Characterization of a Vanadate-Based Transition-State-Analogue Complex of Phosphoglucomutase by Kinetic and Equilibrium Binding Studies. Mechanistic Implications†

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ABSTRACT: The inhibitor complex produced by the binding of α-D-glucose 1-phosphate 6-vanadate to the dephospho form of muscle phosphoglucomutase exhibits an unusually small dissociation constant: about 15 fM for the Mg^{2+} enzyme at pH 7.4, when calculated in terms of the tetraanion. Such tight binding suggests that the enzyme/vanadate/glucose phosphate complex mimics a state that at least approaches the transition state for (PO_3^-) transfer in the normal enzymic reaction. This hypothesis also is supported by the observation that replacement of Mg^{2+}, the normal metal ion activator, by Li^+, a poor activator, substantially reduces the binding constant for the glucose phosphate/vanadate mixed diester. Other indicators that support this hypothesis are described. One is the derived equilibrium constant for replacement of a PO_4^{2-} group in bound glucose bisphosphate by VO_4^{2-}: 3×10^6 when the replaced group is the phosphate at the (PO_3^-) transfer site of the Mg^{2+} enzyme—in contrast to about 10 for the same replacement (of PO_4^{2-} by VO_4^{2-}) in an aqueous solution of a phosphate ester. Another is the greatly decreased rate at which Mg^{2+} dissociates from the glucose phosphate/vanadate complex of the enzyme, relative to the rate at which it dissociates from the corresponding bisphosphate complex (rate ratio $\leq 3 \times 10^{-4}$), presumably because Mg^{2+} binds more tightly to the glucose phosphate/vanadate complex than to the corresponding bisphosphate complex. This apparent increase in Mg^{2+} binding occurs in spite of what appears to be a reduced charge density at the bound vanadate grouping, relative to the bound phosphate grouping, and in spite of the somewhat weaker binding of Mg^{2+} by dianionic vanadate than by the phosphate dianion. Although a direct assessment of the binding constant for Mg^{2+} was not possible, the equilibrium constant for Mg^{2+}/Li^+ exchange could be evaluated for the complexes of dephospho enzyme with glucose bisphosphate or glucose 1-phosphate 6-vanadate. The results suggest that the glucose phosphate/vanadate complex of the Mg^{2+} enzyme mimics a state about halfway between the ground state and the transition state for (PO_3^-) transfer. This estimate also is in accord with the binding of glucose phosphate/vanadate relative to that expected for transition-state binding of glucose bisphosphate. A possible scenario for the (PO_3^-) transfer catalyzed by the Mg^{2+} form of phosphoglucomutase is discussed, on the basis of these observations, together with possible reasons why the bound vanadate group appears to mimic an intermediate state for (PO_3^-) transfer rather than the ground state for phosphate binding.

In aqueous solution, inorganic vanadate or V(V) resembles inorganic phosphate in many respects. Even the pK_a values for all three ionizations of vanadic acid are similar to those of phosphoric acid, although increased somewhat in each case (Pope & Dale, 1968; Kepert, 1973; Chasteen, 1983).[1] However, a major difference is the exceedingly rapid rate at which solutions of vanadate and various alcohols produce equilibrium mixtures of esters, presumably due to a greatly increased tendency of vanadate to undergo addition reactions, relative to that of inorganic phosphate, which in turn is due to the ability of vanadium(V) to expand its coordination sphere (Pope & Dale, 1968; Kepert, 1973). A minor difference or, from another standpoint, a similarity is the equilibrium constant for formation of vanadate esters in dilute aqueous solution from V_i[2] and the corresponding alcohol: it is only slightly more favorable for vanadate than for phosphate esters (Tracey et al., 1988). Actually, a precise comparison of formation constants would be complex, because the thermodynamic stability of vanadate and phosphate esters exhibits a different sensitivity to the pK_a of the parent alcohol (Bourne & Williams, 1984; Tracey et al., 1988). But for the many alcohols of biochemical interest, whose pK_a lies between 15 and 16, the formation constant for vanadate esters is about 0.1 M^{-1} and is relatively independent of pH, because the formation constant is approximately the same for $HOVO_3^{2-}$ and $(HO)_2VO_2H^-$ (Tracey

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[1] Although it has been stated (Crans & Shin, 1988) that the pK_a values for phosphates and vanadates differ by less than 1 unit, this claim may have arisen from comparisons made at different ionic strengths, since pK_{2a} for vanadic acid at $\mu = 0.02$ appears to be about 8.6 (see Results) and thus is approximately 1.6 pK_a units above that of phosphate at the same ionic strength [cf. Bates and Acree (1943)]. A difference of 1.6 units in pK_{1a} for phosphoric and vanadic acids also has been reported (Yuchi et al., 1979).

[2] Abbreviations: E-P and E, the phospho and dephospho forms of phosphoglucomutase; E-V the vanado enzyme analogous to E-P; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate—equilibrium mixture of α and β anomers unless otherwise specified; Glc-P_2 or P-6-Glc-1-P, α-D-glucose 1,6-bisphosphate; V_i, inorganic vanadate; V-6-Glc-1-P and V-1-Glc-6-P, the 6-vanadate ester of Glc-1-P and the α-1-vanadate ester of Glc-6-P; E*V*6-Glc-1-P or E*V*6-Glc-1-P(Mg^{2+}) and E*V*1-Glc-6-P or E*V*1-Glc-6-P(Mg^{2+}), the inhibitor complexes produced by treating the Mg^{2+} complex of the dephospho enzyme with V_i plus Glc-1-P or Glc-6-P, respectively; E·V-6-Glc-1-P or E·V-6-Glc-1-P-(Li^+), the corresponding complex produced from the Li^+ complex of the dephospho enzyme; V_2 and V_4, dimeric and tetrameric forms of V_i, respectively.

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et al., 1988). The formation constants for dianionic phosphate esters of alcohols with pK_a values of about 15.5 are close to 10^{-2} M (Bourne & Williams, 1984). Hence, from a thermodynamic standpoint, dianionic vanadate esters of such alcohols are only about 10-fold more stable toward hydrolysis than the corresponding phosphate esters. The unusual stability of the vanadate-based inhibitor complex of phosphoglucomutase, described herein, is even more remarkable in view of this consideration (see Discussion).

That vanadate has a marked effect on the activity of phosphoglucomutase originally was observed by Layne and Najjar (1979), but the mechanism of this interaction first was worked out by Gresser and co-workers and is described in an accompanying paper (Percival et al., 1990). The inhibition is competitive with Glc-P_2 and thus is due to the binding of the mixed diester, V-6-Glc-1-P, to the dephospho enzyme [see also the second paper in this series (Ray et al., 1990)]. Because of the very tight binding of this inhibitor, Percival et al. also suggest that the vanadate ester group of V-6-Glc-1-P may form a covalent, pentacoordinate adduct with the active-site serine hydroxyl group and that, if so, this complex might serve as an analogue of the transition state for the enzymic (PO_3^-)-transfer step.[3]

In a subsequent study [second paper in this series (Ray et al., 1990)] ultraviolet difference spectroscopy was used to show that the phosphoglucosyl grouping of V-6-Glc-1-P binds to the dephospho enzyme in the same way that glucose 1-phosphate binds to the phospho enzyme—with the phosphate at the distal binding site. Hence, as was postulated by Percival et al. (1990), the vanadate ester group must bind at the proximal or (PO_3^-)-transfer site [see Scheme I in the second paper of this series (Ray et al., 1990)]. On the other hand, the spectrum of the oxyvanadium constellation in what we refer to as the E*V*6-Glc-1-P complex is not what is expected for a pentacoordinate adduct of vanadate(V), either from studies of model compounds or from theory, since in such an adduct coordination of V(V) is *increased* above that characteristic of tetrahedral VO_4^{3-} (Ray & Post, 1990, the third paper in this series). In fact, spectral studies suggest that rather than an increase in coordination on formation of the inhibitor complex, the coordination of V(V) in E*V*6-Glc-1-P actually *decreases* relative to simple vanadate esters—as though on binding the $ROVO_3^{2-}$ group were distorted in the direction of $RO^- + VO_3^-$.

Because the vanadate-based inhibitor binds so tightly to phosphoglucomutase, and because assay components produce complex inhibition patterns when Glc-P_2 is subsaturating [cf. Ray and Roscelli (1966a)], it is difficult to assess the true binding constant for V-6-Glc-1-P under steady-state conditions like those employed by Percival et al. (1990). Hence, the present studies were initiated to determine how tenaciously the analogue binds to the enzyme in an equilibrium mixture, as well as the relationship between metal ion and inhibitor binding. The results are considered in terms of the possible structure of the presumed transition-state-analogue complex and what one might infer about the normal (PO_3^-)-transfer step on the basis of this structure.

Experimental Procedures

Materials. Most materials are described in the accompanying papers (Ray et al., 1990; Ray & Post, 1990). Bovine serum albumin was from Pentex. Glucose-6-phosphate dehydrogenase (Boehringer) was treated in the manner previously described (Magneson et al., 1987); Knox gelatin was used.

Procedures. Spectra were accumulated in the manner described in the second paper of this series (Ray et al., 1990). Data were manipulated with the PECSS program of the Perkin-Elmer Lambda 6 spectrophotometer.

Stock solutions of 10 mg/mL of demetalated dephospho enzyme (stable for several days at 0 °C) were appropriately diluted in 40 mM imidazole, pH 7.0–8.0, that contained 0.15 mg/mL serum albumin (excess of V_i over enzyme) or 1 mg/mL gelatin (excess of enzyme over V_i) plus additives. Aliquots of 0.01 mL of the treated enzyme were added to 1.0 mL of assay solution at 25 °C that contained 40 mM imidazole/imidazolium chloride, 2 mM $MgCl_2$, 1 mM EDTA, pH 7–8, 5 mM Glc-1-P, and 10–100 μM Glc-P_2, plus 0.25 mM NAD, 0.01 mg/mL glucose-6-phosphate dehydrogenase, and 0.15 mg/mL serum albumin. In some cases, a second assay was initiated immediately by adding 0.01 mL of the initial assay mixture to 1.0 mL of a second solution that contained all assay components except for the enzyme. The optical density change at 340 nm (that accompanied the oxidation of Glc-6-P formed in the assay by the NAD and dehydrogenase that also were present) was monitored. The amount of phosphoglucomutase in the assay was limited by prior dilution to that which produced an optical density change no greater than 0.1 in 10 min.

The equilibrium binding of Li^+ to the dephospho enzyme complex was assessed by means of its competitive binding with Mg^{2+} at 25 °C in a mixture that contained 20 mM Tris/Tris·HCl, pH 7.5, 0.15 mg/mL serum albumin, 0.01 mg/mL (metal-free) phosphoglucomutase, dephospho form, and 0–80 mM LiCl, plus 0.1 mM $MgCl_2$/0.05 mM EDTA or 2 mM $MgCl_2$/1 mM EDTA. Aliquots of 0.02 mL were removed from such mixtures and added to 2.0 mL of a stirred assay solution that contained 5 mM Glc-1-P, 50 μM Glc-P_2, and excess EDTA, plus NADP and Glc-6-P dehydrogenase [cf. Magneson et al. (1987)]. The overall optical density change at 340 nm was recorded.[4]

The equilibrium binding of Li^+ to the E·Glc-1-P complex was assessed in a similar manner, except that 10 mM Glc-1-P was present in the equilibrium mixture, and the enzyme concentration was increased (0.01–0.083 mg/mL) as the concentration of LiCl was increased (from 0 to 20 mM). The $MgCl_2$/EDTA concentrations were the same, but Mg^{2+} was added last and the metal ion equilibration time was limited to between 30 s and 2 min (to minimize the conversion of Glc-1-P to Glc-6-P by traces of the phospho enzyme that also were present).

The binding of Glc-1-P, 0–10 mM, to the dephospho enzyme, 32 μM, was assessed at 25 °C in 20 mM Tris/Tris·HCl buffer at pH 7.5, in the presence of 1 mM EDTA. Difference spectra were recorded before and after mixing of the contents of a sectored cell that contained the enzyme and Glc-1-P in separate compartments. The one-cell procedure described in the second paper of this series was used (Ray et al., 1990).

Results

In all inhibitor-binding studies reported herein, the concentration of metal ion, Mg^{2+} or Li^+, was saturating.[5] Since

[3] "(PO_3^-) transfer" is used herein to designate the process $R_1OPO_3^{2-} + R_2OH \rightarrow R_1OH + R_2OPO_3^{2-}$ and refers to the identity of the group transferred, without inference regarding the mechanism of transfer.

[4] The total Glc-6-P produced prior to dissociation of bound Mg^{2+} (in the presence of excess EDTA) is proportional to the fraction of enzyme present as E·Mg·Glc-1-P when the assay is initiated [cf. Ray and Roscelli (1966) and Magneson et al. (1987)].

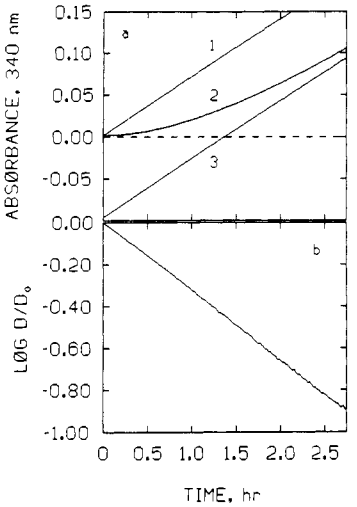


FIGURE 1: Recovery of activity during an enzymic assay initiated with the transition-state-analogue complex. (a) The analogue complex obtained by treating the dephospho enzyme with 5 mM Glc-1-P and 5 μM V_i in the presence of 9 mM $MgCl_2$/1 mM EDTA was diluted 100-fold at time zero into a standard assay solution at pH 7.4 that contained 0.1 mM Glc-P_2 and 5 mM Glc-1-P. (For other details see Experimental Procedures.) Immediately, 0.01 mL of the assay solution was added to 1.0 mL of a second, identical assay solution, and the change in optical density produced by the NAD/Glc-6-P dehydrogenase couple was monitored at 340 nm (plot 2). The optical density change obtained when an identical sample of dephospho enzyme was treated in the same way, but without prior exposure to V_i, also is shown (plot 1). Plot 3, which was used to represent the asymptote of plot 1, was obtained by digital subtraction of 0.098 OD unit from plot 1 (see rationale under Results). (b) Semilog plot (base 10) of the fraction of the maximal distance between plots 2 and 3 in (a) (at $t = 0$) as a function of time. The difference between the two plots was obtained by digital subtraction. The rate constant obtained from the slope of the line is 2.1×10^{-4} s^{-1}.

in most cases the physiological activator, Mg^{2+}, was used [cf. Peck and Ray (1971)], the identity of the bound metal ion usually is omitted when various complexes of the enzyme are represented, except where a distinction between the Mg^{2+} and Li^+ complexes is made. In addition, the complexes of the dephospho enzyme with Glc-1-P plus V_i and with Glc-6-P plus V_i are represented as E*V*6-Glc-1-P and E*V*1-Glc-6-P, respectively, both to emphasize the structural relationships between the inhibitor complexes and those which the normal substrates form with the phospho enzyme, E-P·Glc-1-P and E-P·Glc-6-P, respectively, and to serve as a reminder of uncertainties about the bonding within the oxyvanadium constellation in these complexes [cf. Ray and Post (1990), second paper in this series].

Rate Constant for Dissociation of the Mixed Diester from the Transition-State-Analogue Complex. Figure 1a (plot 2) shows the time course for recovery of activity in a standard assay mixture (saturating Glc-1-P, Glc-P_2, and Mg^{2+}—see Experimental Procedures) initiated with the dephospho form of phosphoglucomutase that previously had been treated with excess Glc-1-P plus V_i in the presence of Mg^{2+} to produce the inhibited enzyme, which was extensively diluted by initiating the assay. Since the initial slope of this plot is zero, the inhibition produced by the preassay treatment is essentially complete. The observed slope after a time interval of 2.75 h (extreme right) is nearly parallel to the slope obtained with a sample of dephspho enzyme that was treated identically, but in the absence of V_i (plot 1 of Figure 1a), and still is increasing with time. Hence, the inhibition by vanadate is completely reversible for practical purposes, and a line parallel to that obtained with the untreated enzyme (plot 1), but displaced downward by an arbitrary distance (plot 3), is used to represent the asymptote of plot 2 (rather than attempting to measure that asymptote experimentally). A logarithmic plot of the percent of the maximal vertical displacement between plots 2 and 3 (at $t = 0$), obtained by digital subtraction, is shown in Figure 1b as a function of assay time. (The arbitrary displacement of plot 1 used to represent the asymptote of plot 2 was selected to maximize linearity in this logarithmic plot.) The plot in Figure 1b is close to linear throughout, as would be expected [cf. Hatfield et al. (1970)] for a first-order recovery of activity: $k_d = 2.1 \times 10^{-4}$ s^{-1} at 25 °C ($\tau_{1/2}$ for dissociation of E-V-6-Glc-1-P: 55 min). Similar results were obtained at pH 7 (not shown), but k_d was larger by a factor of about 7-fold. When the dephospho enzyme was inhibited by treatment with V_i plus Glc-6-P instead of Glc-1-P, recovery of activity under the same conditions as described above for E*V*6-Glc-1-P was nearly an order of magnitude faster (plot not shown).

*Does the Inhibitor Complex, E*V*6-Glc-1-P, Dissociate in a Stepwise Process with Glucose 1-Phosphate Leaving First?* If E*V*6-Glc-1-P(Mg^{2+}) dissociates to E(Mg^{2+}) via E-V-(Mg^{2+}) + Glc-1-P, where E-V is analogous to E-P, it might be possible to slow conversion of the inhibitor complex to E(Mg^{2+}) by increasing the concentration of Glc-1-P so that E-V is "trapped" via the process E-V(Mg^{2+}) + Glc-1-P → E*V*6-Glc-1-P(Mg^{2+}). (The Mg^{2+} bound to the enzyme is omitted in the remainder of this section.) Hence, the differential recovery of activity at 10 and 1 mM Glc-1-P was measured simultaneously in sample and reference cells, respectively, in the manner described above [cf. Burgner et al. (1987)]. However, the results (not shown) require that the rate constant for recovery of activity be almost identical at 1 and 10 mM Glc-1-P. Thus, after a time interval equal to $1/k_d^{app}$ for E*V*6-Glc-1-P (about 80 min), when product accumulation in the sample cell produced $OD_{340nm} = 0.020$, OD_{340nm} in the reference cell differed from that in the sample cell by less than 0.0002 OD. From the independently measured velocity of Glc-1-P → Glc-6-P that is obtained with the uninhibited enzyme under these conditions, 1.9×10^{-5} OD s^{-1}, and the above value of k_d^{app} at 1 mM Glc-1-P, the reduction in k_d^{app} produced by 10 mM Glc-1-P is calculated as less than 1%. However, considering possible pipetting errors and differential temperature effects ($\Delta T \leq 0.1$ °C), a rate difference as large as 2.5% cannot be ruled out.

Under the conditions of the above study, the rate constant for the binding of Glc-1-P to E-P(Mg^{2+}) is at least 8×10^7 M^{-1} s^{-1} (from $k_{cat}/K_m = 630/8$ μM^{-1} s^{-1}). Hence, at the higher assay concentration of Glc-1-P (10 mM) the effective unimolecular rate constant for the binding process should be 8×10^5 s^{-1}. If E*V*6-Glc-1-P dissociates to give Glc-1-P plus E-V, and if Glc-1-P binds to E-V at a rate comparable to that for binding to E-P, a lifetime for E-V of less than 10^{-5} s, i.e., a half-time for the hydrolysis of E-V of less than 7 μs, would be required in order that the net appearance of free E, which is active in the assay (excess Glc-P_2 present), be retarded by only 2.5% when the Glc-1-P concentration is increased from

[5] In the E/Glc-P_2 system, saturation of E with Mg^{2+} requires a sufficiently high Mg^{2+} concentration (K_d = 0.45 mM; Magneson et al., 1987) that the binding of Mg^{2+} to Glc-P_2 (to produce Mg·Glc-P_2, which does not bind to the enzyme) becomes a serious problem [cf. Ray et al. (1966)]. In the case of the E/Glc-1-P/V_i system, 10 mM Mg^{2+} essentially saturates E, but does not significantly alter the extent to which the inhibitor complex forms (W.J.R., unpublished results), viz., does not bind significantly to V-6-Glc-1-P at the pH of the experiment (partly because the vanadate is largely monoanionic at this pH; see Results).

1 to 10 mM. To the extent that such a rapid hydrolysis seems unlikely, the above stepwise dissociation with Glc-1-P leaving first, i.e., E-V-6-Glc-1-P $\rightleftharpoons$ (E_V) + Glc-1-P $\rightarrow$ E + V_i + Glc-1-P, is correspondingly unlikely. [The half-time for hydrolysis of normal vanadate esters is measured in terms of seconds (M. J. Gresser, personal communication).]

An alternative procedure also was used to address this point. This procedure takes advantage of the fact that EDTA forms a very tight complex with V_i (Przyborowski et al., 1965) and can be used as a scavanger to prevent V_i not *initially* bound to the enzyme from forming an inhibitor complex with Glc-1-P and thus to circumvent induction of inhibition *after the assay is initiated*. In fact, the binding of V_i by EDTA is so tight that no inhibition of enzyme activity is observed when the enzymic assay is conducted in the presence of 1 mM V_i and 10 mM Glc-1-P if 10 mM EDTA also is present, although under the same conditions, but in the absence of EDTA, essentially no enzymic activity is observed. [In an assay conducted in the presence of excess EDTA the enzyme is active only as long as the Mg^{2+} at its activation site remains bound; the philosophy of such multiturnover-end point assays is discussed in Magneson et al. (1987).] In addition, a lack of inhibition also is observed when E(Mg^{2+}) is treated with 10–100 mM V_i and assayed by diluting 100-fold into the same assay mixture. Thus, if significant amounts of E-V, analogous with E-P, are formed on treatment of E with high concentrations of V_i [cf. Ray and Post (1990)], E-V cannot be trapped with any degree of efficiency by subsequent binding of Glc-1-P.

*Assay for Free Dephospho Enzyme in the Presence of the Inhibitor Complex, E*V*6-Glc-1-P(Mg(II))*. Because the E*V*6-Glc-1-P(Mg^{2+}) complex dissociates so slowly to E-(Mg^{2+}) (Figure 1), a short-term enzymic assay can provide an estimate of the fraction of enzyme in a mixture of E(Mg^{2+}) plus E*V*6-Glc-1-P(Mg^{2+}) that can react rapidly with excess Glc-P_2 to produce a catalytically competent enzyme/glucose phosphate complex. (As in the previous section, the Mg^{2+} bound to the enzyme is omitted in the remainder of this section and in the following two sections.) In such an assay, one ideally would measure the initial velocity induced by samples of the partially inhibited and the uninhibited enzyme. But since inhibitor binding produces complete inhibition, and since conversion of the E*V*6-Glc-1-P complex to free E is both slow and first order (see Figure 1), the product (P), produced during a suitable time interval under conditions where *complete recovery* of activity *eventually would be obtained*, can be used as a measure of the enzyme initially present as free E.

$$E/E_T = (P_I - P_{I=\infty})/(P_{I=0} - P_{I=\infty}) \quad (1)$$

Here, the subscripts refer to the concentration of inhibitor to which the enzyme was exposed *prior* to initiation of the assay.

Equilibrium Binding of Glucose 1-Phosphate and Inorganic Vanadate to the Dephospho Enzyme. Equilibrating the dephospho enzyme with Glc-1-P plus V_i under conditions that produce a final concentration of active enzyme equal to 20–90% proved difficult because of the slow rate at which equilibrium is obtained at the low concentrations of V_i and Glc-1-P required to only partially inhibit the enzyme. Thus, the rate of approach to equilibrium is a function of two parameters: k_d for the complex and the fractional conversion to E*V*6-Glc-1-P at equilibrium. But k_d for E*V*6-Glc-1-P is so small (about 2×10^{-4} s^{-1}—see above) that during the several-hour equilibration period required to produce $[E^*V^*Glc\text{-}1\text{-}P]_{ep} \approx [E]_{eq}$ the free enzyme converts a significant fraction of the Glc-1-P initially present to Glc-6-P,[6] which then forms V-1-Glc-6-P. But V-1-Glc-6-P binds to the enzyme less tenaciously than V-6-Glc-1-P (W.J.R., unpublished results[7]), and the mixed diester that is bound dissociates more rapidly (see above). Thus, the inhibitor equilibration time that can be used if generation of V-1-Glc-6-P is to be avoided depends on the ratio of free enzyme to Glc-1-P initially present. In fact, a time interval long enough to achieve equilibrium relative to the binding of V-6-Glc-1-P, but without a significant conversion of Glc-1-P to Glc-6-P, could be obtained only at V_i concentration high enough to produce at least 80% inhibition when $[V_i]_{total} \gg [enzyme]_{total}$. (The higher the concentration of V_i used, the lower the concentration of Glc-1-P that produces a given inhibition—see below.) Because of the above effects, when the dephospho enzyme is treated with Glc-1-P and V_i, the fraction of enzyme that is present as free dephospho enzyme first decreases and later increases (as Glc-1-P is converted to Glc-6-P and E*V*1-Glc-6-P accumulates). Hence, the binding of V-6-Glc-1-P was assessed by determining the concentration of Glc-1-P and V_i required to convert approximately 90% of the dephospho enzyme present into the inactive E*V*6-Glc-1-P complex at a time when inhibition was maximal. The time course of this inhibition study, monitored in terms of free E remaining in a mixture of E,

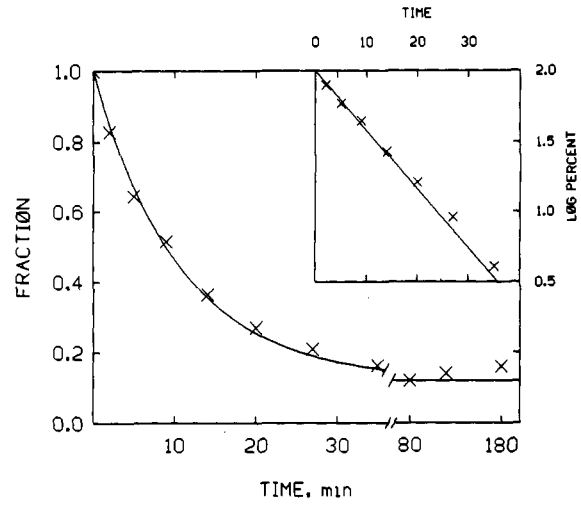


FIGURE 2: Time course for formation of the transition-state-analogue complex. The fraction of enzyme present that can rapidly form a catalytically active complex by reaction with Glc-P_2, viz., the enzyme not present as the transition-state-analogue complex, is shown as a function of time during the reaction of the dephospho enzyme with vanadate and Glc-1-P at concentrations of 0.25 and 150 μM, respectively, and at pH 7.5, under the conditions described in the Figure 1 except that 1 mg/mL of gelatin was used instead of serum albumin. Time aliquots were removed and immediately assayed for the remaining enzyme that had not been converted to the transition-state-analogue complex. A modification of the standard assay procedure described under Results was used. The fraction of enzyme that had not reacted is shown, together with the calculated time course of the reaction if the apparent first-order rate constant for formation of the transition-state-analogue complex is 1.6×10^{-3} s^{-1} under these conditions and the fraction present at equilibrium is 12% (—). Also shown (inset) is the appropriate logarithmic plot (base 10) obtained digitally.

[6] Although the dephospho enzyme is catalytically inactive in the absence of added Glc-P_2, all preparations of dephospho enzyme contain phospho enzyme at levels of about 1%.

[7] Glc-P_2 binds preferentially to the dephospho enzyme (Li^+ form) to give mainly the E-P-6-Glc-1-P complex as opposed to E-P-1-Glc-6-P (W.J.R., unpublished results); hence, the more tenacious binding of V-6-Glc-1-P relative to V-1-Glc-6-P is expected since both mixed diesters bind with their phosphate group at the distal site [see the second paper in this series (Ray et al., 1990)].

Glc-1-P, and V_i, is shown in Figure 2a.

If the maximal inhibition observed in the above study (88%) is the equilibrium inhibition, the rate of approach to the inhibited state is given by

$$d[E_t]/dt = -k_a[V_i][Glc\text{-}1\text{-}P][E_t] + k_d[E^*V^*6\text{-}Glc\text{-}1\text{-}P] \quad (2)$$

$$d[E_t]/dt = -k_d(K_e + 1)[E_t] + k_d[E_0] \quad (3)$$

where $K_e = [E^*V^*6\text{-Glc-1-P}]_e/[E]_e$ and the subscripts, o, t, and e, refer to reaction times of zero, t, and ∞. The value of k_d obtained from the slope of a logarithmic plot of $([E_t] - [E_e])/([E_0] - [E_e])$ versus t, (Figure 2, inset) divided by the function $(1 + K_e)$ is 1.9×10^{-4} s^{-1}, in good agreement with the value of k_d obtained by direct measurement of the rate at which E*V*6-Glc-1-P dissociates (Figure 1), 2.1×10^{-4} s^{-1}. Similar values for the inhibition observed after a 1-h treatment also were obtained when the concentration product, $[Glc\text{-}1\text{-}P][V_i]$, was maintained constant as the concentration of Glc-1-P was doubled and halved, as is expected from the results of Percival et al. (1990). In view of these results, it seems reasonable to use the above data to calculate the value of $[Glc\text{-}1\text{-}P][V_i]$ that would be required to produce 50% inhibition if the inhibition equilibrium could be established before significant conversion of Glc-1-P to Glc-6-P occurred: 5×10^{-12} M^2 (at pH 7.5). Since the concentration of Cl^- in the equilibrium mixture was approximately equal to K_d for the complex of Cl^- with E(Mg^{2+}) (Ray & Roscelli, 1966a), a concentration product equal to about 2.5×10^{-12} M^2 would be obtained in the absence of competitive binding by Cl^-.

Since the inhibitor appears to be the tetraanion of V-6-Glc-1-P (see the following section), the concentration of the tetraanion in equilibrium with the Glc-1-P and V_i present was approximated. This approximation utilized an average value of the equilibrium constant for the reaction of $HOVO_3^{2-}$ with methanol and ethanol, about 0.06 M^{-1}. The difference in pK_a for the 6-hydroxyl group of Glc-1-P and these alcohols, as well as the difference in pK_2 for $HOVO_3^{2-}$ and Glc-6-OVO_3^{2-}, was ignored since the effects of these differences are rather small and would tend to cancel [cf. Tracey et al. (1988)]. Finally, a value of 8.6 for the pK_a of $(HO)_2VO_2^-$ (see section below) was used to calculate the fraction of vanadate present as the dianion at pH 7.5 and the ionic strength where the above studies were conducted. On this basis, K_d for E*V*6-Glc-1-P $\rightleftharpoons$ E + V-6-Glc-1-P^{4-} is about 1.5×10^{-14} M.[8]

*Is the Inhibitor Complex, E*V*6-Glc-1-P, Produced by Direct Binding of V-6-Glc-1-P?* To inquire whether formation of the E*V*6-Glc-1-P complex by direct binding of V-6-Glc-1-P is possible, or whether a stepwise process is necessary, the bimolecular rate constant for the direct, one-step binding process is calculated on the assumption that preformed V-6-Glc-1-P binds to the enzyme. The calculated value then is compared with the diffusion-controlled limit. In this calculation we assume that only the tetraanion of V-6-Glc-1-P binds, since bound P-6-Glc-1-P appears to be tetraanionic (Rhyu et al., 1985) and since the spectrum of the bound vanadate is more easily rationalized in terms of a distorted dianion than a distorted monoanion (Ray & Post, 1989, third paper in this series).

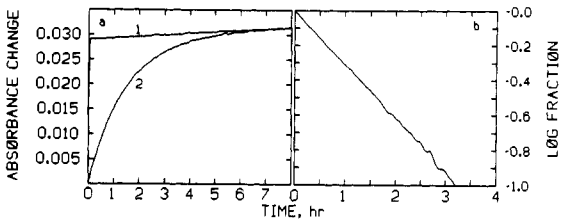


FIGURE 3: Product obtained in multiturnover-end point assays initiated with the Mg^{2+} form of the enzyme–substrate complex or with the transition-state-analogue complex. Prior to the assay, samples of dephospho enzyme were treated as described below. Assays were initiated by adding 0.02-mL aliquots of treated enzyme to 2.0 mL of a stirred solution similar to the standard assay mixture, except that it contained 2 mM EDTA and no Mg^{2+} (see Experimental Procedures), and product accumulation was monitored at 340 nm by means of the NAD/Glc-6-P dehydrogenase couple. (a) (Plot 1) Dephospho enzyme treated prior to the assay with 10 mM Mg^{2+}/1 mM EDTA plus 10 μM Glc-P_2 (in 20 mM imidazole, pH 7.5, that contained 0.2 mg/mL serum albumin). The assay was initiated with a 4-μL aliquot of the treated enzyme. The increased absorbance as a function of time is shown. (Plot 2) Same as plot 1, except that the solution used for treatment prior to the assay also contained 0.5 mM Glc-1-P and 175 μM vanadate, but no Glc-P_2, so that the enzyme was converted entirely to the transition-state-analogue complex. (b) Logarithm (base 10) of the difference between plots 1 and 2 in (a), expressed as a fraction of the difference observed at the 5-min point (differences obtained by digital subtraction). The rate constant calculated from the slope of the line is 2.0×10^{-4} s^{-1}.

From the ratio of k_d for the E-V-6-Glc-1-P complex, 2.1×10^{-4} s^{-1} (see above), and the above value of K_d, an apparent rate constant for the binding of V-6-Glc-1-P^{4-} to the enzyme of greater than 10^{10} M^{-1} s^{-1} is required, which seems too large to represent a complex bimolecular binding process [cf. Eigen and Hammes (1962)], even though the size of the rate constant for diffusion-controlled binding of an ion to a macromolecule depends on the product of the electrostatic charge, or effective electrostatic charge, on the two species (above reference). Thus, the rate constant for binding of Glc-1,6-P_2^{4-} to the dephospho enzyme (Mg^{2+} form), calculated in the same way (i.e., as k_d/K_d), is smaller than the above value by more than an order of magnitude, as is the k_{cat}/K_m value for Glc-1-P (Ray et al., 1989). Of course, if complex formation occurs by binding of the trianion of V-6-Glc-1-P, followed by dissociation of a proton, the required association rate would be smaller by a factor of about 0.1.

On the other hand, Percival et al. (1990) also conclude that formation of the vanadate-based transition-state-analogue complex is stepwise, on the basis of an analysis of pre-steady-state inhibition by V_i (in the presence of Glc-1-P). In fact, binding of the trianion of V-6-Glc-1-P might account for the "loose" complex of V-6-Glc-1-P with E(Mg^{2+}) that they observed.

$$E + V\text{-}6\text{-}Glc\text{-}1\text{-}P \underset{k_d}{\overset{k_a}{\rightleftharpoons}} E{\cdot}V\text{-}6\text{-}Glc\text{-}1\text{-}P \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} E^*V^*6\text{-}Glc\text{-}1\text{-}P$$

Because the first step in their model comes to equilibrium rapidly, our studies would measure $k_a^{app} = k_a k_i/k_d$ and $k_d^{app} = k_{-i}$.

Dissociation of Mg(II) from the Transition-State-Analogue Complexes. Rate Studies. Figure 3a shows product/time plots obtained when an enzymic assay is initiated with either the catalytically competent E·P-6-Glc-1-P(Mg^{2+}) complex[9]

[8] The complex E*V*6-Glc-1-P can dissociate via a pathway other than that by which it forms, viz., via isomerization to give E*P-1-Glc-6-V followed by (PO_3^-) transfer and a dissociation step to produce E-P + Glc + V_i. In fact, an equilibrium mixture of E*V*6-Glc-1-P + E*V*1-Glc-6-P does react in this manner: $k^{app} \approx 1.2 \times 10^{-4}$ s^{-1} (W.J.R., unpublished results). But the production of E-P likely involves E*V*1-Glc-6-P to a substantially greater extent than E*V*6-Glc-1-P, so that the true equilibrium between E*V*6-Glc-1-P and E + V-6-Glc-1-P is not substantially smaller than that reported.

(plot 1) or an equal quantity of the inactive E*V*6-Glc-1-P-(Mg^{2+}) complex (plot 2) in the presence of excess EDTA and excess Glc-1-P plus Glc-P_2 (as opposed to Figure 1, where saturating Mg^{2+} was maintained). Previous studies with E-P(Mg^{2+}) show that under these conditions the first-order constant that defines the rate at which enzymic activity approaches zero is the rate constant for dissociation of Mg^{2+} from the enzyme—or, to be more precise, from the central complexes (Ray & Roscelli, 1966b). Thus, the total product produced prior to the complete dissociation of Mg^{2+} in a reaction initiated with E-P(Mg^{2+}) (saturating Glc-1-P), or with E·P-6-Glc-1-P(Mg^{2+}),[9] is given by

$$[P_\infty] = k_{cat}[E_0]/k_d \quad (4)$$

where k_d is the rate constant in question and the product, $k_{cat}[E_0]$, is V_{max}. Surprisingly, plot 2 shows that the inactive complex, E*V*6-Glc-1-P(Mg^{2+}), produces essentially the same value of $[P_\infty]$ as that obtained with the catalytically active complex, E·P-6-Glc-1-P(Mg^{2+}) (plot 1). [The slow increase in product with time after the 5-min point in assays initiated with E·P-6-Glc-1-P(Mg^{2+}) is due to the finite amount of Mg^{2+} present in the assay mixture; essentially the same increase is observed after several hours in assays initiated with E*V*6-Glc-1-P(Mg^{2+}) (see Figure 3a).] Observing the same value of $[P_\infty]$ can only mean that Mg^{2+} dissociates from E*V*6-Glc-1-P(Mg^{2+}) much more slowly than does V-6-Glc-1-P so that the reaction sequence in eq 5 represents the nearly ex-

$$\text{E*V*6-Glc-1-P}(Mg^{2+}) \rightarrow \text{E}(Mg^{2+}) + \text{V-6-Glc-1-P} \quad (\text{or } V_i + \text{Glc-1-P}) \quad (5)$$

clusive dissociation pathway. (The alternative pathway that produces E·V-6-Glc-1-P plus Mg^{2+} produces "irreversibly" inactivated enzyme.) Thus, the E(Mg^{2+}) produced via the above process rapidly reacts with Glc-P_2 to produce the catalytically active central complexes, which remain catalytically active until Mg^{2+} dissociates. Because E(Mg^{2+}), the precursor to active enzyme, is formed very slowly from E*V*6-Glc-1-P(Mg^{2+}), the steady-state concentration of catalytically active complexes is quite low throughout the reaction under the conditions used to obtain plot 2 (Figure 3a). Nevertheless, for the process in eq 5, $\int_0^\infty [CC]\,dt$ must equal $[E^*V^*6\text{-Glc-1-P}]_0$ for the values of $[P_\infty]$ to be the same. In addition, the rate constant obtained by plotting the logarithm of the difference between plots 1 and 2 after 5 min, as in Figure 3b, 2.0×10^{-4} s^{-1}, is essentially the same as the rate constant observed for the dissociation of V-6-Glc-1-P from E*V*6-Glc-1-P(Mg^{2+}) in the presence of excess Mg^{2+} (see the first section) as is required by the pathway in eq 5. [The difference between the two plots in used to correct for the slow background reaction in both systems (see above).] In view of this agreement, it is probable that no more than 5% of the E*V*6-Glc-1-P(Mg^{2+}) present initially dissociates via the alternative pathway in which Mg^{2+} leaves first. Thus, the true value of $k_{d(Mg)}$ cannot be larger than about 10^{-5} s^{-1} and may be much smaller than this. By contrast, the rate constant for dissociation of Mg^{2+} from the steady-state mixture of central enzyme–substrate/product complexes is about 3×10^{-2} s^{-1} (Ray & Roscelli, 1966), or larger by a factor of at least 3000-fold.

Binding of Mg(II) to Inorganic Vanadate. Figure 4a shows a spectral titration of 20 μM V_i in the presence of glycylglycine as a supporting buffer[10] at an ionic strength of 0.02, together with a theoretical plot calculated on the basis that the limiting absorbances (20 μM V_i) are 0.0315 and 0.0725 and that the pK_a for $(HO)_2VO_2^-$ is 8.65. [The low concentration of V_i was

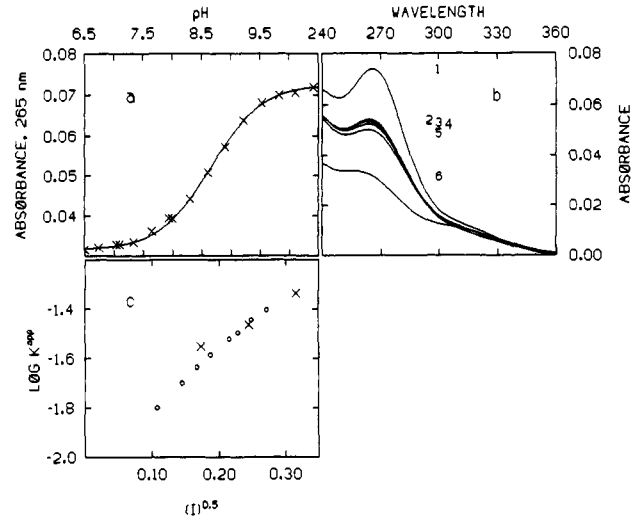


FIGURE 4: Binding of Mg(II) by dianionic vanadate. (a) pH titration of vanadate monitored spectrally (at 266 μm). A solution of 20 mM glycylglycine/20 mM KCl was adjusted to the required pH with 20 mM KOH. Aliquots of 1 mL were placed in the sample and reference cells of a double-beam spectrophotometer and base-line spectra accumulated from 440 to 240 nm at 100 nm/min; slit = 4 mm. Subsequently, 4 μL of 5 mM vanadate or water was mixed into the sample and reference cells, respectively, and a second spectrum obtained. The two spectra were superimposed within ±0.0001 absorbance unit in the 400–440-nm region of the spectrum before they were subtracted digitally. The absorbance obtained at 266 nm, after smoothing, is shown as a function of pH (×). The solid line is the theoretical plot for a process that increases the absorbance of the solution from 0.0315 to 0.0725 as the result of a single ionization with $pK_a = 8.65$. (b) Spectral change produced by the binding of Mg^{2+} to the dianion of vanadate. Spectra were accumulated as in (a) and are numbered according to the absorbance at 266 nm. Solutions containing 20 mM glycylglycine, 20, 15, 10, or 0 mM $MgCl_2$ at $I = 0.1$ (maintained with KCl) and pH 8.60 were used to obtain spectra 2, 3, 4, and 5, respectively. Spectra 1 and 6 were obtained at the same ionic strength as above but at pH 10.6 (20 mM CHES buffer) and pH 6.6 (20 mM imidazole buffer), respectively. Values of K_d for $MgHOVO_3 \rightleftharpoons Mg^{2+} + HOVO_3^{2-}$ were calculated as 46, 44, and 43 mM from these spectra (see Results). (c) Effect of ionic strength on K_d for the dissociation constant in (b). Spectra obtained as in (b), but at $I = 0.06$ and 0.03, were employed to calculate values of K_d at these ionic strengths. The logarithm of the average calculated value is shown as function of $I^{1/2}$ (×). Also shown are constants from Clarke et al. (1954) for K_d of $MgHOPO_3 \rightleftharpoons Mg^{2+} + HOPO_3^{2-}$, which, for display, have been increased by 0.447 logarithmic unit. [Clarke et al. report their results in terms of ionic activities; these were converted to molarities in the standard way (cf. the original paper) for comparison with the results obtained here.]

[9] The E·P-6-Glc-1-P(Mg^{2+}) added to the assay also contained equilibrium amounts of E-P·Glc-1-P(Mg^{2+}) and E-P·Glc-6-P(Mg^{2+}) [cf. Ray and Long (1976b)].

[10] After completion of this study, a question arose as to whether the glycylglycine buffer used in studies described in this section (at a concentration of 20 mM) might have perturbed the results by forming a complex with V_i (Crans et al., 1989). Hence, the results were checked with two buffers that appear to have a reduced capacity toward forming such complexes. When the titration described below was repeated with 20 mM *N*-ethylmorpholine as the supporting buffer, no difference in pK_a (±0.03 unit) was observed. The midpoint of the titration curve also was checked with 20 mM Tris buffer. Again no significant difference was observed. The binding of V_i at 27 mM Mg^{2+} ($\mu = 0.1$) also was compared at the approximate midpoint of all three titration curves. No significant differences in binding were observed between Tris and glycylglycine buffers (20 mM). However, the binding of Mg^{2+} to $HOVO_3^{2-}$ appeared to be stronger by about 30% in *N*-ethylmorpholine. While a difference of this size is of no consequence with regard to conclusions drawn here, it does raise the question of whether our values of pK_{a2}^{app} for $(HO)_2VO_2^-$ and k_d^{app} for $Mg{\cdot}HOVO_3^-$ are entirely independent of the buffer.

employed to obviate the formation of polymeric vanadates [cf. Kepart (1973) and Chasteen (1983)].] Since formation of the Ca^{2+} complex of $HOVO_3^{2-}$ at a 4 M concentration of $CaCl_2$ does not substantially alter the absorbance of the dianion (Ray & Post, 1989) and since Mg^{2+} in the concentration range 3–20 mM is not expected to bind significantly to $(HO)_2VO_2^-$ [by analogy with the phosphate system [cf. Clark et al. (1954)]], the binding of Mg^{2+} to $HOVO_3^{2-}$ was assessed spectrally as a function of ionic strength at a pH close to where $[HOVO_3^{2-}] = [(HO)_2VO_2^-]$ or [Di] ≈ [Mono]. Figure 4b shows spectra obtained at $\mu = 0.1$ (maintained by added KCl) in the presence of 20, 15, 10, and 0 mM $MgCl_2$ (spectra 2, 3, 4, and 5, respectively). The fractional change in the dianion present that is produced by added Mg^{2+} can be represented as $[(Di + MgDi)_{Mg} - Di_0]/[Di_0]$, where the subscripts 0 and Mg refer to the absence and presence of Mg^{2+}, respectively. This fractional change can be calculated as

$$\text{fractional change} = (A_{Mg} - A_0)/(A_0 - A_{lo}) \quad (6)$$

where A refers to absorbance and the subscripts hi and lo refer to the extremes of the pH titration curve, viz. to spectra 1 and 6 of Figure 4b. Similarly, K_d^{app} for $MgHOVO_3 \rightleftharpoons Mg^{2+} + HOVO_3^{2-}$, expressed in terms of molarities, can be calculated by use of

$$K_d^{app} = [Mg^{2+}][(A_{hi} - A_{Mg})(A_0 - A_{lo})/(A_{hi} - A_{lo})(A_{Mg} - A_0)] \quad (7)$$

Figure 4c shows a plot of log K_d^{app} versus $I^{1/2}$ at three different ionic strengths (×). At ionic strengths of 0.1 and 0.06, the highest and lowest concentrations of Mg^{2+} used differed by 2-fold and the standard error of the calculated value for K_d^{app}, obtained from single measurements at three Mg^{2+} concentrations, was 2–3% (even though the measured differences were quite small and a standard error of about 5% might have been expected). At the lowest ionic strength the four Mg^{2+} concentrations used differed in concentration at most by only 1.4-fold and the standard error of the average value for K_d^{app} was 13%.

Also shown in Figure 4c are data from the study of Clark et al. (1954) on the binding of Mg^{2+} to $HOPO_3^{2-}$, recalculated for presentation with the above data. The data of Clark et al. are displaced upward by 0.477 log unit (3-fold increase in K_d^{app}) to show the similarity in the effect of ionic strength on the activity coefficients of $HOPO_3^{2-}$ and $HOVO_3^{2-}$. The apparent difference in slopes for the two data sets is not significant in view of the uncertainties in our constants—especially at the lowest ionic strength. However, our data are sufficiently accurate to show that $HOVO_3^{2-}$ binds Mg^{2+} almost as well as $HOPO_3^{2-}$ and that the extent of binding is highly dependent on ionic strength.

Binding of V-6-Glc-1-P to the Li(I) Form of the Dephospho Enzyme. Since formation of the E·V-6-Glc-1-P(Li^+) complex is less favorable, thermodynamically, than formation of the corresponding Mg^{2+} complex [see the second paper in this series (Ray et al., 1990)], for simplicity, an assessment of the stability of this complex was conducted at sufficiently high concentrations of Glc-1-P and Li^+ that the only enzymic species present in solution to a significant extent were E·Glc-1-P(Li^+) and E·V-6-Glc-1-P(Li^+) (see following section). [Because the complex of V-6-Glc-1-P with E(Li^+) does not exhibit the unusual spectral properties of the E*V*6-Glc-1-P(Mg^+) complex, it is formulated, as above, like an ordinary enzyme–inhibitor complex.] Figure 5a shows the spectral changes in the ultraviolet that occur when E·Glc-1-P(Li^+) is

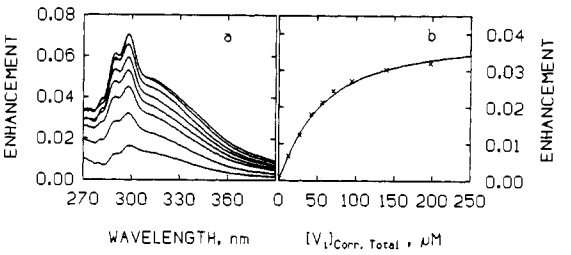


FIGURE 5: Binding of inorganic vanadate by the dephospho enzyme/glucose 1-phosphate/Li^+ complex as assessed by spectral enhancement measurements. Spectra were accumulated as in Figure 4a, except that a spectral range from 540 to 240 nm and a scan rate of 60 nm/min were used; a 1-cell procedure also was employed [cf. the second paper of this series (Ray et al., 1990)]. Aliquots, 0.8 mL, of a solution containing 30 mM LiCl, 20 mM imidazole/imidazolium chloride, pH 7.5, 10 mM Glc-1-P, and either 2 mg/mL of the dephospho enzyme (0.032 mM) or no enzyme were used. An aliquot, 4 μL, of 6–80 mM inorganic vanadate was mixed into the solution and a second spectrum accumulated. The spectral increase produced by the added vanadate in the absence of the enzyme was subtracted from the analogous increase in the presence of the enzyme. The smoothed difference, which is related to $\epsilon(V_i)_{bound} - \epsilon(V_i)_{free}$, is the spectral enhancement. The enhancement, as a function of wavelength, is shown in (a) at total concentrations of added vanadate equal to 15, 30, 45, 60, 75, 100, 150, and 250 μM. A plot of the enhancement at 340 nm (maximal enhancement taken as 0.04) versus the ratio of total vanadate to total enzyme present is shown in (b) (×) along with the fractional enhancement calculated by assuming that K_d for the E*V*6-Glc-1-P(Li) complex is 36 μM (—) (for details, see Results).

treated with V_i in the presence of saturating Glc-1-P [cf. Figure 3c of Ray et al. (1990)]. These changes are shown in terms of the *enhancement* in absorbance as a function of the vanadate added to the system, viz., the *increase* in absorbance on addition of vanadate to a solution containing 32 μM E·Glc-1-P(Li^+), minus the *increase* observed on addition of the same amount of vanadate to a solution that otherwise was identical, except that no enzyme was present. Figure 5a suggests that the system exhibits saturation-type behavior under these conditions, although at the higher V_i concentrations complexities arise, especially at the shorter wavelengths, presumably as a result of the $2V_i \rightleftharpoons V_2$ and $4V_i \rightleftharpoons V_4$ equilibria, which become significant above 100 μM free V_i [cf. Tracey and Gresser (1988)]. Because plots of OD_{340nm} versus $[V_i]_{total}$ in the absence of the enzyme were essentially linear up to 250 μm $[V_i]_{total}$, the enhancement of vanadate absorbance in the presence of the enzyme was investigated at this wavelength.

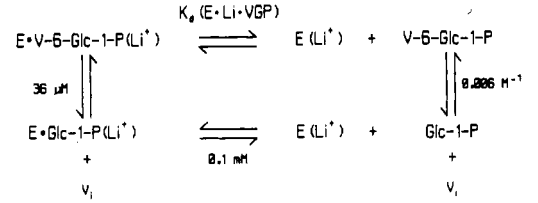
Figure 4b shows the enhancement produced by added V_i under the conditions described above. Also shown is the expected enhancement if the maximal enhancement is 0.04 (at 32 μM enzyme), and the apparent equilibrium constant for eq 8 is 36 μM (at pH 7.5). In addition to fitting the absor-

$$E\cdot V\text{-}6\text{-}Glc\text{-}1\text{-}P(Li^+) \rightleftharpoons E\cdot Glc\text{-}1\text{-}P(Li^+) + V_i \quad (8)$$

bance data to the required expression for bound vanadate,[11] the concentration of added vanadate was corrected iteratively for the presence of V_2 and V_4 by using the formation constants reported by Tracey et al. (1988) under similar conditions: 280 M^{-1} and 2.5×10^8 M^{-3}, respectively. (The sum of V_i present as V_2 plus V_4 was estimated as 50, 8, 3, 2, and 1 μM at 250, 150, 100, 75, and 60 μM *total* vanadate, respectively.) In view of the complexities of the system, the values of maximal enhancement and equilibrium constant for eq 8 obviously are

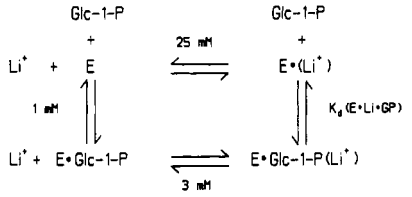
[11] The expression for bound V_i is the positive root of the equation, $x^2 - bx + c = 0$, where $b = [E]_{total} + [V_i]_{total} + K$, $c = [V_i]_{total}$, x is the concentration of the complex in question, and K is the equilibrium constant for eq 8.

Scheme I: Thermodynamic Box Showing How the Equilibrium Constant for the Dissociation of V-6-Glc-1-P from E·V-6-Glc-1-P(Li$^+$), K_d(E·Li·VGP), Was Evaluated[a]



[a] Equilibrium constants, except that for the formation of V-6-Glc-1-P, are formulated as dissociation constants and refer to the dianionic forms of V_i and Glc-1-P and the tetraanionic form of V-6-Glc-1-P that are present at pH 7.5 (see Results).

Scheme II: Thermodynamic Box Showing How the Equilibrium Constant for Dissociation of Glc-1-P from E·Glc-1-P(Li$^+$), K_d(E·Li·Glc-P), Was Evaluated[a]



[a] All equilibrium constants are formulated as dissociation constants.

not unique. However, substantially poorer fits were obtained by using values of this constant equal to 24 and 48 μM, irrespective of the value chosen for the maximal enhancement.

Under conditions of saturating Glc-1-P and Li$^+$, used above, the equilibrium dissociation constant for E·V-6-Glc-1-P(Li$^+$) $\rightleftharpoons$ E(Li$^+$) + (V-6-Glc-1-P)$^{4-}$ can be approximated from the thermodynamic box in Scheme I, which provides the values of the required constants. Thus, the dissociation constant for E·V-6-Glc-1-P(Li$^+$), about 2×10^{-11} M, is about 1400-fold larger than the value of 1.5×10^{-14} M for the corresponding process in the case of the Mg^{2+} enzyme.

Binding of Glucose 1-Phosphate and Li(I) to the Dephospho Enzyme. To make comparisons with the Mg^{2+} enzyme, the equilibrium constants for the separate and simultaneous binding of Glc-1-P and Li$^+$ to the dephospho enzyme (Scheme II) were assessed. The binding of Li$^+$ was measured in a competitive binding experiment with Mg^{2+}, at Mg^{2+} concentrations equal to 0.1 and 2 times that of K_d(E·Mg). The fraction of the enzyme present as its Mg^{2+} complex was assessed in an assay that contained excess Glc-P_2, to convert E(Mg^{2+}) to the catalytically active complex, and excess EDTA, to prevent any E or E(Li$^+$) initially present from being converted into the active Mg^{2+} complex. The general philosophy of this type of assay is discussed in Magneson et al. (1986), and conditions are given under Experimental Procedures. The values of K_d(E·Li) obtained from plots of reciprocal activity versus [Li$^+$] were 30 mM and 23 M at 0.05 and 1.0 mM Mg^{2+}, respectively [K_d(E·Mg) = 0.5 mM; Magneson et al., 1986]. Thus, Mg^{2+} binds to the dephospho enzyme some 50-fold more tenaciously than does Li$^+$.

The binding of Glc-1-P to the dephospho enzyme was assessed by measuring the spectral change at 290 nm produced by the binding process under conditions given under Experimental Procedures. [The overall spectral change is similar to that for binding of Glc-1-P to the phospho enzyme (Ma and Ray, 1980) but about half as intense.] Double-reciprocal plots of $\Delta\epsilon_{290nm}$ versus [Glc-1-P]$^{-1}$ provided a value of K_d(E·Glc-1-P) = 1.0 mM, which is in reasonable agreement with the value of about 0.6 mM reported previously on the basis of an indirect kinetic evaluation (Ray et al., 1966).

The binding of Li$^+$ to E·Glc-1-P was evaluated in the same way as the binding of Li$^+$ to the free dephospho enzyme, except that the Mg^{2+}/Li$^+$ equilibrium was established in the presence of a saturating concentration of Glc-1-P [10 mM or 10K_d(E·Glc-1-P)]. The results provide a value of the equilibrium constant for dissociation of Li$^+$ from the E·Glc-1-P(Li$^+$) complex equal to 3 mM and, in comparison with the results obtained in the absence of Glc-1-P [K_d(E·Li) ~ 25 mM], show that binding of Li$^+$ and Glc-1-P to the dephospho enzyme is markedly cooperative [as opposed to the binding of Mg^{2+} and Glc-1-P, which at best exhibits only a minor degree of cooperativity (less than 2-fold)].

Because of the weak binding of Li$^+$ to the dephospho enzyme and problems attendant with anion binding if a sufficiently high concentration of a Li$^+$ salt were used to saturate the dephospho enzyme with Li$^+$ (Ray & Roscelli, 1966), no attempt was made to directly measure binding of Glc-1-P to E(Li$^+$). Instead, values of the other three equilibrium constants in Scheme II were used to approximate K_d for dissociation of Glc-1-P from the E·Glc-1-P(Li$^+$) complex, about 0.1 mM.

Binding of Li(I) to the Complex of the Dephospho Enzyme and Glucose 1,6-Bisphosphate. Isotopic exchange studies conducted at chemical equilibrium in the presence of saturating concentrations of Mg^{2+}, 3 mM, and of glucose mono- and bisphosphates [cf. Ray and Long (1976)] showed that in the presence of 3 mM Li$^+$ the rate of the catalytic interconversion of Glc-1-P and Glc-6-P was approximately half that in its absence, i.e., approximately half of the enzyme was present as inactive Li$^+$ complexes of E_P·Glc-1-P, E·Glc-P_2, and E_P·Glc-6-P. Since unreported data from a study of the equilibrium between E-P·Glc-1-P(Li$^+$) and E·Glc-P_2(Li$^+$) (Ray et al., 1989) show that the fraction of enzyme present as the bisphosphate complex is not significantly altered by replacing bound Mg^{2+} by Li$^+$, the equilibrium constant for E·Glc-P_2(Mg^{2+}) + Li$^+$ $\rightleftharpoons$ E·Glc-P_2(Li$^+$) + Mg^{2+} must be close to the value observed for the Mg^{2+}/Li$^+$ replacement when the equilibrium mixture of complexes is involved (about 1). Thus, Li$^+$ and Mg^{2+} bind to E·GlcP_2 with approximately equal affinity. [An approximately equal affinity of Li$^+$ and Mg^{2+} for the mixture of enzyme substrate/intermediate/product complexes present in the steady-state reaction was reported previously (Ray et al., 1978).]

Discussion

There are a number of reasons for suspecting that V-6-Glc-1-P bound to the dephospho form of phosphoglucomutase might represent a transition-state analogue for the (PO_3^-)-transfer process catalyzed by this enzyme. These are summarized below. However, it seems reasonable to state our final conclusion in advance: the complex of the mixed diester mimics a state normally encountered during the catalytic (PO_3^-)-transfer process that is well above the ground state, energetically, but still is substantially below the transition state. On the other hand, bound V-6-Glc-1-P comes far closer to mimicking the transition state for the phosphoglucomutase reaction than the run-of-the-mill complexes loosely dubbed transition-state complexes in the current literature. Hence, for brevity we refer to the E*V*6-Glc-1-P(Mg^{2+}) complex as a transition-state-analogue complex. (As is indicated, below, V-6-Glc-1-P probably is not a transition-state analogue; however, its complex with the enzyme does appear to have properties that generally are taken as characteristic of a transition-state-analogue complex.) We also suggest a possible origin for the quantitative differences between the interactions of vanadate and phosphate groupings at the active site of the

enzyme and the significance of these differences with regard to the mechanism of (PO_3^-) transfer utilized by the enzyme.

The Vanadate Group of the Analogue Complex Is Bound Preferentially at the (PO_3^-)-Transfer Site of the Enzyme. The basis for considering the V-6-Glc-1-P complex as a transition-state-analogue complex for the (PO_3^-) transfer catalyzed by phosphoglucomutase is detailed in the second paper of this series (Ray et al., 1990). This study shows that part, if not all, of the nonreacting hydroxyl groups of the glucose moiety, whose disposition is critical to efficient catalysis (Lowry & Passonneau, 1969), interact with the enzyme in essentially the same way in E·P-6-Glc-1-P and E*V*6-Glc-1-P and that, given a choice, the dephospho enzyme exhibits a high preference for binding the vanadate rather than the phosphate group of V-6-Glc-1-P at its proximal or (PO_3^-)-transfer site—as opposed to its distal site [Scheme I of Ray et al. (1990)]. [Similar conclusions are drawn by Percival et al. (1990) from a detailed kinetic analysis.]

The Vanadate Group in the Analogue Complex Is Bound in an Unusual Environment. The value of k_{cat}/K_m for (PO_3^-) transfer from bound P-6-Glc-1-P to the active-site hydroxyl group of the enzyme is quite large relative to the rate of an uncatalyzed (PO_3^-)-transfer process, e.g., from a simple phosphate ester dianion to a hydroxyl group of water, which is taken to mean that transition-state binding interactions in the normal enzymic reaction are quite large. Hence, *if* E*V*6-Glc-1-P(Mg^{2+}) mimics the transition state for the enzymic reaction, one would expect that binding interactions would substantially alter the chemical properties of the vanadate group. In fact, the ultraviolet spectrum of V(V) indicates that vanadium is in an unusual environment in this complex (Ray & Post, 1990).

The Mixed Diester Binds with Unusual Affinity. The dissociation constant for E*V*6-Glc-1-P(Mg^{2+}), calculated in terms of V-6-Glc-1-P^{4-} (see Results), is among the smallest known for an enzyme/small-molecule complex—about 1.5×10^{-14} M. [The properties of the system that allow the more or less direct evaluation of such a small dissociation constant are discussed in Percival et al. (1990).] As might be expected for a complex with such a small dissociation constant, the binding of V-6-Glc-1-P apparently occurs in a stepwise manner, via formation of a tight complex followed by conversion into an even tighter one on a time scale of a few seconds (Percival et al., 1990). What changes accompany this conversion are not known, but the two-step mechanism is consistent with the observation herein that the association rate is too fast to easily rationalize in terms of a one-step process. However, it is possible that the trianion of V-6-Glc-1-P, which is the predominant species at the pH of these studies, binds to the enzyme, initially, and is only slowly converted to the tetraanion.

The Preference for Binding the Vanadate Group Relative to the Phosphate Group at the (PO_3^-)-Transfer Site Is Large. Although the binding constant for V-6-Glc-1-P is unusually small when calculated in terms of the tetraanion, admittedly the mixed diester is structurally similar to an intermediate in the normal enzymic reaction that itself binds quite tenaciously to the enzyme. Thus, K_d for dissociation of Glc-P_2 from E·P-6-Glc-1-P(Mg^{2+}) is about 1×10^{-8} M (Ray & Long, 1976b). On the other hand, it is unusual for a substitution of the type

$$\text{E·6-P-Glc-1-P} + HOVO_3^{2-} \rightarrow \text{E*V*6-Glc-1-P} + HOPO_3^{2-} \quad (9)$$

to produce such a large increase in binding. Thus, the equilibrium constant for the hypothetical reaction in eq 9, in which the PO_4^{2-} group at the 6-position of bound P-6-Glc-1-P is replaced by a VO_4^{2-} group, can be estimated as 6×10^6.[12] Only a small fraction of the value of $\Delta G°$ for eq 9 (about 9.4 kcal/mol) can be attributed to the greater tendency for alcohols to form esters with $HOVO_3^{2-}$ than with $HOPO_3^{2-}$, since the equilibrium constant for eq 10 is about 10 for alcohols with

$$\text{Glc-6-}P^{2-} + HOVO_3^{2-} \rightleftharpoons \text{Glc-6-}V^{2-} + HOPO_3^{2-} \quad (10)$$

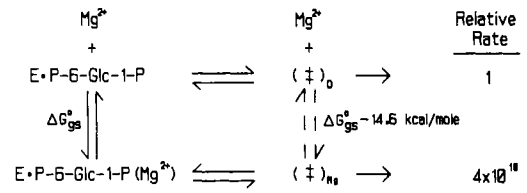
pK_a values in the range of about 15.5 (see the introduction). Hence, the intrinsic binding energy of the vanadate group of V-6-Glc-1-P with respect to the (PO_3^-)-transfer site of phosphoglucomutase is more negative than that of the corresponding phosphate group by at least 8 kcal/mol, and possibly more than 9. (In view of our suggesting about the structure of E*V*6-Glc-1-P, below, the use of the equilibrium in eq 9 to calculate relative affinities of PO_4^- and VO_4^{2-} in E·P-6-Glc-1-P and E*V*6-Glc-1-P may well constitute an overcorrection.)

How Close Is the Transition-State-Analogue Complex to the Actual Transition State for (PO_3^-) Transfer? To obtain an estimate of how close the E*V*6-Glc-1-P(Mg^{2+}) complex is to the actual transition state for (PO_3^-) transfer, $(\ddagger)_{Mg}$, one might compare the increased binding energy of E*V*6-Glc-1-P(Mg^{2+}), relative to E·P-6-Glc-1-P(Mg^{2+}), about 8 kcal/mol, with the increased binding energy in the actual transition state, relative to the catalytically competent complex, E·P-6-Glc-1-P(Mg^{2+}). Of course, the latter value cannot be measured directly. However, a crude estimate of this increase can be obtained by comparing the rate constant for E·P-6-Glc-1-P(Mg^{2+}) $\rightarrow$ $(\ddagger)_{Mg}$ with that of a reference reaction where there is little or no increase in binding interactions in the transition state. Since neither of these rate constants is known, we use a *maximal estimate* of the above increase, 18 kcal/mol, obtained as in the Appendix. On this basis, if the increased interactions in E*V*6-Glc-1-P(Mg^{2+}), relative to E·P-6-Glc-1-P(Mg^{2+}), were entirely the result of a structure for E*V*6-Glc-1-P(Mg^{2+}) that is displaced in the direction of the transition state for (PO_3^-) transfer, the system would be $^8/_{18}$ of the way to the transition state—and more if distortion of the normal bonding in E(Mg^{2+}) and V-6-Glc-1-P is required to realize these increased binding interactions. Fortunately, in the present system one also can assess progress toward the transition state in terms of metal ion binding, as is described in the following sections.

The Binding of Mg(II) to the Analogue Complex Appears To Be Stronger Than to Substrate/Product Complexes. The effect of metal ion binding on formation of the transition-state-analogue complex is in accord with what might be expected if factors which affect $(\ddagger)_{Mg}$ are reflected in the stability of the E*V-6-Glc-1-P(Mg^{2+}). Thus, bound Mg^{2+} increases the rate of (PO_3^-) transfer, i.e., binding interactions in the transition state, by some (4×10^{10})-fold relative to transfer by the metal-free system (W.J.R., unpublished results). According to the transition-state binding paradigm of catalysis, Mg^{2+} in $(\ddagger)_{Mg}$ must be bound much more tenaciously than in E·P-6-Glc-1-P(Mg^{2+}), as is illustrated in Scheme III. By analogy, if the properties of E*V*6-Glc-1-P(Mg^{2+}) accurately reflect those of $(\ddagger)_{Mg}$, Mg^{2+} in the glucose phosphate/*vanadate* complex also should be bound more tenaciously than in the bisphosphate complex by about (4×10^{10})-fold. In fact, E·V-6-Glc-1-P does not form readily in the absence of Mg^{2+},

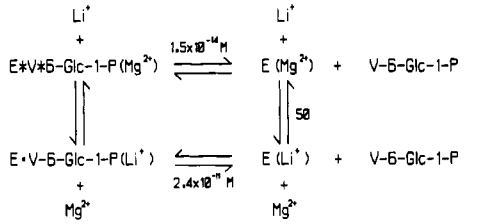
[12] This estimate involves a comparison of the dissociation constants for E·Glc-P_2, 1×10^{-8} M (Ray & Long, 1976b), and E*V*6-Glc-1-P, 1.5×10^{-14} M (see above), with the eqilibrium constant for $ROPO_3^{2-} + HOVO_3^{2-} \rightleftharpoons ROVO_3^{2-} + HOPO_3^{2-}$, about 10 (see the introduction).

Scheme III: Thermodynamic Box Showing How the Relative Binding of Mg^{2+} in $(\ddagger)_{Mg}$ and in the Ground State, E·P-6-Glc-1-P(Mg^{2+}), Was Approximated[a]



[a] Subscript, gs, refers to the ground state.

Scheme IV: Thermodynamic Box Showing How the Relative Binding of Mg^{2+} in E*V*6-Glc-1-P(Mg^{2+}) and That of Li^+ in E·V-6-Glc-1-P(Li^+) Were Compared



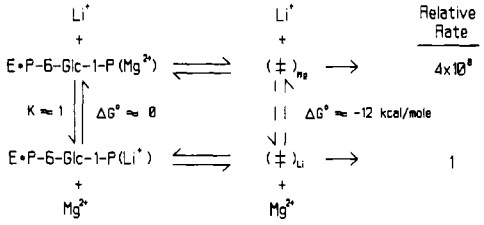
although at present we do not know how to quantitate the formation of such a complex [cf. the second paper of this series (Ray et al., 1990)]. On the other hand, it is possible to show that the *rate* of dissociation of Mg^{2+} from the latter complex, relative to the rate of its dissociation from E·P-6-Glc-1-P-(Mg^{2+}), is slowed by a factor equal to or less than 3×10^{-4} (Figure 3). In fact, in contrast with the substrate/product complexes, where neither the rate nor the extent of Mg^{2+} binding is substantially affected by the presence of bound substate/product, Mg^{2+} does not dissociate from the E*V*6-Glc-1-P(Mg^{2+}) complex at a rate distinguishable from that at which V-6-Glc-1-P dissociates. While it is not possible to demonstrate that the reduced dissociation rate of Mg^{2+} in the transition-state-analogue complex is produced solely by tighter binding, the results are consistent with such a rationale.

The Relative Binding of Mg(II) and Li(I) to the Analogue Complex Seems To Reflect Their Relative Effect on the Catalytic Process. Bound Li^+ increases the rate of (PO_3^-) transfer in the E-P·Glc-1-P complex only by about 100-fold, compared with at least (4×10^{10})-fold for bound Mg^{2+} (W.J.R., unpublished results). In comparison with Mg^{2+}, Li^+ thus has little effect on the transition state for (PO_3^-) transfer relative to the metal-free enzyme. Because of this small effect and because the Li^+ enzyme is easier to handle experimentally than the metal-free enzyme, the Li^+ enzyme was used as a reference in comparative binding studies. Other reasons for this choice are that Li^+, which is almost the same size as Mg^{2+}, not only binds to the enzyme competitively with Mg^{2+} and other bivalent metal ion activators but also produces nearly the same change in the ultraviolet spectrum of aromatic residues in the enzyme (Ray, 1978). Because formation of E·V-6-Glc-1-P(Li^+) can be monitored spectrally (cf. Figure 5 and Results), the equilibrium dissociation constant for the process

$$E\cdot V\text{-}6\text{-}Glc\text{-}1\text{-}P(Li^+) \rightleftharpoons E(Li^+) + V\text{-}6\text{-}Glc\text{-}6\text{-}P$$

can be evaluated as about 2×10^{-11} M. As is indicated under Results, this dissociation constant is some (1.4×10^3)-fold larger than the corresponding constant for the Mg^{2+} enzyme [1.5×10^{-14} M (see previous section)]. Since Mg^{2+} binds to the dephospho enzyme approximately 50-fold more tenaciously than does Li^+ (see Results), the thermodynamic box in Scheme IV can be used to show that Mg^{2+} in E*V*6-Glc-1-P(Mg^{2+}) is bound some (8×10^4)-fold more tenaciously than is Li^+ in E·V-6-Glc-1-P(Li^+). By comparing this ratio with an estimate of the equilibrium constant for $(\ddagger)_{Li} + Mg^{2+} \rightleftharpoons (\ddagger)_{Mg} + Li^+$, 4×10^8 M, one can approximate progress toward the transition state that has occurred in E*V*6-Glc-1-P(Mg^{2+}) (cf. the thermodynamic box in Scheme V and the Appendix). This approach places the state mimicked by E*V*6-Glc-1-P(Mg^{2+}) approximately halfway to $(\ddagger)_{Mg}$, in reasonable agreement with the estimate based on overall binding interactions in a previous section.[13]

Scheme V: Thermodynamic Box Showing How the Relative Binding of Mg^{2+} in $(\ddagger)_{Mg}$ and That of Li^+ in $(\ddagger)_{Li}$ Were Compared



Crude as the above estimates are, their agreement lends support to the suggestion that one may be able to use properties of the E*V*6-Glc-1-P complex to deduce the nature of $(\ddagger)_{Mg}$, as in the following sections.

Implications Relative to an Associative Mechanism for (PO_3^-) Transfer. Earlier studies (Post et al., 1989) provided relatively similar values of the rate constant for (PO_3^-) transfer by the Mg^{2+} and Cd^{2+} complexes of phosphoglucomutase, in spite of the rather disparate nature of these activating metal ions—except for electrostatic charge—as well as a huge difference between the catalytic activity of the Mg^{2+} and Li^+ forms of the enzyme (Ray et al., 1989)—in spite of several similar physical properties of Mg^{2+} and Li^+—except for charge. On this basis we suggested that the charge at the metal ion binding site becomes more negative in the transition state and that at least part of the role of the bivalent metal ion activator is to stabilize the increased charge. Moreover, since the activating metal ion is bound to the phosphate group in the phospho enzyme (Rhyu et al., 1984), an increase in electrostatic charge at the metal ion binding site might well be caused by an increased charge on the (PO_3^-) group undergoing transfer as the system approaches the transition state. The enhanced intrinsic binding energy of vanadate at the active site of the Mg^{2+} enzyme, relative to the Li^+ enzyme, and the related preferential binding of Mg^{2+} relative to Li^+ by the E/V_i/Glc-1-P complexes are in accord with this proposal, i.e., with an associative mechanism for (PO_3^-) transfer [cf. Knowles (1980)], where the hydrogen of the incoming hydroxyl is largely removed during approach to the transition state.

Implications Relative to a Dissociative Mechanism for (PO_3^-) Transfer. The above scenario produces a fundamental problem with respect to the timing of bond breaking/bond

[13] While bound V-6-Glc-1-P appears to mimic some state between E·P-6-Glc-1-P and the transition state leading to E-P·Glc-1-P, none of the available evidence suggests that E*V*6-Glc-1-P mimics intermediate states between that transition state and E-P·Glc-1-P. Thus, there is no evidence that E-V, analogous to E-P, ever forms [cf. Ray et al. (1990)], and treatment of the dephospho enzyme with V_i followed by Glc-1-P does not lead to E*V*6-Glc-1-P (see Results). Nor does E-V appear to be formed by dissociation of E*V*6-Glc-1-P, although these experiments are inconclusive because of various kinetic considerations. If states on the E·P-6-Glc-1-P side of the transition state only are mimicked in E*V*6-Glc-1-P, this complex might better be represented as E·V*6-Glc-1-P. Why a complex that, in the same vein, would be represented as E*V·Glc-1-P fails to form remains to be seen.

making during (PO_3^-) transfer. If, on binding to the enzyme, the PGlcO–PO_3^{2-} bond is distorted in the direction of PGlcO$^-$ + PO_3^-, as is suggested by the spectral studies of the vanadate-based transition-state-analogue complex (Ray & Post, 1990), the electrostatic charge on the (PO_3^-) group should *decrease* and thus set up the system for (PO_3^-) transfer via a primarily *dissociative* process which mimics that in model systems: extensive bond breaking with little bond making (Allen & Haake, 1980; Bourne & Williams, 1984; Skoog & Jencks, 1984; Jencks, 1985; Herschlag & Jencks, 1987, 1989a,b; Williams, 1987). Thus, the results of both the previous and current thermodynamic studies seem more nearly in accord with an associative mechanism for (PO_3^-) transfer, while attempts to assess the nature of the bonding within the oxyvanadium constellation of the transition-state-analogue complex seem to indicate a dissociative process.

One way around the above dilemma would be to show that the enzyme is constructed so that it provides a couple between increased binding of the activating metal ion and (PO_3^-) transfer—one that allows an increase in binding interactions between the activating metal ion and groups *other than the* (PO_3^-) *group being transferred* and allows that increase only as the system progresses along the reaction coordinate toward the transition state. Whether such coupling represents a viable possibility, or whether some of our observations will require a basically different interpretation, remains to be seen. But as long as such a possibility remains, the seemingly less ambiguous spectral studies weight our judgment in favor of a *dissociative mechanism* for PO_3^- transfer in the phosphoglucomatase reaction.

Why the Difference between Phosphate and Vanadate? A basic question, unanswered by the present results, is why a molecule structurally as similar to P-6-Glc-1-P as V-6-Glc-1-P should serve as a model of a state that approaches the *transition state* for (PO_3^-) transfer instead of mimicking the *ground state* binding of the bisphosphate. Although V–O bonds tend to be some 0.15 Å or about 10% longer than the corresponding P–O bonds [cf. VanEtten et al. (1974)], such a difference, per se, seems insufficient to provide a rationale for the large differences in binding energy produced by substituting vanadate for phosphate in the systems involving phosphoglucomutase that are described herein. Neither can the difference between interactions of the active-site metal ion (Mg^{2+}) with phosphate and vanadate provide such a rationale, since such differences are minimal, at least in aqueous solution (see Results). On an a priori basis, the suggestion of Percival et al. (1990) that the stability of E*V*6-Glc-1-P(Mg^{2+}) is due to the formation of a pentacoordinate adduct of V-6-Glc-1-P represents a more palatable explanation than increments in bond length of 0.15 Å. Indeed, apart from the spectral studies in the third paper of this series (Ray & Post, 1990), there would be no reason to question this suggestion.

An alternative possibility based on other chemical differences between phosphates and vanadates also seems feasible. Although not extensively documented, a substantial increase in the RO–VO_3^{2-} bond distance may not be nearly as demanding, energetically, as in the case of RO–PO_3^{2-}. Whereas the more remarkable examples of unusually long coordinate bonds to V(V) involve octahedral complexes (Holloway & Melnik, 1985), even in the case of tetrahedral vanadates, V–O bonds substantially longer than the nominal V–O bond are found, e.g., in $^{2-}O_3V$–O–VO_3^{2-}, where the central V–O bond distance may be as long as 1.89 Å [in $Na_4V_2O_7 \cdot 18H_2O$; cf. Holloway and Melnik (1986)]. In fact, the ultraviolet spectrum of $V_2O_7^{4-}$(aq) is closer to that of the oxyvanadium constellation in E*V*6-Glc-1-P(Mg^{2+}) (Ray & Post, 1990) than any other of the model V(V) derivatives studied to date, in terms of intensity, as well as band shape and position (W.J.R., unpublished results).

In pentacoordinate complexes of V(V), one of the O^{2-} groups in VO_4^{3-} [or in $V^{5+}(O^{2-})_4$, a representation of V_i that frequently is used by inorganic chemists [see footnote 6 in Ray and Post (1990)]] is replaced by two oxyanions. Because such derivatives tend to form polymers, V–O bond lengths in $(RO)_2VO_3^{3-}$, for example, are not available. However, when two O^{2-} groups are replaced by four such anions, as in $[VO_2(oxalate^{2-})_2]^{3-}$, the length of the V–O bonds opposite the O^{2-} ligands increases to approximately 2.20 Å [cf. Holloway and Melnick (1986)]. Although such vanadates are rather different, structurally, from those studied here, what such models show is that V(V) can form relatively stable interactions with oxygen ligands at a considerable distance. This being the case, it seems reasonable to suggest on the basis of the spectral studies in the third paper of this series that "ground-state" binding of V-6-Glc-1-P by the dephospho form of phosphoglucomutase (Mg^{2+} form) involves a substantially stretched V–O bond.

The same spectral studies suggest that bond formation to V(V) involving the hydroxyl group of Ser^{116}, which is the potential (PO_3^-) acceptor group in the case of bound P-6-Glc-1-P, does not compensate for the decreased bond order of the critical vanadium–oxygen bond in bound V-6-Glc-1-P. If, as is suggested herein, there is a close relationship between E*V*6-Glc-1-P(Mg^{2+}) and a state partway between E·P-6-Glc-1-P(Mg^{2+}) and $(\ddagger)_{Mg}$, the transition state for (PO_3^-) transfer would have a distinctly dissociative character in the phosphoglucomutase reaction. A transition state with dissociative character also has been suggested on the basis of the ease with which the dephospho enzyme accepts a PO_3^- group from the 6-thiophosphate analogue of P-6-Glc-1-P (Knight et al., 1984). On the other hand, how the enzyme utilizes increased binding of Mg^{2+} to achieve such a state still is a mystery.

APPENDIX

Approximation of the Increase in Overall Binding Interactions and the Increased Mg^{2+}/Li^+ Binding in the Transition State for the (PO_3^-) Transfer Catalyzed by Phosphoglucomutase. The rate constant for the process E·P-6-Glc-1-P(Mg^{2+}) → E-P(Mg^{2+}) + Glc-1-P is about 400 s^{-1} (Ray et al., 1989). Since it seems unlikely that the rate of product release is less than 0.1 that of the chemical process, for a *maximal* estimate we take 4000 s^{-1} as the rate constant for the (PO_3^-) transfer step in this reaction.

By contrast, in the case of the Mg^{2+} enzyme the rate constant for E-P $\xrightarrow{H_2O}$ E + P_i, is about 2.2×10^{-8} s^{-1} (Ray et al., 1976). Here, the enzyme is acting as a (PO_3^-) donor rather than an acceptor, as in the forward phase of the process, E-P·Glc-1-P(Mg^{2+}) ⇌ E·P-6-Glc-1-P(Mg^{2+}). But since the equilibrium constant in the normal donor/acceptor reaction is close to unity (Ray & Long, 1976), and since in E-P → E + P_i there must be a much smaller increase in binding interactions in the transition state than in the normal transfer process, we take 2×10^{-8} s^{-1} as the *upper* limit rate constant for the reference reaction. But even in this slow reference reaction, increased interactions between the (PO_3^-) group being transferred and peripheral groups of the enzyme may occur. In fact, the rate of the process E-P → E + P_i is some 10–100-fold faster than expected for the comparable hydrolysis reaction involving *N*-acetylserinamide phosphate dianion. Hence, for a *lower* limit value of the rate constant for the

reference reaction we use 2×10^{-10} s^{-1}. The ratio of the rate constants, $4000/2 \times 10^{-10}$, provides the basis for our estimate of the *maximal* increase in binding interactions that might accompany the enzymic (PO_3^-) transfer process: 18 kcal/mol.

A comparison of (PO_3^-)-transfer rates for the Mg^{2+} and Li^+ enzymes with the equilibrium binding of these metal ions to the E·Glc-P_2 complex (see Results) shows that Li^+ interactions in $(\ddagger)_{Li}$ are weaker than Mg^{2+} interactions in $(\ddagger)_{Mg}$ by some (4×10^8)-fold, equivalent to a Gibbs energy difference of nearly 12 kcal/mol (see Scheme V). Hence, in the unlikely event that E*V*6-Glc-1-P(Mg^{2+}) accurately mimics $(\ddagger)_{Mg}$ and that E·V-6-Glc-1-P(Li^+) accurately mimics $(\ddagger)_{Li}$, the expected equilibrium constant for the metal ion exchange process in eq 11 also would be about 4×10^8 which is

$$\text{E·V-6-Glc-1-P}(Li^+) + Mg^{2+} \rightleftharpoons \text{E*V*6-Glc-1-P}(Mg^{2+}) + Li^+ \quad (11)$$

equivalent to a Gibbs energy difference of about 12 kcal/mol. In fact, the observed equilibrium constant for eq 11 is about 8×10^4 M, or about 7 kcal/mol. We use these values to assess relative progress toward the respective transition states as follows.

If progress toward a transition state is represented by a fractional distance, for example, between E·Glc-P_2(Mg^{2+}) and $(\ddagger)_{Mg}$, estimating progress toward $(\ddagger)_{Mg}$ that occurs in E*V*6-Glc-1-P(Mg^{2+}) is equivalent to placing a marker denoting the intermediate state mimicked by E*V*6-Glc-1-P-(Mg^{2+}) along the upper horizontal arrows in Scheme V. If both E·V-6-Glc-1-P(Li^+) and E*V*6-Glc-1-P(Mg^{2+}) mimic states halfway between the ground and transition states for the Li^+ and Mg^{2+} systems, respectively, $-\Delta G°$ for the metal ion interchange reaction in eq 11 would be about 6 instead of 12 kcal/mol (see Scheme V). But E·V-6-Glc-1-P(Li^+) likely is substantially less distorted than the Mg^{2+} complex (Ray & Post, 1990) and thus mimics a state closer to the ground state. This could add about 1 kcal/mol to the expected value of $-\Delta G°$ for eq 11 [since Li^+ in $(\ddagger)_{Li}$ binds only about 100-fold more tenaciously than in E·P-6-Glc-1-P(Li^+) (W.J.R., unpublished results)]. Thus, if E*V*6-Glc-1-P(Mg^{2+}) mimics a state halfway to $(\ddagger)_{Mg}$ and E·V-6-Glc-1-P(Li^+) mimics a state close to E·P-6-Glc-1-P(Li^+), $-\Delta G°$ for eq 11 should be about 7 kcal/mol, which is the observed value.

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