Inhibition of Phosphoglucomutase by Vanadate†

M. David Percival, Kevin Doherty, and Michael J. Gresser*

Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6, and The Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Québec, Canada H9R 4P8

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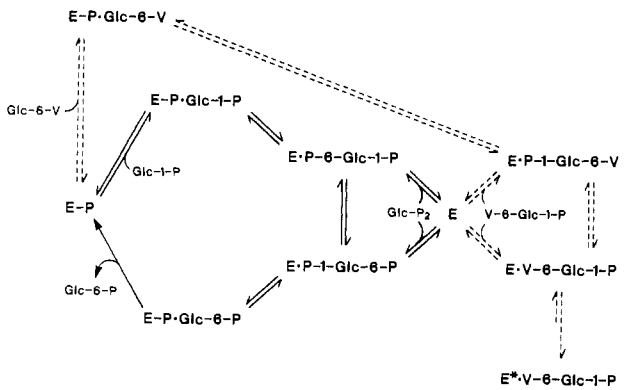
ABSTRACT: Phosphoglucomutase is inhibited by a complex formed from α-D-glucose 1-phosphate (Glc-1-P) and inorganic vanadate (V_i). Both the inhibition at steady state and the rate of approach to steady state are dependent on the concentrations of both Glc-1-P and V_i. Inhibition is competitive versus α-D-glucose 1,6-bisphosphate (Glc-P_2) and is ascribed to binding of the 6-vanadate ester of Glc-1-P (V-6-Glc-1-P) to the dephospho form of phosphoglucomutase (E). The inhibition constant for V-6-Glc-1-P at pH 7.4 was determined from steady-state kinetic measurements to be 2×10^{-12} M. The first-order rate constant for approach to steady state increases hyperbolically with inhibitor concentration. The results are consistent with rapid equilibrium binding of V-6-Glc-1-P to E, with dissociation constant 1×10^{-9} M, followed by rate-limiting conversion of the E·V-6-Glc-1-P complex to another species, E*·V-6-Glc-1-P, with first-order rate constant 4×10^{-2} s^{-1}. The rate constant determined for the reverse reaction, conversion of E*·V-6-Glc-1-P to E·V-6-Glc-1-P, is 2.5×10^{-4} s^{-1}. Formation of E*·V-6-Glc-1-P can also occur via binding of glucose 6-vanadate to the phospho form of phosphoglucomutase (E-P) followed by phosphoryl transfer and rearrangement of the enzyme–product complex.

Vanadate and vanadate-containing complexes are of considerable interest as mechanistic probes of enzymes that catalyze phosphoryl-transfer reactions. The ease with which vanadium can expand its coordination sphere allows the facile formation of vanadium(V) complexes which are thought to resemble the transition states for enzymatically catalyzed phosphoryl-transfer reactions (Knowles, 1980; Chasteen, 1983). This is presumably the reason why vanadium(V) complexes are very strong inhibitors of some enzymes that catalyze phosphoryl-transfer reactions, which is in turn the most likely explanation for the insulin-like and mitogenic effects of vanadium (Gresser et al., 1987).

A number of enzymes that accept phosphate esters as substrates, and catalyze reactions other than phosphoryl transfer, readily accept the corresponding vanadate esters as substrates (Nour-Eldeen et al., 1985; Drueckhammer et al., 1989). Thus, it is likely that for some phosphoryl-transfer enzymes, vanadate esters in solution, which closely resemble the substrates, bind to the enzyme as substrate analogues and only then are converted to transition-state analogues. Since the formation of vanadate esters from V_i and alcohols comes to equilibrium within milliseconds with equilibrium constants on the order of 0.2 M^{-1} (Gresser & Tracey, 1985; Tracey et al., 1988), very low yet constant concentrations of vanadate esters can be maintained even in solutions in which they bind tightly to higher concentrations of enzymes, because the esters are "buffered" by the presence of relatively high concentrations of their hydrolysis products.

To be able to effectively use vanadate esters as mechanistic probes and to work toward understanding the mechanisms of the physiological effects of vanadate, it is reasonable to carry out detailed studies of the interactions of vanadate with relatively well understood phosphoryl-transfer enzymes. For this purpose, the well-studied enzyme phosphoglucomutase (PGM)[1] was chosen (Post et al., 1989, and references cited therein). Vanadate is known to strongly inhibit the enzyme (Climent et al., 1982; Carreras et al., 1988; Ninfali et al., 1983), for which the kinetic mechanism shown in Scheme I has been established by extensive studies (Post et al., 1989, and references cited therein). The pathways indicated by dashed arrows represent the ways in which two vanadate esters, Glc-6-V and V-6-Glc-1-P, would be expected to interact with the enzyme as analogues of the corresponding phosphate esters. In the species E·V-6-Glc-1-P the vanadate moiety is bound at the phosphotransfer site and is a potential transition-state analogue

Scheme I: Kinetic Mechanism of Phosphoglucomutase[a]



[a] Dashed arrows represent the proposed interactions of Glc-6-V and 6-V-Glc-1-P with the enzyme.

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* Correspondence should be addressed to this author at The Merck Frosst Centre for Therapeutic Research.

[1] Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; V_i, inorganic vanadate; Glc-P_2, α-D-glucose 1,6-bisphosphate; V-6-Glc-1-P, α-D-glucose 1-phosphate 6-vanadate; E, the dephospho form of phosphoglucomutase; E-P, the phospho form of phosphoglucomutase; Glc, D-glucose, equilibrium mixture of α and β anomers; Glc-6-V, D-glucose 6-vanadate, equilibrium mixture of α and β anomers; NAD, β-nicotinamide adenine dinucleotide; BSA, bovine serum albumin; PGM, phosphoglucomutase.

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for the step in which E-P-Glc-1-P and E-P-6-Glc-1-P are interconverted. In the species E-P-1-Glc-6-V the phosphate moiety is bound at the phosphotransfer site, and the ligand would be expected to bind similarly to Glc-P_2. The species E-P-1-Glc-6-V would be expected to form if Glc-6-V bound to E-P as a Glc-6-P analogue and became phosphorylated by the phosphoenzyme. E-P-1-Glc-6-V could then isomerize to the potential transition-state-analogue complex E-V-6-Glc-1-P in the same way that E-P-1-Glc-6-P isomerizes to complete the normal catalytic cycle. The results reported in this paper are consistent with the mechanism outlined in Scheme I but prompt some questions concerning the detailed mechanisms of interconversion of E-P-1-Glc-6-V and E-V-6-Glc-1-P and the "tightening down" of E-V-6-Glc-1-P into the presumed transition-state-analogue complex.

Experimental Procedures

Materials. Glc-P_2 (Sigma) was used without further purification. Glc-1-P (Sigma) was purified by an ion-exchange chromatography procedure described elsewhere (Ray et al., 1990) to remove traces of Glc-P_2. The concentrations of stock solutions of Glc-1-P and Glc-P_2 were determined by acid-labile phosphate analysis (Bartlett, 1959). PGM (the phospho form) was a gift from Professor W. J. Ray, Jr. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Boehringer) was dialyzed twice for 5 h at 4 °C in buffer at pH 7.4, containing 20 mM Tris-HCl. After dialysis, the enzyme was concentrated by using a Centricon device, to approximately 1 mg/mL, and stored as droplets in liquid nitrogen. Both bovine serum albumin (catalog no. 238031) and superoxide dismutase were from Boehringer Mannheim. A 0.10 M stock solution of inorganic vanadate (NaH_2VO_4) was prepared by dissolving V_2O_5 (Aldrich, gold label) in 2 molar equiv of 1.0 M NaOH and diluting to volume after the solution had become colorless.

Rate Measurements. All of the experiments were done with solutions maintained at 25 °C, pH 7.40, with 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 1.3 mM EDTA, 0.03 mg/mL BSA, 0.2 mM NAD, 5 μg/mL glucose-6-phosphate dehydrogenase, and 60 units/mL superoxide dismutase, total volume 1.50 mL. The superoxide dismutase was added to inhibit the vanadate-activated oxidation of NADH. Concentrations of other solutes were as given in the figure legends and with the other descriptions of the experiments. In all cases PGM was activated as described elsewhere (Peck & Ray, 1971) prior to the experiment. Concentrations of PGM were determined by using E_{278} = 7.0 for a 1% w/v solution and a molecular weight of 61 600 (Ray et al., 1983). The enzyme had an activity of 800 units/mg. Reactions were followed by measuring the absorbance of solutions at 340 nm as a function of time with a Hewlett-Packard 8452 spectrophotometer. Values of k_{obs} were determined by fitting the equation expressing product concentration as a function of time for a first-order approach to steady state (Morrison & Walsh, 1988) to the data by using an iterative procedure supplied with the Hewlett-Packard data station.

Results

When the phosphoglucomutase-catalyzed conversion of Glc-1-P to Glc-6-P was initiated by addition of the phospho form of the enzyme (E-P) to reaction mixtures containing Glc-1-P, Glc-P_2, and V_i, the initial rate, at V_i concentrations up to 35 μM, was similar to that observed when V_i was not present. The rate decreased with time in a first-order manner to a steady-state level, with k_{obs} values for this inactivation process which decreased with increasing Glc-P_2 and increased

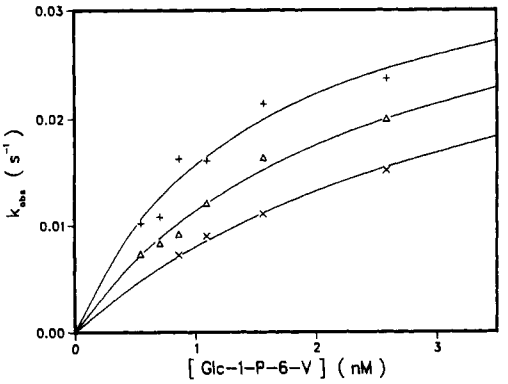

FIGURE 1: Effect of inhibitor and Glc-P_2 concentrations on k_{obs} for approach to the steady-state rate of PGM-catalyzed conversion of Glc-1-P to Glc-6-P. Reaction mixtures contained 0.02 unit of PGM, 5.15×10^{-4} M Glc-1-P, 5.0×10^{-8} M Glc-P_2 (+), 1.6×10^{-7} M Glc-P_2 (△), or 3.3×10^{-7} M Glc-P_2 (×), and V_i as indicated, and other conditions and procedures were as described under Experimental Procedures. The points are experimental values; the lines were calculated from eq 1 by using the values $k_6 = 0\ s^{-1}$, $k_5 = (3.8 \pm 0.3) \times 10^{-2}\ s^{-1}$, $K_i = (1.0 \pm 0.4) \times 10^{-9}$ M, and $K_a = (1.20 \pm 0.23) \times 10^{-7}$ M. The values for the constants with indicated standard deviations were obtained from a nonlinear least-squares fit of eq 1 to the data.

with increasing V_i or Glc-1-P concentrations. The effects of changes in V_i and Glc-P_2 concentrations on the magnitude of k_{obs} are shown in Figure 1. The lines drawn through the experimental points were calculated from eq 1 by assuming

$$k_{obs} = k_6 + k_5\left(\frac{[I]/K_I}{1 + [A]/K_a + [I]/K_I}\right) \qquad (1)$$

that $k_6 \ll k_{obs}$ under the conditions used and using the values for k_5, K_i, and K_a given in the figure legend, which were obtained from a nonlinear least-squares fit of eq 1 to the data. Equation 1 was derived on the basis of the mechanism shown in eq 4. The various rate constants are defined in eq 4, $K_a = k_2/k_1$ and $K_i = k_4/k_3$.

In eq 1, A represents Glc-P_2 and I represents the inhibitor, which is considered to be the 6-vanadate ester of Glc-1-P (V-6-Glc-1-P) formed spontaneously from V_i and Glc-1-P. The concentrations of V-6-Glc-1-P were calculated by using a value of 0.17 M^{-1} for the equilibrium constant (K_f) for formation of V-6-Glc-1-P. This value was obtained from the pK_a value of 14.8 for the 6-hydroxyl group of glucose (Ballinger & Long 1960) and the relationship of the equilibrium constant for vanadate ester formation to the pK_a of the alcohol (Tracey et al., 1988). This value of $K_f = 0.17\ M^{-1}$ is the formation constant for the trianion of V-6-Glc-1-P from vanadate monoanion and Glc-1-P dianion. Initial estimates for the values of k_5, K_a, and K_i were obtained from plots of k_{obs}^{-1} vs $[I]^{-1}$, which intersect at a common point on the vertical axis equal to k_5^{-1}, when analyzed according to eq 1, assuming $k_6 \ll k_{obs}$. A replot of the slopes of the double-reciprocal plots versus [Glc-P_2] was also linear with vertical intercept equal to K_i/k_5 and the slope equal to $K_i/(k_5K_a)$. Similar trends were observed when $[V_i]$ was held constant and [Glc-1-P] and [Glc-P_2] were varied. The values and standard deviations obtained for the constants in this series of experiments were $k_5 = (5.3 \pm 0.5) \times 10^{-2}\ s^{-1}$, $K_i = (1.0 \pm 0.3) \times 10^{-9}$ M, and $K_a = (2.30 \pm 0.72) \times 10^{-7}$ M.

Figure 2 shows the results of a similar experiment in which Glc-P_2 was maintained at a fixed concentration and [Glc-1-P] was varied at two different fixed concentrations of V_i. The lines were calculated from eq 1 by using the contants $k_5 = 4 \times 10^{-2}\ s^{-1}$, $K_i = 1.0 \times 10^{-9}$ M, and $K_a = 2.3 \times 10^{-7}$ M. The double-reciprocal plots of k_{obs}^{-1} vs $[\text{Glc-1-P}]^{-1}$ were linear with

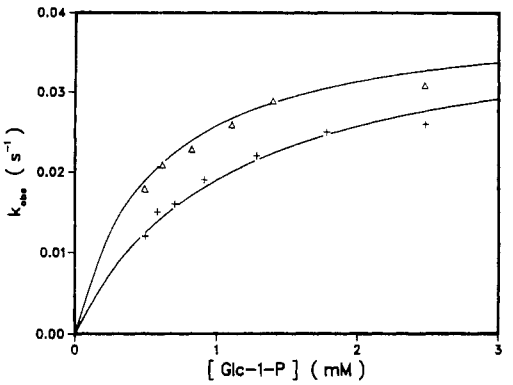

FIGURE 2: Effect of V_i and Glc-1-P concentrations on k_{obs} for approach to the steady-state rate of PGM-catalyzed conversion of Glc-1-P to Glc-6-P. Reaction mixtures contained 0.03 unit of PGM, 1.5×10^{-7} M Glc-P_2, 1.0×10^{-5} M V_i (+) or 2.0×10^{-5} M V_i (Δ), and Glc-1-P as indicated, and other conditions and procedures were as described under Experimental Procedures. The lines were calculated from eq 1 by using the values $k_6 = 0\ s^{-1}$, $k_5 = 4.0 \times 10^{-2}\ s^{-1}$, $K_i = 1.0 \times 10^{-9}$ M, and $K_a = 2.3 \times 10^{-7}$ M.

a common intercept of the two lines on the vertical axis, which is required by eq 1. The implications of this result are discussed below.

The effect of changes in $[V_i]$ and $[Glc\text{-}P_2]$ on the steady-state rate of the reaction were determined in a series of experiments in which Glc-P_2 and V_i concentrations were varied within the ranges $0.4\text{–}2.5 \times 10^{-7}$ and $0\text{–}1.0 \times 10^{-5}$ M, respectively. Reaction mixtures contained 0.01 or 0.02 unit of PGM and 1.0×10^{-4} M Glc-1-P. The linear plots of velocity^{-1} vs $[Glc\text{-}P_2]^{-1}$ obtained are consistent with competitive inhibition. The results were analyzed in terms of eq 2, which was

$$\text{rate} = V_m[Glc\text{-}P_2]/([Glc\text{-}P_2] + K_m(1 + [V_i]/K_v + [Glc\text{-}1\text{-}P]/K_{GP} + [Glc\text{-}1\text{-}P][V_i]K_f/K_{GPV})) \quad (2)$$

derived on the basis of the assumption that V_i, Glc-1-P, and V-6-Glc-1-P all compete with Glc-P_2 for the dephospho form of phosphoglucomutase (E) with inhibition constants K_V, K_{GP}, and K_{GPV}, respectively. The values of the kinetic constants, $K_{GPV} = (1.9 \pm 0.1) \times 10^{-12}$ M, $K_v = K_{GP} = \infty$, and $K_m = (4.8 \pm 0.3) \times 10^{-8}$ M, were obtained from a nonlinear least-squares fit of eq 2 to the data in which initial estimates were obtained from a replot of the slopes of the double-reciprocal plots (of velocity^{-1} vs $[Glc\text{-}P_2]^{-1}$) versus $[V_i]$. The replot was linear and intersected the origin, indicating that the K_v term could be ignored. Similar analysis of a study in which $[V_i]$ was held constant and three different fixed Glc-1-P concentrations were used gave a value of $(1.6 \pm 0.1) \times 10^{-12}$ M for K_{GPV}, which is in close agreement with the value obtained above. In the latter study it was found that the K_{GP} term could be ignored, consistent with the published value of 5×10^{-4} M for K_{GP} (Ray et al., 1966). In this additional experiment the three fixed concentrations of Glc-1-P were 4.10×10^{-4} M, 1.03×10^{-3} M, and 2.07×10^{-3} M. Glc-P_2 concentration was varied from 4.0×10^{-8} to 2.5×10^{-7} M, and $[V_i]$ was constant at 5.0×10^{-7} M.

Equations 1 and 2 both ignore the presence of other inhibitors, such as Cl^- and SO_4^{2-} (Ray & Roscelli, 1966), which bind to E and therefore reduce the concentration of free E that is available to react with V-6-Glc-1-P. Evidence of this is indicated by the calculated value of K_m (Glc-P_2), 1.20×10^{-7} M (Figure 1), which is larger, by a factor of about 10, than the accepted value (Ray et al., 1966). The result of this is that the calculated estimates of K_I and K_{GPV} are somewhat higher than their true values. However, the differences are small compared to the overall magnitude of the constants and have little effect on the conclusions drawn.

Even though the calculated concentrations of the inhibitor 6-V-Glc-1-P present in these experiments are comparable to the enzyme concentrations, depletion of the inhibitor in solution need not be considered, contrary to the case with normal tight binding inhibitors (Williams & Morrison, 1979). If depletion of the inhibitor in solution due to its binding to the enzyme did occur, then plots of rate in the presence of inhibitor versus PGM concentration would curve upward as the concentration of enzyme exceeded that of inhibitor (Ackerman & Potter, 1949). It was found that in the presence of 1×10^{-4} M V_i, 9.6×10^{-5} M Glc-1-P, and 2.5×10^{-7} M Glc-P_2 and other conditions as given under Experimental Procedures, the steady-state rate increased linearly with PGM concentration up to at least 5.3×10^{-9} M PGM. Under these conditions and using the value 0.17 M^{-1} for the K_f, the calculated concentration of V-6-Glc-1-P was 1.63×10^{-9} M.

When the dephospho form of PGM (E, 0.012 mg/mL) was incubated with Glc-1-P (2.5×10^{-3} M) and V_i (9.5×10^{-6} M) and then diluted (15 000-fold) into an assay mixture (containing 6.0×10^{-4} M Glc-1-P and 2.0×10^{-5} M Glc-P_2) such that the concentration of inhibitor was too low to cause detectable inhibition, the rate increased from essentially zero to the steady-state value in a first-order manner over a period of several hours. The final rate was almost identical with that of a control incubated in the absence of V_i. Similar results were obtained when the phospho form of PGM (E-P, 0.008 mg/mL) was incubated with Glc (0.17 M) and V_i (15.0×10^{-6} M). However, in this case, the initial rate after dilution was approximately 50% of the final rate. In each case the first-order rate constant for approach to the uninhibited steady state was $(2.5 \pm 0.5) \times 10^{-4}\ s^{-1}$. The rate constants for approach to steady state in these and the pre-steady-state studies described earlier did not vary over a 7-fold range of [PGM]. When E was incubated with Glc and V_i, or E-P was incubated with Glc-1-P and V_i before dilution into an assay mixture, no significant inhibition was observed.

Formation of the inhibited enzyme by incubating E-P with Glc plus V_i is rationalized as shown in Scheme I in terms of binding of Glc-6-V to E-P as a substrate analogue, followed by transfer of the phosphate group from the enzyme to the bound Glc-6-V and a subsequent change in the mode of binding of the resulting P-1-Glc-6-V so that the vanadate group occupies the phosphotransfer site. Other investigators have reported that V_i strongly enhances the rate of transfer of phosphate from E-P to Glc, although their observations were rationalized in terms of a different mechanism from that described above (Layne & Najjar, 1979).

The glucose-enhanced V_i inhibition could not be studied at steady-state, because of the difficulty of separating the inhibition due to Glc plus V_i and Glc-1-P plus V_i. The reason for this is that to reach steady-state before substrate depletion became a problem, the concentrations of Glc-1-P and V_i that had to be used resulted in very high levels of inhibition. However, useful information was obtained from a study of the pre-steady-state inhibition by Glc plus V_i. High concentrations of Glc were used to obtain a relatively fast approach to a "steady-state" rate, which was essentially zero. When a high Glc-P_2 concentration and a relatively low Glc-1-P concentration were used, the time-dependent inhibition due to Glc-1-P plus V_i was sufficiently slow to be insignificant on the time scale of the experiments. The k_{obs} for onset of the inhibition due to Glc plus V_i increased hyperbolically with Glc concentration, was not affected by changes in Glc-P_2 concentration, and decreased with increasing Glc-1-P concentration. The

Table I: Expressions for the First-Order Rate Constants for Approach to Steady State, k_{obs}, for the Mechanisms of Equations 3–5

eq	case	assumptions	k_{obs}
3	1	$k_2/k_1 = K_a$, always at equil $k_4/k_3 = K_i$, rate limiting	$(k_4(1 + [A]/K_a + [I]/K_i))/(1 + [A]/K_a)$
3	2	apply steady-state approximation to E, assume $[E] \ll [E_{tot}]$	$(k_1k_4[A] + k_2k_3[I])/(k_1[A] + k_3[I])$
4	3	$k_2/k_1 = K_a$, always at equil $k_4/k_3 = K_i$, always at equil k_5, k_6, rate limiting	$k_6 + k_5(([I]/K_i)/(1 + [A]/K_a + [I]/K_i))$
4	4	$k_2/k_1 = K_a$, always at equil $k_5/k_6 = K_{eq}$, always at equil k_3, k_4, rate limiting	$k_4/(1 + K_{eq}) + k_3[I]/(1 + [A]/K_a)$
4	5	$k_2/k_1 = K_a$, always at equil apply steady-state approximation to EI, assume $[EI] \ll [E_{tot}]$	$k_4k_6/(k_4 + k_5 + k_6) + k_3(k_5 + k_6)[I]/(k_4 + k_5 + k_6)(1 + [A]/K_a)$
5	6	$k_2/k_1 = K_a$, always at equil $k_3/k_4 = K_{eq}$, always at equil k_5/k_6, rate limiting	$(k_6(1 + ([A]/K_a(1 + K_{eq})) + ([I]/K_i(1 + 1/K_{eq}))))/(1 + ([A]/K_a(1 + K_{eq})))$
5	7	$k_2/k_1 = K_a$, always at equil $k_6/k_5 = K_i$, always at equil k_3/k_4, rate limiting	$k_4K_i/(K_i + [I]) + k_3/(1 + [A]/K_a)$
5	8	$k_2/k_1 = K_a$, always at equil apply steady-state approximation to E*, assume $[E^*] \ll [E_{tot}]$	$(k_4(1 + [A]/K_a) + k_3[I]/K_i)/((1 + [A]/K_a)(k_4/k_6 + [I]/K_i))$

results were quantitatively analyzed in terms of eq 1, where A is Glc-1-P. When $[Glc\text{-}P_2] = 9.3 \times 10^{-6}$ M, $[V_i] = 6.5 \times 10^{-5}$ M, [Glc-1-P] = 2.5×10^{-5} M, and [Glc] was varied from zero to 6.5×10^{-2} M, using $K_a = 8.0 \times 10^{-6}$ M, the values $K_i = (8 \pm 2) \times 10^{-8}$ M and $k_5 = (4.7 \pm 0.4) \times 10^{-2}$ s^{-1} were obtained. K_a is the Michaelis contant of Glc-1-P, which was determined in an independent experiment. These values, together with $k_6 = 2.5 \times 10^{-4}$ s^{-1}, from k_{obs} for recovery of activity after incubation of E-P with Glc plus V_i and dilution into an assay mixture, yield an overall dissociation constant for the equilibrium E-P + Glc-6-V $\rightleftharpoons$ E*·V-6-Glc-1-P of 4.3×10^{-10} M (see eq 4 and Scheme I). This can be compared with the overall dissociation constant for the equilibrium E + V-6-Glc-1-P $\rightleftharpoons$ E*·V-6-Glc-1-P, which is referred to as K_{GPV} (eq 2) of 1.9×10^{-12} M. Since these two equilibria are assumed to yield a common product, the quotient of the two dissociation constants, 2.2×10^2, should be equal to the equilibrium constant for the reaction in which the two sets of reactants are interconverted, E + V-6-Glc-1-P $\rightleftharpoons$ E-P + Glc-6-V. From published values for the free energy of hydrolysis of E-P, −1.0 kcal mol^{-1} (Ray & Long, 1976) and for hydrolysis of Glc-1-P, −4.2 kcal mol^{-1} (Peck et al., 1968) both at pH 7.5 and 25 °C, a value of 2.3×10^2 can be estimated for this equilibrium constant. This value is in good agreement with that calculated from the two dissociation constants.

Discussion

When a kinetic mechanism to account for the first-order approach to steady state in the presence of inhibitor was selected, the three basic mechanisms shown as eqs 3–5 were

$$EA \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} E \underset{k_4}{\overset{k_3I}{\rightleftharpoons}} EI \quad (3)$$

$$EA \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} E \underset{k_4}{\overset{k_3I}{\rightleftharpoons}} EI \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E^*I \quad (4)$$

$$EA \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} E \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^* \underset{k_6}{\overset{k_5I}{\rightleftharpoons}} E^*I \quad (5)$$

considered. In general, none of these mechanisms requires a first-order approach to steady state. Only in certain limiting cases will a first-order process by observed (Szabo, 1969). Eight such cases, along with the corresponding expressions for the first-order rate constant, k_{obs}, for approach to steady state are summarized in Table I. Two additional cases, which could result from application of the steady-state approximation to E in eqs 4 and 5, were not considered. Other investigators have discussed the expressions for k_{obs} corresponding to cases 1, 3, and 6 (Cha, 1976), case 2 (Nakamura & Abeles, 1985), and case 7 (Shapiro & Riordan, 1984). The other cases, 4, 5, and 8, do not appear to have been considered, at least not in the recent literature on time-dependent inhibition. Only cases 2, 3, and 8 are consistent with the nonlinear increase of k_{obs} with increasing [I] shown in Figure 1. Case 8 is consistent with a nonlinear increase or decrease of k_{obs} with increasing [I], depending upon the relative magnitudes of k_6 and $k_3/(1 + [A]/K_a)$, but this case can be ruled out because it requires that at saturating [I], k_{obs} decrease with increasing [A]. In terms of case 2, the decreased sensitivity of k_{obs} to changes in [I] at high [I] is due to the dissociation of A becoming rate limiting. This requires, in terms of the data shown in Figure 1, that $k_2 = 4 \times 10^{-2}$ s^{-1}, which is not consistent with the value of 11 s^{-1} determined independently for k_2 under similar conditions (Ray et al., 1989). Thus, of the cases considered, only case 3 can account for the results reported here. Case 3 is considered by some authorities (Morrison & Walsh, 1988) to rationalize all time-dependent inhibition by slow binding inhibitors. While this may well be true, it is worth remembering that other cases exist and that in general the interaction of slow binding inhibitors with enzymes need not result in a first-order approach to steady state.

In terms of eq 4, the value of K_{GPV} obtained from steady-state experiments should be equal to K_ik_6/k_5, thus allowing the calculation of k_6, which should agree with the value for k_{obs} obtained for the recovery of activity when dephospho enzyme incubated with Glc-1-P and V_i was added to assay medium such that the inhibitor was diluted to a concentration far below K_{GPV} and $k_{obs} \simeq k_6$. From the values for K_{GPV}, K_i, obtained from the steady-state experiments, and k_5, given in the legend of Figure 1, $k_6 = K_{GPV}k_5/K_i = (1.9 \times 10^{-12} \times 3.8 \times 10^{-2})/1.0 \times 10^{-9} = 0.73 \times 10^{-4}$ s^{-1}, which is in reasonable agreement with the value of 2.5×10^{-4} s^{-1} obtained as described above for k_6.

In the experiments in which Glc plus V_i formed a complex that bound to E-P, the proposed enzymic phosphorylation of Glc-6-V to yield a transition-state-analogue inhibitor is analogous to the interaction of methionine sulfoximine with glutamine synthetase (Rowe et al., 1969) and of various sulfoximes with δ-glutamylcysteine synthetase (Griffith, 1982).

Binding of Glc-1-V should occur as well, and probably does, leading to formation of species analogous to those proposed below and in Scheme I. However, the present results do not require the proposal of separate intermediates arising from reaction of Glc-6-V and Glc-1-V with E-P, so it will be assumed for the present that if both classes of intermediates exist in significant amounts, they behave similarly kinetically.

The species EI in eq 4 can be considered to correspond, in terms of Scheme I, to a rapidly interconverting mixture of E·V-6-Glc-1-P and E·P-1-Glc-6-V. In terms of this model, the k_5 process corresponds to the tightening down of the E·V-6-Glc-1-P complex to the E*·V-6-Glc-1-P complex. It is proposed that this tight complex has a structure analogous to that of the transition state for the interconversion of E-P·Glc-1-P and E·P-6-Glc-1-P. This transition-state analogue is most probably a pentacoordinate vanadate complex, in which the active-site serine hydroxyl occupies one apical coordination site and the 6-hydroxyl of Glc-1-P the other. There is no obvious reason why this tightening down process should be slow, if it is true that the vanadate esters interact with the enzyme as substrate analogues and are converted to the transition-state analogue only after binding at the catalytic site. A possible cause could be the tendency of the "wrong" nucleophile to move into the coordination sphere of the bound V-6-Glc-1-P. There is evidence that when chymotrypsin catalyzes the hydrolysis of highly reactive esters, the histidine imidazole moiety, which is thought to normally function as a general base, acts as a nucleophile (Hubbard & Kirsch, 1972; Fife, 1972). The formation of E*I from EI could involve the replacement of the wrong nucleophile in the vanadium coordination sphere with the serine hydroxyl which is phosphorylated in the normal catalytic cycle. The observation that the K_i value obtained from the pre-steady-state studies for the binding of 6-V-Glc-1-P to E, 1×10^{-9} M, is well below the K_m for Glc-P_2 under the same conditions, 4.8×10^{-8} M, indicates that the inhibitor in the EI complex binds more tightly than does Glc-P_2.

A second possibility is that the formation of E·V-6-Glc-1-P from E, Glc-1-P, and V_i involves stepwise binding of Glc-1-P and V_i rather than binding of performed V-6-Glc-1-P. This possibility in its simplest form can be ruled out on the basis of the results of the time-dependent inhibition studies and the expression for k_{obs} derived for this mechanism. The stepwise mechanism is represented as eq 6, where X and Y represent

$$EA \underset{K_A}{\overset{A}{\rightleftharpoons}} E \underset{K_X}{\overset{X}{\rightleftharpoons}} EX \underset{k_6}{\overset{k_5Y}{\rightleftharpoons}} EI \qquad (6)$$

the two components, V_i and Glc-1-P, of V-6-Glc-1-P binding to E in either order. Assuming that the k_5,k_6 step is rate limiting and the other two steps are at equilibrium, with dissociation constants K_a and K_X, leads to the expression

$$k_{obs} = k_6 + k_5[Y]\left(\frac{[X]/K_X}{1 + [A]/K_a + [X]/K_X}\right) \qquad (7)$$

for k_{obs}. From this equation it is predicted that, assuming $k_6 \ll k_{obs}$, the intercept of a plot of $1/k_{obs}$ versus $1/[X]$ will decrease with increasing [Y] and a plot of $1/k_{obs}$ versus $1/[Y]$ will intersect the origin. Double-reciprocal plots of data shown in Figures 1 and 2 are not consistent with the mechanism shown in eq 6 for either possible assignment of X and Y as both produce lines that intersect at a common point on the k_{obs}^{-1} axis (25 s).

The similar values obtained for k_{obs} for recovery of activity (k_6) after E-P incubated with Glc plus V_i, or E incubated with Glc-1-P plus V_i, was diluted into an assay mixture support the hypothesis that E*·6-V-Glc-1-P was formed in both cases. The similar values of k_{obs} for development of the time-dependent inhibition at saturating inhibitor concentrations (k_5) due to Glc plus V_i and that due to Glc-1-P plus V_i is consistent with a common rate-limiting step in formation of E*·6-V-Glc-1-P by both pathways. The results reported here are consistent with that common rate-limiting step being reaction of E·V-6-Glc-1-P or a rapidly equilibrating mixture of E·V-6-Glc-1-P and E·P-1-Glc-6-V to yield E*·V-6-Glc-1-P. Since the kinetic model used to interpret the time-dependent inhibition studies involves equilibrium formation of a common intermediate from both E plus V-6-Glc-1-P and E-P plus Glc-6-V, some "mixing" of the pathways is expected to occur. For example, some of the EI formed from E plus V-6-Glc-1-P should react to form E-P plus Glc-6-V before it has time to react to form E*·V-6-Glc-1-P in the rate-limiting step. Thus, the EI complex has an alternative pathway with which to reenter the catalytic cycle. This behavior would result in a value of K_i larger than the true dissociation constant for E·V-6-Glc-1-P to yield E plus V-6-Glc-1-P, but would have no consequences observable to pre-steady-state kinetic experiments of the type shown in Figure 1. Some evidence of this occurrence has been detected in single-turnover experiments. When E is incubated with V_i and Glc-1-P, complete inhibition takes several hours to be achieved. This is probably due to the formation of E-P (via E·P-1-Glc-6-V and E-P·Glc-6-V), the Glc-P_2 subsequently produced by reaction of E-P with Glc-1-P protecting E from inhibition by the mixed diester. In addition, the observation that incubation of E-P with Glc plus V_i does not result in complete inhibition is evidence that the E·P-1-Glc-6-V and E·V-6-Glc-1-P complexes thus produced can partition to give either E*V-6-Glc-1-P or E plus V-6-Glc-1-P.

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