.3 Hz, H-6), 2.81 (dq, J = 7, 7 Hz, H-11), 2.57 (dddd, J = 11, 7, 6.5, 4.3 Hz, H-7), 2.05 (s, OAc), 1.90 (m, H-3), 1.80–1.40 (m, H-1, H-2, H-8$_\alpha$), 1.51 (ddd, J = 13.4, 11.2, 11 Hz, H-8$_\beta$), 1.40 (s, H-15), 1.16 (d, J = 7 Hz, H-13), 1.11 (s, H-14); ^{13}C NMR, see Table I; MS m/z (relative intensity) 308 (M^+, 14), 265 (M^+ – $COCH_3$, 100), 248 (16), 233 (9), 223 (51), 209 (79), 205 (62), 175 (35), 131 (59), 119 (54), 55 (38); HRMS m/z calcd for $C_{17}H_{24}O_5$ 308.1624, found 308.1624.

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Supplementary Material Available: ^1H NMR spectra of compounds **7**, **8**, **9**, **11**, **12**, **14**, **15**, **16**, **18**, **19**, and **20** (22 pages). Ordering information is given on any current masthead page.

Synthesis and Testing of Sugar Phosphofluoridates and Cyclic Phosphates as Inhibitors of Phosphoglucomutase

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Three aldose phosphofluoridates, D-glucose 6-phosphofluoridate, α-D-mannopyranosyl phosphofluoridate, and 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphofluoridate, have been synthesized from the parent phosphate and 2,4-dinitrofluorobenzene, and the mechanism of fluorination has been investigated. Another modified aldose phosphate, α-D-glucopyranosyl 4,6-cyclic phosphate [phosphate] has also been synthesized as an analogue of 6-phospho-α-D-glucopyranosyl phosphate. These compounds were tested as possible mechanism-based inactivators of rabbit muscle phosphoglucomutase, but no time-dependent inactivation was observed. They were, however, found to be reversible inhibitors of phosphoglucomutase, and comparison of their dissociation constants with those of the parent phosphates revealed that the removal of a single negative charge weakens ground-state binding by approximately 11 kJ/mol. Further, the absence of any detectable phosphorylation of these analogues reveals that this second charge is even more important for transition-state interactions, contributing at least 40 kJ/mol to transition-state stability. This suggests that the parent substrates bind to the enzyme and react in their dianionic forms, and it provides a measure of the value of charge–charge interactions at the active site of this key metabolic enzyme.

A complete understanding of the specificities and mechanisms of enzymes that utilize substrates or cofactors containing ionizable groups requires a knowledge of the charge state of the enzyme-bound species. In the cases of enzymes utilizing phosphate monoesters for example, both monoanions and dianions are present in solution at physiological pH and in principle either could be the active species. Studies on the reaction's pH dependence[1] and, in the case of phosphate-containing ligands, the ^{31}P NMR

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chemical shift of the bound ligand[2] can provide information on the charge state of a bound ligand that has multiple ionization states. However, these techniques have disadvantages,[3,4] and less ambiguous information can often be gained using chemically modified ligands. Ideally, a modification would only alter the charged group itself, either by changing the pK_a values or by addition or deletion of a whole charge, and not affect the remainder of the molecule to any great degree. The pK_a values of phosphate esters have been modified by forming analogues such as thiophosphates, imidophosphates, phosphonates, α-halo phosphates, and α-halo phosphonates.[5,6] Alternatively, a whole charge can be deleted by replacing a phosphate hydroxyl with a nonionizable group such as hydrogen, to form a phosphite,[7] or with fluorine, to form a phosphofluoridate,[8] or by cyclization to form a cyclic phosphate.[9]

Good mechanism-based inactivators of phosphoryl-transferring enzymes would be extremely valuable tools in the study of the mode of action and biological function of this extremely important class of enzymes. Suitably designed phosphofluoridates and cyclic phosphates offer some potential in this regard for enzymes which operate through a phosphoryl–enzyme intermediate since, should they be recognized by the enzyme in question, then attack of the enzymic nucleophile may result in displacement of the fluoride or opening of the cyclic phosphate rather than the normal phosphoryl-transfer reaction. Inactivation would result due to the formation of a phosphodiester-derivatized enzyme.

This report describes the synthesis and use of phosphofluoridate and cyclic phosphate analogues of α-D-glucopyranosyl phosphate (glucose-1-P)[10] and D-glucose 6-phosphate (glucose-6-P) as inhibitors and as possible inactivators of the enzyme phosphoglucomutase from rabbit muscle. Phosphoglucomutase is an enzyme of molecular weight 61 600 Da, which is responsible for the interconversion of glucose-1-P and glucose-6-P.[11] It contains a phosphoserine residue in its active site and is dependent on the presence of both Mg^{2+} and 6-phospho-α-D-glucopyranosyl phosphate (glucose-1,6-diP) for full enzymatic activity. Its mechanism of action is fairly well established as involving two consecutive phosphoryl-transfer reactions. In the first reaction glucose-1-P binds at the active site with its 6-hydroxyl adjacent to the phosphoserine residue and a phosphoryl transfer occurs with formation of a dephosphoenzyme–glucose-1,6-diP complex. This complex then rearranges such that the 1-phosphate is transferred to the active-site serine residue in a second step, regenerating the phosphoenzyme and releasing the glucose-6-P.

The compounds D-glucose 6-phosphofluoridate (glucose-6-PF) (1) and α-D-glucopyranosyl phosphofluoridate (glucose-1-PF) (2) are good candidates for covalent inactivators of this enzyme since it is reasonable to expect that they would bind to the enzyme and be phosphorylated by the serine phosphate in the initial step exactly as with the parent substrate. Subsequent attack of the serine hydroxyl group on the phosphofluoridate moiety might well result in formation of the phosphodiester with displacement of fluoride, thus inactivation. Good potential for such inactivation exists since another class of enzymes containing an active-site serine nucleophile, the serine proteases, is efficiently inactivated by diisopropyl phosphofluoridate.[12] In addition, it has been reported that thymidine 3′-phosphofluoridate irreversibly inhibits ribonuclease, possibly by reaction of the phosphofluoridate moiety with a reactive enzyme group.[13] Inactivation of phosphoglucomutase might also be expected with the cyclic phosphate analogues for the same reason. If such inactivation does not occur, the reversible binding of these compounds would still be of interest since comparison of the affinities of these analogues with the affinities of the corresponding substrates should provide valuable insight into the contribution of charge–charge interactions to their overall binding affinity.

Results

Synthesis. The fluorination of glucose-6-P with 2,4-dinitrofluorobenzene (DNFB) proceeded smoothly, yielding glucose-6-PF (1) which was characterized by ^1H, ^{19}F, and ^{13}P NMR as well as by elemental analysis and FAB-MS. The synthesis of glucose-6-PF has been reported previously,[14] but no analytical data confirming the structure of the product were provided in that case. Attempts to prepare glucose-1-PF (2) by a similar procedure were unsuccessful and resulted only in the formation of glucose 1,2-cyclic phosphate, as evidenced by the ^{31}P and ^1H NMR spectra obtained for the product which were identical to those of authentic material prepared previously[15] by the method of Zmudzka and Shugar.[9] The probable mechanism of formation of this material is of interest. Previous work on the synthesis of nucleotide phosphofluoridates[16] has suggested that the formation of a phosphofluoridate under these conditions involves an initial nucleophilic attack of the phosphate ester onto DNFB to yield a 2,4-dinitrophenyl ester intermediate with release of fluoride. This intermediate subsequently undergoes attack by fluoride to give the phosphofluoridate with expulsion of 2,4-dinitrophenolate. Presuming this mechanism holds true for these pyranosyl phosphates, then the formation of glucose 1,2-cyclic phosphate rather than the desired glucose-1-PF must arise from attack of the 2-hydroxyl upon the phosphate, either in the dinitrophenyl ester intermediate or after it has formed the phosphofluoridate product. If the attack occurs only on the intermediate the desired product, glucose-1-PF (2) might be obtainable if the 2-hydroxyl could be protected during the reaction and only exposed when the product phosphofluoridate had been formed.

Confirmation of the mechanism of fluorination for these sugars was first sought by examining a pyranosyl phos-

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(10) Abbreviations used: glucose-1-P, α-D-glucopyranosyl phosphate; glucose-6-P, D-glucose 6-phosphate; glucose-1-PF, α-D-glucopyranosyl phosphofluoridate; glucose-6-PF, D-glucose 6-phosphofluoridate; mannose-1-P, α-D-mannopyranosyl phosphate; mannose-1-PF, α-D-mannopyranosyl phosphofluoridate; 2-fluoroglucose-1-PF, 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphofluoridate; glucose-1,6-diP, 6-phospho-α-D-glucopyranosyl phosphate; glucose-4,6-cyclic-P-1-P, α-D-glucopyranosyl 4,6-cyclic phosphate [phosphate]; xylose-1-P, α-D-xylopyranosyl phosphate; DNFB, 2,4-dinitrofluorobenzene; FAB-MS, fast atom bombardment mass spectrometry; Tris, tris(hydroxymethyl)aminomethane.
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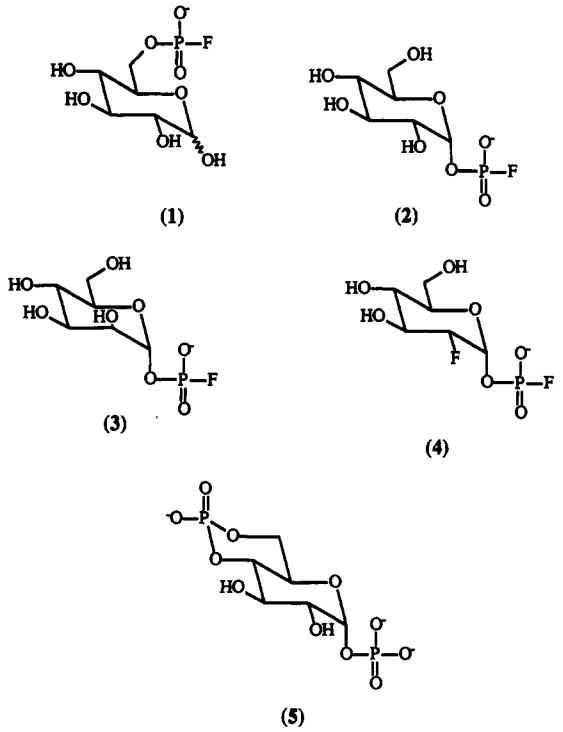


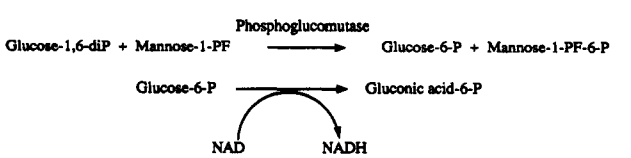
phate which could not undergo this transformation. α-D-Mannopyranosyl phosphate (mannose-1-P) was chosen since the trans relationship of the 2-hydroxyl and the anomeric phosphate precludes formation of a cyclic phosphate. Synthesis of the dinitrophenyl ester of mannose-1-P was achieved by means of a dicyclohexylcarbodiimide-mediated coupling of mannose-1-P and 2,4-dinitrophenol. This material could be purified by DE-52 ion-exchange chromatography but proved to be relatively unstable, decomposing with release of dinitrophenol. Nonetheless, addition of an excess of potassium fluoride to samples of this intermediate resulted in the rapid release of dinitrophenol and the formation of a stable α-D-mannopyranosyl phosphofluoridate (mannose-1-PF) (3) product whose identity was confirmed by comparison of spectroscopic data with that of an authentic sample (vide infra).

This result clearly indicates the same mechanism of phosphofluoridate formation as that found for the nucleotide phosphofluoridate. Further, the observed high reactivity of the dinitrophenyl ester of mannose-1-P and the relative stability of mannose-1-PF (3) would suggest that the unwanted cyclization process during the attempted synthesis of glucose-1-PF (2) likely occurs at the dinitrophenyl ester intermediate stage. On the basis of this, attempts were made to prepare a protected glucose-1-P derivative which could be used in the synthesis of glucose-1-PF. Synthesis of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl phosphate was achieved both by acetylation of glucose-1-P with acetic anhydride in pyridine[17] and by the MacDonald phosphorylation of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose followed by neutralization.[18] However, subsequent fluorination of this compound by DNFB was not successful, as was evidenced by ^{31}P NMR, the reaction resulting in a complex mixture of compounds, none of which exhibited the expected characteristic P–F splitting of approximately 950 Hz. Attempts to prepare a per-O-benzylated glucose-1-P derivative which might not degrade upon attempted fluorination were unsuccessful. Since glucose-1-PF could not be synthesized by the routes attempted, alternative compounds for the enzymic experiments were sought. Those chosen were mannose-1-PF (3) and 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphofluoridate (2-fluoroglucose-1-PF) (4) since neither of these compounds can possibly cyclize and since previous work suggested that both would likely be bound by phosphoglucomutase.[19,20] These were both successfully synthesized using DNFB, and the products were characterized by NMR and elemental analysis.

The aldose phosphofluoridates were all stable for 3–6 months at −20 °C when stored in a dessicator over calcium chloride. However glucose-6-PF (1) partially decomposed over several weeks at room temperature on exposure to air, turning black and becoming highly acidic. Investigation of the product mixture by ^{31}P NMR revealed that the major decomposition product was a phosphate monoester and did not contain any of the anticipated glucose 4,6-cyclic phosphate. It is therefore unlikely that this facile decomposition is caused by intramolecular attack of the C-4 glucose hydroxyl on the phosphofluoridate group with displacement of fluoride, but not impossible since initial formation of the cyclic phosphate could have been followed by its hydrolysis to form a monoester.

Synthesis of glucose-4,6-cyclic-P-1-P (5) was achieved in a two-step reaction from glucose-6-P. In the first step glucose-6-P was converted into its 4,6-cyclic phosphate using dicyclohexylcarbodiimide, according to the published protocol[9] and purified by ion-exchange chromatography. This material was then phosphorylated essentially according to MacDonald[18] to yield the desired diphosphate product after ion-exchange chromatography.

Enzymology. Inactivation Tests. Incubation of each of the phosphofluoridate substrate analogues with the phosphoenzyme form of phosphoglucomutase for periods of up to 6 h resulted in no time-dependent decrease in enzyme activity, the activity of the enzyme remaining identical to that of a control. There are two most likely reasons for the absence of inactivation, based upon the fact that inactivation by these compounds would require the inactivator to first bind to the phosphoenzyme and be phosphorylated by the active site serine phosphate before the enzymic nucleophile is free to attack the phosphofluoridate. Lack of inactivation could therefore result either from the inability of the enzyme to perform the initial phosphorylation step or, if this has occurred, from the inability of the enzymic nucleophile to attack the phosphofluoridate with release of the fluoride. These two possibilities have been distinguished by assaying for enzyme-catalyzed phosphorylation of the sugar phosphofluoridate. This was achieved using a coupled assay (glucose-6-P dehydrogenase) which can measure the enzyme-catalyzed release of glucose-6-P from the glucose-1,6-diP which is consumed in rephosphorylating the enzyme after the phosphofluoridate has been converted.



No detectable production of glucose-6-P took place even at phosphoglucomutase concentrations of 1 mg/mL and

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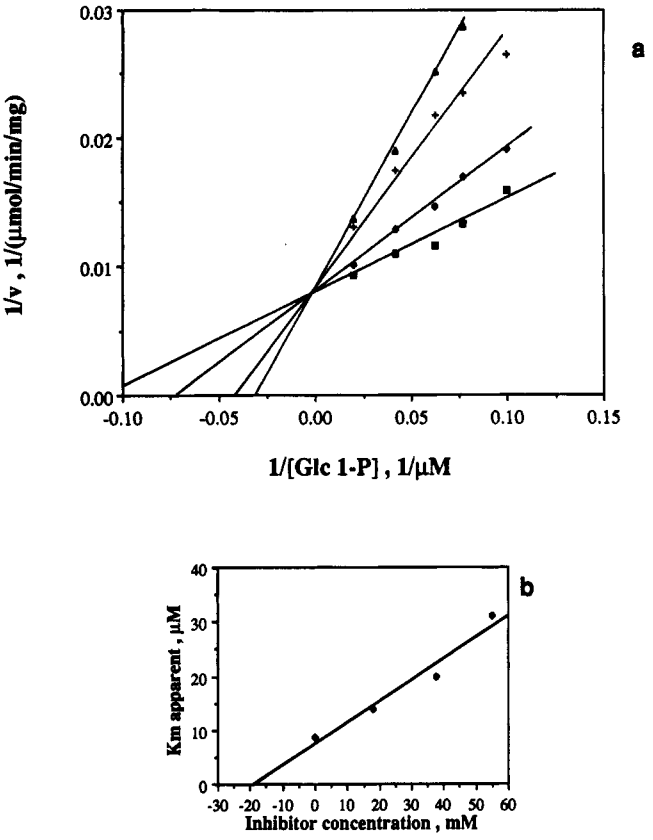

Figure 1. Inhibition of phosphoglucomutase by mannose-1-PF. (a) Lineweaver–Burk plot of the inhibition; inhibitor concentrations were 0 (■), 18 (◆), 38 (+), and 55 mM (▲). (b) Replot of the $K_{m_{app}}$ values obtained from (a) versus inhibitor concentration.

phosphofluoridate monoester concentrations of 5 mM. Under these conditions a phosphorylation rate of approximately 5×10^{-5} μmol/min per mg would have been detected, which is approximately 10^{-7} times the rate of glucose-1-P conversion. The viability of the above assay system was demonstrated by the addition of xylose-1-P to a similar assay mix when a rapid release of glucose-6-P was observed. This is due to the well-known ability of xylose-1-P to promote hydrolysis of the serine phosphate moiety from the phosphoenzyme.[21] Absence of inactivation in these cases is therefore due to the inability of the enzyme to effect phosphoryl transfer to the aldose phosphofluoridate. The possibility of completely bypassing the problem of phosphorylation of the substrate by direct treatment of the dephosphoenzyme with the aldose phosphofluoridate was considered. However, since the dephosphoenzyme is known to bind glucose-1-P and glucose-6-P only very weakly,[21] no attempt was made to inactivate the dephosphoenzyme with these phosphofluoridates.

Glucose-4,6-cyclic-P-1-P (**5**) was also tested as a possible inactivator of phosphoglucomutase by incubating it with the dephosphoenzyme for periods of up to 6 h. Once again no time-dependent inactivation of the enzyme could be detected, even though in this case no initial phosphoryl-transfer step was required. Indeed, when such inactivation mixtures were assayed in the absence of glucose-1,6-diP, a time-dependent *activation* of the enzyme was observed due to transfer of a phosphate from the glucose-4,6-cyclic-P-1-P to the dephosphoenzyme. (In the normal inactivation experiments, glucose-1,6-diP was included in the assay mixture: this essentially instantaneously rephosphorylates the enzyme.) A similar effect of reactivation by rephosphorylation of the dephosphoenzyme has been observed previously with 1,3-diphosphoglycerate.[23,24] The lack of inactivation with glucose-4,6-cyclic-P-1-P therefore cannot be due to an ineffective phosphorylation step and must result from the inability of the serine hydroxyl to attack the cyclic phosphate.

Table I. Dissociation Constants of Phosphoglucomutase with Substrate Analogues

inhibitor	K_i (mM)	$\Delta\Delta G°$ [a] (kJ/mol)
glucose-4:6-cyclic-P-1-P	0.065	16.3[b]
glucose-6-PF	7	12.1[c]
mannose-1-PF	19	11.0[d]
2-fluoroglucose-1-PF	17	10.9[e]

[a] Values calculated from the expression $\Delta\Delta G° = RT \ln (K_1/K_2)$, where T = 303 K, R = 8.314 J/K per mol, K_2 is the K_i of the substrate analogue and K_1 is the K_m of the corresponding substrate. [b] K_m, glucose-1,6-diP = 0.1 μM. [c] K_d, glucose-6-P = 57 μM.[25] [d] K_m, mannose-1-P = 245 μM.[19] [e] K_m, 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate = 222 μM.[20]

Reversible Inhibition. Measurement of the inhibition afforded by mannose-1-PF (**3**) revealed this to be a competitive inhibitor with respect to substrate glucose-1-P as is demonstrated by the double-reciprocal plot in Figure 1a. The replot of the apparent K_m values versus inhibitor concentrations (Figure 1b) allowed calculation of an inhibition constant of K_i = 19 mM. Measurement of precise data for glucose-6-PF (**1**) was extremely difficult due to the fact that it is a substrate, albeit poor, for the coupling enzyme glucose-6-P dehydrogenase, thus a full determination of K_i at a series of concentrations of substrate was not carried out. Problems associated with the limited quantity of inhibitor available also precluded a full K_i determination for 2-fluoroglucose-1-PF (**4**). In both cases the K_i determination was based upon measurement of rates at a fixed (9.7 μM = K_m) concentration of substrate with different concentrations of inhibitor. Results were then plotted in the form of a Dixon plot, the K_i value being determined from the intersection point of the plot obtained with the horizontal line drawn through $1/V_{max}$. In our hands we have found this method to give K_i values within a factor of two of that ultimately determined by a full K_i measurement.

The K_i value for the binding of glucose-4,6-cyclic-P-1-P (**5**) to the dephosphoenzyme was determined similarly by measuring the rate at a series of inhibitor concentrations while keeping the concentration of glucose-1,6-diP fixed at a value (0.1 μM) near its dissociation constant. Evidence for activation of the dephosphoenzyme by glucose-4,6-cyclic-P-1-P also showed up in these experiments at high "inhibitor" concentrations as a higher reaction rate than expected.

The values of K_i for each inhibitor and the calculated loss of binding energy compared to that of the parent substrate are presented in Table I. For these calculations the dissociation constant (K_d) of the parent substrate was assumed to be equal to its K_m as has been demonstrated previously for this enzyme.[25]

Discussion

The absence of any detectable inactivation by either the phosphofluoridates or the cyclic phosphate is somewhat

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surprising in light of previous results. In the case of the phosphofluoridates it would seem to be due to the fact that these derivatives are not phosphorylated by the enzyme at any significant rate, thus the necessary dephosphoenzyme/phosphorylated hexose phosphofluoridate complex is not generated in significant quantities. Again the absence of phosphorylation is surprising given that the analogues clearly bind to the enzyme and that the phosphorylation site is relatively remote from the modified phosphate site. It is even more surprising given that it has been reported previously[21] that D-glucose-6-sulfate, also a monoanionic species, is quite efficiently phosphorylated by the phosphoenzyme, at almost half the rate at which glucose-6-P is phosphorylated. Further, within the work reported here it has been shown that glucose-4,6-cyclic-P-1-P can undergo a phosphoryl-transfer reaction with the dephosphoenzyme. This is simply the reverse of the same phosphoryl-transfer reaction as that which failed with the sugar phosphofluoridates, but again with a monoanionic phosphate bound at the "remote" site. Reasons for the apparent inactivity of these extremely sterically conservative analogues are not at all clear, especially since other acceptors such as 1,4-butanediol phosphate and glucose are reportedly phosphorylated by phosphoglucomutase.[21] However, these alternate acceptors are phosphorylated at very low rates, approximately 10^7–10^9 times slower than is glucose-1-P, so it is quite possible that the phosphofluoridate analogues are phosphorylated by phosphoglucomutase, but that the rates are too low to be detected by the assay used in this study. If small quantities of the phosphorylated hexose phosphofluoridate are indeed produced during the extended (6 h) incubation of the attempted inactivation experiment, they clearly do not inactivate the enzyme. This is presumably either because the binding of this diphosphate species is too weak to allow a sufficient concentration of the complex to form, or because the compound is intrinsically unreactive.

The problem of prior phosphorylation could be avoided by chemical synthesis of the necessary phosphorylated hexose phosphofluoridate. However, this would be an extremely challenging synthetic target given the relative labilities of phosphofluoridates, and probably not worth attempting given that the probability of successful inactivation was rather low. The alternative synthetic target was the more accessible glucose-4,6-cyclic-P-1-P (**5**), which was indeed successfully synthesized. However, once again it proved not to be effective as an inactivator, even though the correct complex could be formed. There would appear to be two likely reasons for the ineffectiveness of this inactivator, and also of the phosphofluoridates if sufficient of the potential inactivator had indeed been generated in the latter case. One of these is related to the fact that this is a Mg^{2+}-dependent enzyme, and the metal ion has been shown previously to coordinate directly to the enzymic phosphate in the phosphoenzyme form.[26] On the basis of this, and of recent kinetic studies,[27] along with ample literature precedent for enzyme-catalyzed phosphoryl-transfer reactions, it seems likely that the metal ion coordinates to the transferred phosphate during catalysis. It is therefore quite probable that these monoanionic phosphate species cannot coordinate the metal effectively, thus no phosphoryl-transfer reaction occurs, despite the presence of a reactive leaving group (fluoride) in the case of the phosphofluoridates. Previous work has indeed shown that the transferred phosphate is in its dianionic form both prior and subsequent to transfer.[28] The second reason relates to the strict conformational requirements of enzymic phosphoryl-transfer reactions. Enzymic phosphoryl-transfer reactions are believed to occur via an in-line associative mechanism in which the leaving group departs from an apical position, in line with the incoming nucleophile, with no pseudorotations.[29] The constraints of the rigid cyclic phosphate group and the preordained binding mode could prevent the correct geometry for phosphoryl transfer from being attained. This could also be true for the phosphofluoridates, since for covalent binding to occur the fluoride leaving group would have to be in line with the incoming serine nucleophile, yet the enzyme has evolved to bind the substrate with the C-6 carbon–oxygen bond in line. The efficient inactivation of serine proteases by diisopropyl phosphofluoridate is presumably a consequence both of its higher inherent reactivity and of fortuitous transition-state interactions of the inactivator with the enzyme. Since it is quite unrelated in structure to the normal substrates is is less constrained by the binding and stereoelectronic requirements of the enzyme active site.

Study of the reversible binding of the phosphofluoridate substrate analogues with phosphoglucomutase reveals that a loss of enzyme–ligand binding free energy ($\Delta\Delta G°$) of approximately 11 kJ/mol occurs on the deletion of a negative charge from the substrate's phosphate functional group. A slightly larger value of 16 kJ/mol was obtained for the cyclic phosphate analogue of glucose-1,6-diP. This result suggests that the substrate dianion is the active species and is therefore consistent with results from NMR studies on phosphoglucomutase in which it was reported that the tetraanion of glucose-1,6-diP binds to the dephosphoenzyme.[28] The strong interaction between the dianionic substrate and the enzyme is likely due to the presence of three cationic arginine residues that have been identified in the active site by X-ray crystallographic analysis.[30] The higher value for $\Delta\Delta G°$ of 16 kJ/mol obtained for the cyclic phosphate analogue may reflect a greater dependence on salt bridge interactions in the dephosphoenzyme/glucose-1,6-diP complex or, more likely, either the effect of an unfavorable steric interaction of the cyclic phosphate moiety with the enzyme or interference with the normal hydrogen bonding interactions at the 4-hydroxyl. Such effects are unlikely with the phosphofluoridate analogues since replacement of hydroxyl by fluorine is a structurally conservative change and both phosphofluoridate and phosphate salts have tetrahedral structures.[31] The inability of the hexose phosphofluoridates to undergo the initial phosphorylation step by the phosphoenzyme suggests that the second negative charge of the nontransferred phosphate is even more important at the transition state for phosphoryl transfer than at the ground state, despite the fact that it is rather remote. Indeed a $\Delta\Delta G°$ value of at least 40 kJ/mol can be assigned to this interaction at the transition state based upon the fact that phosphoryl transfer, if it occurs at all, must take place at least 10^7 times slower than with the parent substrates. This value is very large but is quite consistent with recent estimates of the contributions of interactions at single groups to the stability of protein/ligand complexes.[44]

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The $\Delta\Delta G°$ value (11 kJ/mol) obtained in this study for the deletion of a single charge compares favorably with those previously reported for ground-state salt bridge interactions.[7,32-34] Such values are not simple to interpret in terms of the actual energy of interaction associated with the charges within the enzyme/substrate complex since changes in other interactions caused by the charge removal also have to be considered. In particular, differences in hydrogen-bonding energies between the substrate and solvent water will be quite significant. Nevertheless these numbers are of considerable value since they represent the true contribution of charge–charge interactions to the stability of the complex.

Experimental Section

Syntheses. Chemicals were obtained from Aldrich or Sigma Chemical Co. DMF was dried by heating over CaH_2 and distilled at reduced pressure. Pyridine was dried over and distilled from KOH. Tri-*n*-butylamine was dried and freed of primary amines by distillation from *p*-toluenesulfonyl chloride. Thin-layer chromatography of phosphate and phosphofluoridate esters was performed using cellulose PEI-F plates (J. T. Baker Co.) developed with an aqueous solution of lithium chloride (1–2 M). Compounds were visualized using a dipping solution specific for phosphates[35] followed by development with UV light. The color of phosphofluoridate-containing species developed more slowly than that of phosphates, presumably because of the necessity for prior hydrolysis to a phosphate. The ^{31}P NMR chemical shifts are referenced relative to 85% orthophosphoric acid in D_2O and ^{19}F NMR chemical shifts relative to $CFCl_3$, signals at higher field being given a positive value. NMR spectra other than ^1H were measured at pH 6.8 in buffer containing 1 mM EDTA and 50% D_2O.

D-Glucose 6-[Ammonium phosphofluoridate] (1). The general method was that of Wittmann.[36] D-Glucose-6-[disodium phosphate] (1.0 g, 2.8 mmol) was converted to its tri-*n*-butylammonium salt by dissolving in water and passing through a column (4 °C) of Dowex 50W-X8 (H^+) resin into an excess of tri-*n*-butylamine. The solvent was evaporated, and the material was then further dried in vacuo. The gum was dissolved in DMF (10 mL) containing tri-*n*-butylamine (2.0 mL, 8.4 mmol) and DNFB (0.60 mL, 4.8 mmol) and stirred for 24 h while protected from moisture with a calcium chloride guard tube. Diethyl ether was added to cloud point and the product precipitated by addition of cyclohexylamine (1.1 mL, 10 mmol). After the solvent was decanted, the gum was washed well with diethyl ether, and the precipitate was dissolved in water, extracted with diethyl ether four times, and finally lyophilized to yield a gummy yellow solid. Dissolution of this material in a minimum volume of methanol and precipitation by addition of diethyl ether was repeated three times after which most of the color was removed from the precipitate. The precipitate was then dissolved in water (500 mL), the pH adjusted to 8.0, and the solution applied to a column (1.8 × 30 cm) of DE-52 cellulose at 4 °C which had been previously equilibrated with 10 mM ammonium bicarbonate. After the column was washed with water (200 mL), the products were eluted with a linear gradient (0–50 mM ammonium bicarbonate, 2 L) and fractions (20 mL) collected at a flow rate of 2 mL/min. The fractions containing the desired product, eluting at a salt concentration of approximately 30 mM, were identified by a "spot" test for reducing sugars.[37] After pooling the fractions, the buffer was removed by repeated lyophilization to yield a mixture of the α- and β-anomers of 1 as a pale yellow foam (0.41 g, 1.5 mmol, 53%): ^{31}P NMR δ 2.93 (dt, $J_{P,F}$ = 932, $J_{P,6+6'}$ = 6.0 Hz), 2.98 (dt, $J_{P,F}$ = 932, $J_{P,6+6'}$ = 6.0 Hz); ^{19}F NMR δ 78.63 (d, $J_{F,P}$ = 932 Hz), 78.72 (d, $J_{F,P}$ = 932 Hz); FAB mass spectrum (M + glycerol) 371.

Anal. Calcd for $C_6H_{15}O_8NPF$: C, 25.82; H, 5.42; N, 5.02. Found: C, 25.58; H, 5.55; N, 5.61.

α-D-Mannopyranosyl [Bis(cyclohexylammonium) phosphate]. 1,2,3,4,6-Penta-*O*-acetyl-α-D-mannopyranose[38] (18.0 g, 46 mmol) was phosphorylated according to the method of MacDonald[18] and the bis(cyclohexylammonium) salt crystallized from water/acetone to yield colorless crystals (10.6 g 23 mmol, 50%): ^1H NMR δ 5.31 (dd, 1 H, $J_{1,P}$ = 8.6, $J_{1,2}$ = 1.6 Hz, H-1), 3.95–3.66 (m, 5 H, H-2,3,5,6,6'), 3.58 (t, 1 H, $J_{4,5}$ = 9.5, $J_{4,3}$ = 9.5 Hz, H-4), 3.13 (m, 2 H, 2 NH_3^+CH), 2.00–1.18 (m, 20 H, 2 cyclohexyl); ^{31}P NMR δ −4.70 (d, $J_{P,1}$ = 8.5 Hz).

α-D-Mannopyranosyl [Ammonium phosphofluoridate] (3). α-D-Mannopyranosyl [bis(cyclohexylammonium) phosphate] (1.5 g, 3.2 mmol) was converted to the bis(tributylammonium) salt and treated, appropriately scaled, exactly as in the synthesis of glucose-6-PF. The product was purified by DE-52 cellulose column chromatography, the fractions containing the desired products being identified by a colorimetric assay for acid-labile phosphate.[39] Repeated lyophilizations yielded **3** as a white foam (0.49 g, 1.76 mmol, 55%): ^1H NMR δ 5.51 (dd, 1 H, $J_{P,1}$ = 6.8, $J_{1,2}$ = 1.8 Hz, H-1), 4.02 (t, 1 H, $J_{1,2}$ = 2.0, $J_{2,3}$ = 2.0 Hz, H-2), 3.92–3.74 (m, 4 H, H-3,5,6,6'), 3.70 (t, 1 H, $J_{4,3}$ = 9.5, $J_{4,5}$ = 9.5 Hz, H-4); ^{31}P NMR δ 5.79 (dd, $J_{P,F}$ = 940, $J_{P,1}$ = 6.7 Hz); ^{19}F NMR δ 76.00 (d, $J_{F,P}$ = 940 Hz); FAB mass spectrum (M + 1) 279, (M + glycerol) 371.

Anal. Calcd for $C_6H_{15}O_8NPF$: C, 25.82; H, 5.42; N, 5.02. Found: C, 25.64; H, 5.70; N, 5.30.

α-D-Mannopyranosyl [Tri-*n*-butylammonium] 2,4-Dinitrophenyl Phosphate. A solution of α-D-mannopyranosyl [bis(cyclohexylammonium) phosphate] (1.4 g, 2.2 mmol) was converted to the bis(tributylammonium) salt and dried as described previously. The dry gum was dissolved in DMF (25 mL) containing 2,4-dinitrophenol (4.05 g, 22 mmol) and dicyclohexylcarbodiimide (9.06 g, 44 mmol). After the mixture was stirred for 2 h under anhydrous conditions, the solvent was evaporated, the residue suspended in water, and the mixture extracted four times with diethyl ether. After filtration the solution was lyophilized to yield the crude product: ^{31}P NMR (32.4 MHz, D_2O) δ 6.12 (d, $J_{P,1}$ = 6.8 Hz).

2-Deoxy-2-fluoro-α-D-glucopyranosyl [Ammonium phosphofluoridate] (4). 2-Deoxy-2-fluoro-α-D-glucopyranosyl [bis(cyclohexylammonium) phosphate][40] (0.205 g, 0.44 mmol, containing 20% β-anomer) was treated, appropriately scaled, exactly as in the synthesis of glucose-6-PF. DE-52 column chromatography yielded a white powder (0.088 g, 0.30 mmol, 68%) but did not separate the α- and β-anomers. α-Anomer (4): ^1H NMR δ 5.75 (dd, 1 H, $J_{1,P}$ = 6.8, $J_{1,2}$ = 3.6 Hz, H-1), 4.46 (ddt, 1 H, $J_{2,F}$ = 48.5, $J_{2,3}$ = 9.5, $J_{2,1}$ = 3.2, $J_{2,P}$ = 3.2 Hz, H-2), 3.98 (dt, 1 H, $J_{3,F}$ = 13.1, $J_{3,2}$ = 9.4, $J_{3,4}$ = 9.4 Hz, H-3), 3.90–3.75 (m, 3 H, H-5,6,6'), 3.51 (t, 1 H, $J_{4,3}$ = 9.5, $J_{4,5}$ = 9.5 Hz, H-4); ^{31}P NMR δ 5.41 (ddd, $J_{P,F}$ = 940, $J_{P,1}$ = 6.6, $J_{P,2}$ = 2.7 Hz); ^{19}F NMR δ 66.77 (d, $J_{F,P}$ = 942 Hz, fluorophosphate), 192.86 (dd, $J_{F,2}$ = 49, $J_{F,3}$ = 13 Hz, F-2). β-Anomer: ^1H NMR δ 5.25 (dt, 1 H, $J_{1,P}$ = 7.5, $J_{1,2}$ = 7.5, $J_{1,F}$ = 2.8 Hz, H-1), 4.22 (dt, 1 H, $J_{2,F}$ = 51.0, $J_{2,1}$ = 7.5, $J_{2,3}$ = 7.5 Hz, H-2), 3.90–3.75 (m, 4 H, H-3,5,6,6'), 3.48 (dd, 1 H, $J_{4,3}$ = 9.5, $J_{4,5}$ = 9.5 Hz, H-4); ^{31}P NMR δ 5.83 (dd, $J_{P,F}$ = 942, $J_{P,1}$ = 7.6 Hz); ^{19}F NMR δ 66.96 (d, $J_{F,P}$ = 935 Hz, fluorophosphate), 192.37 (dd, $J_{F,2}$ = 51, $J_{F,3}$ = 15 Hz, F-2); FAB mass spectrum (M + 1) 282.

Anal. Calcd for $C_6H_{14}O_7NPF_2$: C, 25.63; H, 5.02; N, 4.98. Found: C, 25.19; H, 5.17; N, 5.01.

D-Glucose [Ammonium 4,6-cyclic phosphate]. D-Glucose 6-(dihydrogen phosphate) (0.86 g, 3.3 mmol) was cyclized according to the method of Zmudzka and Shugar,[9] and the product was chromatographed exactly as in the synthesis of glucose-6-PF. Repeated lyophilizations yielded a colorless foam (0.72 g, 2.8 mmol, 85%): ^{31}P NMR $\{^1H\}$ δ −5.05, −5.08 (α- and β-anomers).

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α-D-Glucopyranosyl 4,6-Cyclic Phosphate [Tris(ammonium) phosphate] (5). This is a modification of the method of Hanna and Mendocino.[41] A solution of D-glucose [ammonium 4,6-cyclic phosphate] (0.70 g, 2.73 mmol) was converted to the pyridinium salt via passage down a Dowex 50W-X8 (H^+) resin column into a solution of pyridine, and the water was removed by evaporation. The gum was further dried in vacuo and then successively dissolved in pyridine followed by evaporation of solvent three times in order to completely dry the gum. Pyridine (6 mL) and acetic anhydride (2 mL) were added, and the mixture was stirred for 2 h at room temperature until all the gum had dissolved. After reacting at 4 °C for 2 days the solvent was removed by evaporation, and the gum was further dried in vacuo. After addition of anhydrous phosphoric acid (2.0 g, 20 mmol) the mixture was heated under vacuum at 55 °C for 2 h, ice-cold 1 M lithium hydroxide (60 mL) was added, and the pH was adjusted to a value of 11 with further base. The solution was left at room temperature for 2 days to saponify the esters and precipitate excess phosphate, the pH being occasionally readjusted to a value of 11.0. After filtration through Celite the pH was adjusted to 8.0 using Dowex 50W-X8 (H^+) resin and the solution applied to a column (1.8 × 30 cm, 4 °C) of DE-52 cellulose which had been previously equilibrated with 40 mM ammonium bicarbonate. After washing with water (200 mL) the material was eluted with a salt gradient (0–0.25 M ammonium bicarbonate, 2 L), the desired product eluting in a symmetric peak at a salt concentration of approximately 0.13 M and identified by colorimetric assay for acid-labile phosphate.[39] After pooling the fractions, the buffer was removed by multiple lyophilizations to yield **5** as a white foam (0.32 g, 0.86 mmol, 31%): ^{1}H NMR δ 5.42 (dd, 1 H, $J_{1,P}$ = 7.0, $J_{1,2}$ = 2.5 Hz, H-1), 4.15–3.90 (m, 2 H), 3.72 (t, 1 H, $J_{3,4}$ = 9.0, $J_{3,2}$ = 9.0 Hz, H-3), 3.60–3.45 (m, 3 H); ^{31}P NMR δ 4.12 (dd, $J_{P,1}$ = 7.2, $J_{P,2}$ = 1.5 Hz, P-1), −4.51 (m, $P_{4,6}$).

Enzymic Methods. Phosphoglucomutase was isolated from rabbit muscle according to an isolation procedure kindly supplied by Prof. W. J. Ray, Jr., Purdue University. All buffers and substrates were obtained from Sigma Chemical Company. In all cases phosphoglucomutase was preactivated prior to assaying.[39] The concentration of phosphoglucomutase solutions used for kinetic measurements was determined by measuring the absorbance at 278 nm; a 1% w/v solution having an absorbance of 7.7.[39]

Assays for Irreversible Inactivation. Phosphoglucomutase (0.6 units/mL) was incubated at 30 °C with the phosphofluoridate (5 mM), glucose-1,6-diP (0.1 mM), magnesium chloride (2 mM), and EDTA (1 mM) in Tris buffer (25 mM, pH 7.4), plus a control in which no inactivator was included. Aliquots were removed at time intervals over a total time period of 6 h and diluted into the assay buffer detailed below to measure residual enzyme activity. Identical conditions were used for the assay of glucose-4,6-cyclic-P-1-P except that the enzyme was dephosphorylated[42] prior to incubation, and a lower concentration (3.5 mM) of inactivator was employed.

Assays for Reversible Inhibition. Phosphoglucomutase activity was determined spectrophotometrically at 30 °C by coupling the production of glucose-6-P to NADPH formation with glucose-6-P dehydrogenase. The assay mixture used was 0.4 mL assay buffer, pH 7.4 containing 2.5 mM Tris buffer, 2.5 mM $MgCl_2$, 1.3 mM EDTA, 30 μM NADP, 0.4 units/mL of glucose-6-P dehydrogenase (Sigma, G5760), and 1.3 μM glucose-1,6-diP. To this was added 0.1 mL of phosphoglucomutase (ca. 0.006 units/mL) in activation buffer, inhibitor (0 to 50 μL) in 25 mM Tris, pH 7.4, and the total volume made up to 0.6 mL with Tris buffer. These reactants were incubated for 10 min in cuvettes in the spectrophotometer prior to the initiation of the reaction by addition of glucose-1-P. Control experiments were performed to ensure that none of the reagents was present in rate-limiting quantities and that the observed rate was not limited by the rate of anomerisation of glucose-6-P. Concentrations of glucose-1-P and inhibitor employed were as follows: mannose-1-PF (0, 18, 38, 55 mM), glucose-1-P (10, 13, 16, 24, 50 μM); glucose-6-PF (0, 6, 9, 12, 15, 21, 24 mM), glucose-1-P (97 μM); 2-fluoroglucose-1-PF (0, 4, 8, 12, 16 mM), glucose-1-P (9.7 μM); glucose-4,6-cyclic-P-1-P (0, 5.6, 56, 175, 280 μM), glucose-1-P (70 μM, glucose-1,6-diP (0.1 μM). Values of $K_{m_{app}}$ and K_i were determined by weighted linear regression analysis of the data according to Wilkinson.[43]

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