Comparison of Rate Constants for (PO_3^-) Transfer by the Mg(II), Cd(II), and Li(I) Forms of Phosphoglucomutase†

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Received April 29, 1988; Revised Manuscript Received August 16, 1988

ABSTRACT: Net rate constants that define the steady-state rate through a sequence of steps and the corresponding effective energy barriers for two (PO_3^-)-transfer steps in the phosphoglucomutase reaction were compared as a function of metal ion, M, where M = Mg^{2+} and Cd^{2+}. These steps involve the reaction of either the 1-phosphate or the 6-phosphate of glucose 1,6-bisphosphate (Glc-P_2) bound to the dephosphoenzyme (E_D) to produce the phosphoenzyme (E_P) and the free monophosphates, glucose 1-phosphate (Glc-1-P) or glucose 6-phosphate (Glc-6-P): E_P·M + Glc-1-P ← E_D·M·Glc-P_2 → E_P·M·Glc-6-P_6. Before this comparison was made, net rate constants for the Cd^{2+} enzyme, obtained at high enzyme concentration via ^{31}P NMR saturation-transfer studies [Post, C. B., Ray, W. J., Jr., & Gorenstein, D. G. (1989) *Biochemistry* (preceding paper in this issue)], were appropriately scaled by using the observed constants to calculate both the expected isotope-transfer rate at equilibrium and the steady-state rate under initial velocity conditions and comparing the calculated values with those measured in dilute solution. For the Mg^{2+} enzyme, narrow limits on possible values of the corresponding net rate constants were imposed on the basis of initial velocity rate constants for the forward and reverse directions plus values for the equilibrium distribution of central complexes, since direct measurement is not feasible. The effective energy barriers for both the Mg^{2+} and Cd^{2+} enzymes, calculated from the respective net rate constants, together with previously published values for the equilibrium distribution of complexes in both enzymic systems [Ray, W. J., Jr., & Long, J. W. (1976) *Biochemistry 15*, 4018–4025], show that the 100-fold decrease in the k_{cat} for the Cd^{2+} relative to the Mg^{2+} enzyme is caused by two factors: the increased stability of the intermediate bisphosphate complex and the decreased ability to cope with the phosphate ester involving the 1-hydroxyl group of the glucose ring. In fact, it is unlikely that the efficiency of (PO_3^-) transfer to the 6-hydroxyl group of bound Glc-1-P (thermodynamically favorable direction) is reduced by more than an order of magnitude in the Cd^{2+} enzyme. By contrast, the efficiency of the Li^+ enzyme in the same (PO_3^-)-transfer step is less than 4×10^{-8} that of the Mg^{2+} enzyme. The large differences in properties of Mg^{2+} and Cd^{2+} plus the small differences in the above rate of (PO_3^-) transfer, coupled with the similarity in properties of Mg^{2+} and Li^+ plus the enormous differences in the corresponding rate of (PO_3^-) transfer, suggest that the primary role of the metal ion in the transfer process may involve an electrostatic effect and that the metal ion may be positioned close to an atom that becomes substantially more negative in the transition state. Whether that atom might be the bridging oxygen of the phosphate groups, as in a predominantly dissociative mechanism, or is a peripheral phosphate oxygen or oxygens, as in a predominantly associative mechanism, is considered.

The ^{31}P saturation-transfer studies described in the accompanying paper (Post et al., 1989) show that one of the (PO_3^-)-transfer steps[1] facilitated by the Cd^{2+} form of phosphoglucomutase, viz., the transfer of the enzymic (PO_3^-) group to the 6-position of bound Glc-1-P,[2] is surprisingly efficient ($k_{t1}^+ \geq 350\ s^{-1}$), given the relative values of k_{cat} for the Cd^{2+} and Mg^{2+} enzymes (1/100). The present studies were undertaken in an attempt to rationalize the relatively low value of k_{cat} for the Cd^{2+} enzyme, in view of its efficiency in the above transfer step, since understanding metal-specific differences in (PO_3^-)-transfer efficiency may provide clues to the role of the metal ion in the bond-breaking/bond-making process (see accompanying paper).

Experimental Procedures

Materials. The preparation, storage, and demetalation of phosphoglucomutase are described in the accompanying paper (Post et al., 1989). Gelatin (Knox) was demetalated as described by Magneson et al. (1987). ^{31}P phosphoric acid in dilute HCl (New England Nuclear) was evaporated to dryness, treated with dilute ammonia, and reevaporated before use. A 2 M stock solution of high-purity lithium chloride (Alfa) was passed through a column of Chelex (Bio-Rad), Li^+ form, before use. Sucrose phosphorylase was isolated from *Leuconostoc mesenteroides* (Doudoroff, 1955). DEAE-Spectra/Gel M was from Spectrum. Triethylamine was distilled under vacuum and either stored in the dark at −20 °C or converted to the hydrochloride in aqueous solution. Before use in a HCO_3^- buffer system, sufficient amine to produce a 1 M solution was suspended in water at 0 °C and bubbled with CO_2 (vigorous stirring) until the pH of a 1:10 dilution fell to about 7.5. ACS scintillation cocktail was from Amersham.

† This work was supported in part by a research grant from the U.S. Public Health Services (GM08963, to W.J.R.).

[1] "(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 implication about the mechanism of transfer.

[2] Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, α-D-glucose 6-phosphate; Glc-P_2, α-D-glucose 1,6-bisphosphate; E_P and E_D, the phospho and dephospho forms of rabbit muscle phosphoglucomutase; P_i, inorganic phosphate; M, a bivalent metal ion activator (of phosphoglucomutase).

0006-2960/89/0428-0559$01.50/0

Instrumentation. The Varian XL200A NMR spectrometer described in the accompanying paper (Post et al., 1989) was used in the manner indicated.

Procedures. General procedures for handling dilute solutions of metal-free phosphoglucomutase have been described (Magneson et al., 1987). As in that reference, *all* components of solutions that contained the enzyme were specially treated; *all* containers and *all* equipment used for transferring the enzyme were plastic and also were specially treated. All pH values refer to 25 °C. Radioactive phosphate was quantified by scintillation counting of 0.5-mL aliquots in 10 mL of ACS cocktail.

Dilute solutions of [^{32}P]Glc-6-P that also contained ^{32}P-labeled inorganic phosphate were quantified after the latter was precipitated in the presence of 0.15 M $HClO_4$, 1.3 mM ammonium molybdate, and 10 mM Et_3N·HCl [cf. Sugino and Mioshi (1964)] by making the solution 0.2 mM in P_i. After standing 5 min and centrifuging, a second addition of P_i was made (initial concentration 0.1 mM); the same centrifuge tube was used, and the soluble contents were rapidly mixed. After repeating the standing and centrifuging steps, most of the supernatant was transferred to a clean tube for a final centrifugation before aliquots were removed for counting. The total radioactivity initially present was assessed in samples that were treated identically but in the absence of Et_3N.

^{31}P saturation-transfer studies were conducted in the manner described in the accompanying paper with solutions that initially contained 1.62 mM phosphoglucomutase, Cd^{2+} form, 1.62 mM Glc-1-P, 1.4–6.0 mM Glc-P_2, and 20 mM Tris-HCl, pH 7.4. Resonances of the free bisphosphate (at 0.7 and −1.5 ppm) were irradiated, and loss of magnetization from both phosphates of the enzyme-bound Glc-P_2 (at −2.7 or +5.2 ppm) was monitored.

The specific activity of the Cd^{2+} enzyme at 24 °C under initial velocity conditions was determined by (a) treating the metal-free enzyme with 1 mM NTA/0.9 mM Cd^{2+} in the presence of gelatin, 1 mg/mL, and 20 mM Tris-HCl, pH 7.4, and (b) assaying aliquots of the treated enzyme in the presence of 4 mM Glc-1-P, 25 μM Glc-P_2, 10 mM EDTA, and 20 mM Tris-HCl, pH 7.4. Time aliquots were removed, quenched in 0.1 M acid, allowed to stand 5 min, and neutralized with excess Tris base containing sufficient EDTA to produce a final concentration of 10 mM. After centrifugation, aliquots were assayed for Glc-6-P by means of the dehydrogenase reaction (Michal, 1984). Rates observed in the time interval from 4 to 8 min were extrapolated to time zero by constructing plots of $\ln \{([P_{t2}] - [P_{t1}])/(t_2 - t_1)\}$ versus t, where $[P_{t1}]$ and $[P_{t2}]$ are the concentrations of product at the reference time, t_1 (4 min), and at a subsequent time t_2. [Such plots are linear with a slope of $-k_d$, where k_d is the rate constant for dissociation of Cd^{2+} from the enzyme (cf. Ray and Roscelli (1966)).]

Glucose [^{32}P]-1-phosphate with a specific activity of about 200 mCi/mmol and essentially free of Glc-6-P and P_i was prepared via the sucrose phosphorylase reaction by using phosphorylase that previously had been exposed to 10 mM EDTA, pH 7.4, for 1 h at room temperature (to eliminate residual phosphoglucomutase activity). Initial concentrations in the reaction mixture were 0.08 M sucrose, 0.1 mM [^{32}P]-phosphate, 0.2 M Bistris-HCl, pH 7, 10 mM EDTA, 12 mM cysteine–HCl adjusted to pH 7 just before use, and about 0.5 unit of sucrose phosphorylase: final volume, 0.1 mL. The reaction was terminated after about 0.85 of the initial [^{32}P]-phosphate had been converted to organic phosphate (see assay procedure, below) by making the reaction mixture 0.15 M in $HClO_4$, 10 mM in Et_3N·HCl, and 1.3 mM in ammonium molybdate: total volume, 1.0 mL. [^{32}P]Phosphate was precipitated as in the assay for [^{32}P]Glc-6-P, above. The final supernatant was diluted to 20 mL with water, made basic with 1.2 mL of 1 M NH_4OH, and subjected to chromatography on a 0.6 × 1.5 cm column of Spectra/Gel DEAE-resin, HCO_3^- form, using a gradient of Et_3N/HCO_3^- 0–0.5 M in a total of 50 mL: flow rate, 1 mL/min. The Et_3N/HCO_3^- was removed by lyophilization; the [^{32}P]Glc-1-P was dissolved in 1 mL of 1 mM Tris-HCl buffer, pH 7.4, relyophilized, and stored in the dry state at −20 °C until used.

Uniformly labeled [^{32}P]Glc-P_2 was prepared by equilibrating [^{32}P]Glc-1-P with a large excess of Glc-P_2 in the presence of phosphoglucomutase. The bisphosphate was isolated chromatographically, as above, except that the elution gradient was twice as steep.

The transfer of [^{32}P](PO_3^-) from labeled Glc-1-P to Glc-6-P at chemical equilibrium was initiated with enzyme that had been treated with the Cd^{2+}/NTA buffer solution described for initial velocity studies (see above). Assay concentrations were 3.8 mM Glc-6-P, 0.2 mM Glc-1-P, 0.025 mM Glc-P_2, 10 mM EDTA, and 20 mM Tris-HCl, pH 7.4, at 24 °C. After 3.75 min, a trace of [^{32}P]Glc-1-P was added. Time aliquots were removed at the 4-min point and every 45 s thereafter and were quenched by addition to a solution that contained reagents for precipitating P_i (see above). The production of acid-stable organic phosphate was determined via a modification of the procedure described for quantifying [^{32}P]Glc-6-P (see above) in which the precipitating solution (0.15 M in $HClO_4$) was heated at 100 °C for 10 min to hydrolyze all remaining [^{32}P]Glc-1-P. After the solution was cooled to room temperature, the sequential precipitation procedure was conducted as described. Rates measured in the time interval of 4–8 min were extrapolated to time zero in the manner described above for initial velocity studies.

Metal-free, ^{32}P-labeled phosphoglucomutase was prepared from an enzyme solution, 2 mL, at a concentration of about 5 mg/mL of enzyme, that had been extensively dialyzed against 10 mM Tris/MES buffer, pH 6.2, that contained 10^{-4} M Zn^{2+}. After the addition of 0.1 equiv or less of [^{32}P]Glc-1-P, the enzyme was adsorbed on a 0.6 × 1.5 cm column of CM-Sephadex previously equilibrated with the same buffer (but in the absence of added Zn^{2+}). The column was washed with 10 mL of buffer and the enzyme eluted (0.1 mL/min) with 8 mM Tris/6 mM $(Tris)_2SO_4$ adjusted to pH 6.7 with MES. The peak tubes were made 10 mM in EDTA and dialyzed for three 12-h intervals versus 10 mM EDTA/20 mM Tris-HCl, pH 7.4. Except for the dialysis steps (at 4 °C), all steps were conducted at room temperature.

The reaction of (freshly prepared) metal-free [^{32}P]E_P with a saturating concentration of Glc-1-P (20 mM) was conducted in the presence of 22 mM LiCl, 10 mM EDTA, and 1 mM Glc-P_2 at 24 °C and at an enzymic concentration of about 4 mg/mL of enzyme. Time aliquots (0.15 mL) were quenched by addition to 0.15 mL of a freshly prepared solution of 10% TCA containing 1 μmol of Glc-P_2, followed by brief sonication to disperse the precipitate. Water (2 mL) was added and the mixture again subjected to brief sonication. After centrifugation, the supernatant was removed, diluted with 8 mL of 3 mM Tris base (to produce a pH of about 4), and stored at 4 °C, if necessary, prior to analysis. The glucose monophosphate and bisphosphate fractions were separated by chromatography on a 0.6 × 4.5 cm column of Spectra/Gel DEAE, formate form. After a washing with 2 mL of water, the monophosphates were eluted with 10 mL of 0.3 M formic acid adjusted to pH 3 with aqueous ammonia (1 mL/min) and

the bisphosphates with the same buffer at a concentration 5-fold higher. Fractions of 1 mL were collected. The phosphates were located by acid hydrolysis of 0.01-mL aliquots of these fractions and subsequent use of a modified Fiske SubbaRao procedure for inorganic phosphate [cf. Boltz (1972)]. The contents of phosphate-containing tubes were combined, lyophilized in scintillation vials, and subsequently counted.

The rate constant for the dissociation of Glc-P_2 from its complex with E_D·Cd was measured in an equilibrium mixture that contained 4.8 mM Glc-6-P, 0.25 mM Glc-1-P, 0.09 mM Glc-P_2, and 20 mM Tris-HCl, pH 7.4. Prior to the assay, the enzyme was treated as in initial velocity studies (see above) and was used at a final concentration of 3.2 μM; the assay also contained 0.5 mM NTA/0.45 mM Cd^{2+}. In the case of Mg^{2+}, the enzyme previously was treated with 1 mM EDTA/2 mM Mg^{2+} and was used at a final concentration of 3.2 nM; the assay also contained 1 mM EDTA/2 mM Mg^{2+}, and the concentrations of monophosphates and bisphosphate were twice and half the above values, respectively. After 20 min at 24 °C to achieve complete equilibrium, a trace of uniformly labeled [^{32}P]Glc-P_2 was added, time aliquots were removed, and the acid-stable radioactivity was assessed as above.

The equilibrium distribution of enzyme/Li^+/glucose phosphate complexes in a solution at 24 °C that initially contained 40 mg/mL of phosphoenzyme (0.66 mM) plus 20 mg/mL of dephosphoenzyme (0.33 mM), 0.25 mM Glc-1-P, 10 mM Tris-HCl, pH 7.4, 50 mM LiCl, 0.5 mM $MgCl_2$, and 0.25 mM EDTA was estimated by quenching 0.2-mL aliquots in 2.5 mL of rapidly stirred 1 N $HClO_4$ according to the method of Ray and Long (1976a), but using the micro stirring apparatus described by Ray and Puvathingal (1986). After addition of EDTA and ammonium formate to concentrations of 10 and 20 mM, respectively, followed by pH adjustment to 4 with 10 N KOH, most of the $KClO_4$ was separated by precipitation and filtration via centrifugation. After lyophilizing the filtrate, the residue was suspended in 0.3 mL of cold 0.2 M Tris-HCl, pH 8.3, and additional $KClO_4$ separated as above. Aliquots (0.05 mL) of the filtrate were assayed sequentially for Glc-6-P, Glc-1-P, and Glc-P_2 by using a modification of the procedure described previously (Ray & Long, 1976b) in which the change in optical density at 340 nm instead of fluorescence change was used to quantify the NADP produced in the coupled assay.

Results

Most rate constants obtained for the processes described below are summarized in Table I.

Initial Velocity and Isotope-Transfer Rate at Equilibrium for the Cd(II) Enzyme. Because the Mg^{2+} enzyme is approximately 100-fold more efficient than the Cd^{2+} enzyme, a 0.1% contamination of the Cd^{2+} enzyme by the Mg^{2+} form produces a significant error in initial velocity studies. Even the use of a Cd^{2+}/NTA buffer system to convert the demetalated enzyme to the Cd^{2+} form did not entirely eliminate this problem, because NTA is a poor chelator for Mg^{2+}. The Cd^{2+}/NTA buffer also produced a reduced enzymic activity in the assay. Hence, both initial velocity and isotope exchange studies were conducted in the presence of excess EDTA (at saturating substrate concentrations). Under these conditions, the half-time for dissociation of Mg^{2+} is about 25 s (Ray & Roscelli, 1966), while that for Cd^{2+} is about 30 min (Ray, 1969). By ignoring the first 4 min of the reaction, during which time essentially all of the Mg^{2+} dissociates from the enzyme, the activity of the Cd^{2+} enzyme can be accurately assessed.

Table I: Measured and Calculated Rate Constants for Reactions of Phosphoglucomutase from Studies at High and Low Concentrations of Enzyme[a]

rate constants	enzyme concentration/form		
	low/Mg^{2+} (s^{-1})	low/Cd^{2+} (s^{-1})	high/Cd^+ (s^{-1})
k_{cat}^f	630[b]	6.5,[b] {6.5–6.9}[c]	[10.0–10.6][d]
k_{cat}^r	200[e]	{7.0–7.3}[c]	[10.8–11.2][d]
k_{tr}^f	84[f]	1.6,[b] {1.6–1.7}[c]	[2.4],[g] [2.4–2.5][d]
k_{-1}^{app}	[340–400][h]	(7.8)[i]	12[j]
k_{-6}^{app}	[960–1660][h]	(7.1)[i]	11[j]
k_{t1}^+	[≥ 1400(1 + p_1)][k]	{230(1 + p_1)}[k]	
k_{t6}^+	[≥ 1500(1 + p_6)][k]	{100(1 + p_6)}[k]	

[a] Listed parameters refer to steady-state processes: k_{cat}^f and k_{cat}^r describe initial velocities in the forward and reverse directions, respectively; k_{tr}^f describes transfer of [^{32}P]phosphate from Glc-1-P to Glc-6-P at equilibrium; k^{app} and k_t describe processes defined in eq 1, 4, and 5. All constants were determined at pH 7.4 and a temperature close to 24 °C. Some constants were measured both at high enzyme concentration (100 mg/mL; 1.62 mM) and at low enzyme concentrations (< 10^{-4} mg/mL). Values in parentheses were obtained by applying a scaling factor of 0.65 to values measured at high enzyme concentrations (see footnote 3 and Results), those in brackets were obtained indirectly from values measured at low enzyme concentrations, and those in braces were obtained indirectly from values measured at high enzyme concentration and scaled with a factor of 0.65. [b] Measured in this study. [c] Calculated as limits from values of k_{-1}^{app} and k_{-6}^{app} obtained via NMR saturation transfer at high enzyme (Post et al., 1989) plus the equilibrium distribution of central complexes (Ray & Long, 1976c) in the manner described in Appendix 1 and scaled by a factor of 0.65 (see Results). [d] Calculated as in *b*, but without scaling. [e] Calculated from the measured value of k_{cat}^f and the ratio of k_{cat}^f/k_{cat}^r reported by Ray and Roscelli (1966). [f] From Ray and Long (1976c) after correcting for an altered extinction coefficient (Ray et al., 1982). [g] Calculated from rates measured via NMR saturation transfer (Post et al., 1989) according to eq 2. [h] Calculated from values of k_{cat}^f, k_{cat}^r, and the equilibrium distribution of central complexes (Ray & Long, 1976c) in the manner described in Appendix 2. [i] From the corresponding measured value at high enzyme (Post et al., 1988), after scaling by a factor of 0.65. [j] Values obtained obtained via NMR saturation transfer (Post et al., 1989). [k] From eq 9–12, using either values or minimal estimates of k_{-1}^{app} or k_{-6}^{app} in rows 4 and 5 together with C_1 = 4.2 and C_6 = 1.6 or C_1 = 28 and C_6 = 14 for the Mg^{2+} and Cd^{2+} enzymes, respectively (Ray & Long, 1976); p_1 and p_6 are undetermined partition coefficients greater than zero for E_P·M·Glc-1-P and E_P·M·Glc-6-P, respectively, and differ for M = Mg^{2+} or Cd^{2+}.

Sufficiently low concentrations of enzyme were used so that during the time interval from 0 to 8 min an insufficient amount of Glc-1-P was converted to Glc-6-P to cause a decrease in catalytic rate. Hence, the downward curvature for product/time plots during the 4–8-min time interval was caused by the dissociation of Cd^{2+}, which is "irreversible" because of the excess of EDTA present. Under these conditions the activity of the enzyme decreases in a first-order manner (Ray & Roscelli, 1966), which facilitates extrapolation of the activity of the Cd^{2+} enzyme observed in the 4–8-min range to the activity that would have been observed at t = 0 in the absence of contamination by the Mg^{2+} enzyme: extrapolation factor, 1.15 (see Experimental Procedures); k_{cat} = 6.5 s^{-1} (at 24 °C). Under similar conditions [EDTA affects the activity of the Mg^{2+} enzyme only in its role as a metal ion scavenger (Ray & Roscelli, 1966)] k_{cat} = 630 s^{-1} for Mg^{2+} enzyme. Hence, the ratio $(k_{cat})_{Cd}/(k_{cat})_{Mg}$ is 0.0103.

Isotope-exchange studies at equilibrium using [^{32}P]Glc-1-P were conducted in the presence of excess EDTA in a manner similar to that described above for the initial velocity (see Experimental Procedures). Since the decrease with time in the label appearing in the Glc-6-P fraction (which was held to less than 40%) is produced not only by a decrease in the fraction of the enzyme in the Cd^{2+} form but also by the approach to isotopic equilibrium, the slope of the plot used to extrapolate the exchange rate to zero time was somewhat

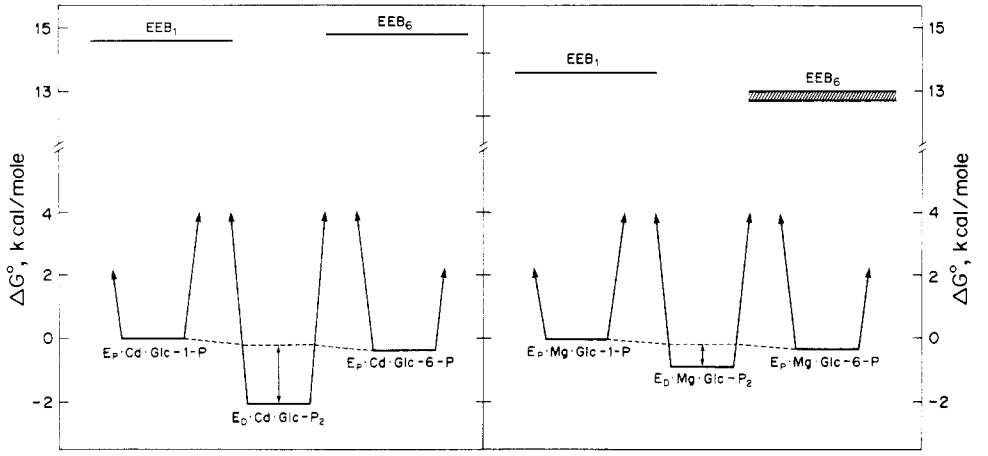


FIGURE 1: Energy level diagram showing the relative values of the Gibbs function for the enzymic complexes present in the phosphoglucomutase reaction; left and right, respectively, values for the Cd^{2+} and Mg^{2+} enzymes. The dashed lines show changes that would produce a monotonic decrease in Gibbs energy for the process E_P·M·Glc-1-P → E_D·M·Glc-P_2 → E_P·M·Glc-6-P. The vertical double-headed arrows show the difference between the Gibbs function for the E_D·M·Glc-P_2 complex and that which would produce a monotonic decrease, viz., the Cd^{2+} hole and the Mg^{2+} hole, respectively (see Discussion). The heights of the effective energy barrier for E_D·M·Glc-P_2 → E_P·M + Glc-1-P, $(EEB)_1$, and for E_D·M·Glc-P_2 → E_P·M + Glc-6-P, $(EEB)_6$, are shown, based on the definition of EEB under Discussion, and values of k_{-1}^{app} and k_{-6}^{app} in columns 2 and 3 of Table I (see also Results).

larger than that stated previously: extrapolation factor, 1.21; $k_{tr}^{f} = 1.6\ s^{-1}$.

Comparison of ^{32}P Isotope-Transfer and ^{31}P Saturation-Transfer Rates for the Cd(II) Enzyme. The accompanying paper (Post et al., 1989) provides values for the rate constants identified in eq 1 under conditions where the concentration

$$E_P\cdot Cd + Glc\text{-}1\text{-}P \underset{k_{-1}^{app}}{\overset{k_1^{app}}{\rightleftharpoons}} E_D\cdot Cd\cdot Glc\text{-}P_2 \underset{k_6^{app}}{\overset{k_{-6}^{app}}{\rightleftharpoons}} E_P\cdot Cd + Glc\text{-}6\text{-}P \quad (1)$$

of enzyme approaches that of the substrate/product pair, viz., under conditions where the *approach* of Glc-1-P to magnetic equilibrium when Glc-6-P is irradiated is not a steady-state process. In fact, a steady-state approach of Glc-1-P to magnetic equilibrium (very small ratio of enzyme to substrate/product pair) could not be obtained under conditions where the time dependence of Glc-1-P magnetization could be measured, because of the overall inefficiency of the Cd^{2+} enzyme coupled with the T_1 values for the various phosphates in the system (see accompanying paper). But the value of k_{tr}^{f}, the constant that specifies the *steady-state* flux of phosphorus nuclei in the forward direction, can be calculated from the results of saturation-transfer studies:

$$k_{tr}^{f} = \frac{k_{-6}^{app}}{2}\,\frac{1}{1 + k_6^{app}K_e/k_1^{app}}\,\frac{[E_D\cdot Cd\cdot Glc\text{-}P_2]}{[E_{total}]} = 2.4\ s^{-1} \quad (2)$$

[The value of k_{-6}^{app} in eq 2 is 11 s^{-1}, and the factor $^1/_2$ is used because transfer requires two catalytic cycles [see accompanying paper, Post et al. (1989), eq 7 and 8]; the second factor, whose value is 0.52, describes the partition of (E_P*·Cd) produced by the first catalytic cycle between reaction with Glc-1-P and with Glc-6-P at chemical equilibrium (where K_e is [α-Glc-6-P]/[Glc-1-P]), in both cases to give E_D·Cd·Glc-P_2. The final factor is the fraction of enzyme present as E_D·Cd·Glc-P_2, 0.85 (see accompanying paper).]

The value measured for k_{tr}^{f} via [^{32}P](PO_3^-) transfer at chemical equilibrium and at a low enzyme/substrate ratio is 1.6 s^{-1} under conditions otherwise similar to those used for NMR saturation-transfer studies, except for enzyme concentration (0.01 instead of 100 mg/mL) and substrate concentration (4 instead of 32 mM). The [^{32}P](PO_3^-)-transfer rate thus is about 0.67 of that expected from the NMR studies, i.e., 1.6 s^{-1}/2.4 s^{-1}.

Comparison of ^{31}P Saturation-Transfer Rates with the Steady-State Catalytic Constant: Cd(II) Enzyme. The nature of the ^{31}P saturation-transfer process, combined with the observed rate constants for the half-reactions involved in that transfer [accompanying paper, Post et al. (1989)], and the equilibrium concentration of the monophosphate and bisphosphate complexes of Cd^{2+} phosphoglucomutase (Ray & Long, 1976) yield a simple but approximate relationship between the rate constants for saturation exchange and the steady-state catalytic constant, k_{cat} (see Appendix 1). In the forward reaction

$$10\ s^{-1} \le k_{cat}^{f} \le 10.6\ s^{-1} \quad (3)$$

The measured value of k_{cat} for the Cd^{2+} enzyme, at 5×10^{-4} instead of 100 mg/mL and at 4 instead of 32 mM glucose phosphates but under conditions otherwise analogous to those used for saturation transfer (see above), is 6.5 s^{-1}. Thus, in accord with the results in the previous section, the rate constant obtained from studies at low enzyme concentration is about 0.63 as large as that obtained from measurements at much higher enzyme concentrations, i.e., 6.5 s^{-1}/10.6 s^{-1}.

In view of the comparisons at high and low enzyme concentration in this and the previous section,[3] the earlier estimates of 350 and 150 s^{-1} for k_{t1}^{+} and k_{t6}^{+} for the following processes (see accompanying paper) are reduced to 230 and 100 s^{-1}, respectively, for comparison with steady-state rate constants obtained at low enzyme concentration (see below).

$$Glc\text{-}1\text{-}P \underset{k_{d1}}{\overset{k_{a1}[E_P\cdot Cd]}{\rightleftharpoons}} (E_P\cdot Cd\cdot Glc\text{-}1\text{-}P) \underset{k_{t1}^{-}}{\overset{k_{t1}^{+}}{\rightleftharpoons}} E_D\cdot Cd\cdot Glc\text{-}P_2 \quad (4)$$

$$Glc\text{-}6\text{-}P \underset{k_{d6}}{\overset{k_{a6}[E_P\cdot Cd]}{\rightleftharpoons}} (E_P\cdot Cd\cdot Glc\text{-}6\text{-}P) \underset{k_{t6}^{-}}{\overset{k_{t6}^{+}}{\rightleftharpoons}} E_D\cdot Cd\cdot Glc\text{-}P_2 \quad (5)$$

[3] The somewhat larger values of the two rate constants calculated from data obtained at high enzyme concentration, relative to studies at low enzyme concentration, might arise from a difference in activity coefficients [cf. Nichol et al. (1983)] from a failure to adequately account for the relative concentrations of the various enzyme/glucose phosphate complexes (Ray & Long, 1976c), or from unrecognized differences in conditions.

Comparison of Net Rate Constants for (PO_3^-) Transfer by the Mg(II) and Cd(II) Enzymes. Figure 1 shows the relative Gibbs energies for the intermediate complexes of the Cd^{2+} and Mg^{2+} enzymes, left and right, respectively. The Gibbs function for the E_P·M·Glc-1-P complex is used as a reference since the equilibrium binding of Glc-1-P to E_P·M is essentially independent of the identity of M^{2+}, not only for Mg^{2+} and Cd^{2+} but also for Zn^{2+}, Co^{2+}, and Mn^{2+} (Ray & Long, 1976c). Also shown in Figure 1 are the effective energy barriers in the two half-reactions of the Cd^{2+} enzyme, EEB_1 and EEB_6, obtained from the values of the *net* rate constants[4] k_{-1}^{app} and k_{-6}^{app} (7.8 and 7.2 s^{-1}, respectively) that were measured via ^{31}P saturation transfer in the accompanying paper and scaled as in the preceding section (Post et al., 1989; see eq 1). Here, EEB is equal to $-0.6 \ln (k^{app}/10^{13})$ (see Discussion).

Analogous saturation-transfer studies cannot be conducted with the Mg^{2+} enzyme because of chemical exchange broadening. But values of k_{-1}^{app} and k_{-6}^{app} (see eq 1) for this form of the enzyme can be approximated from the equilibrium ratios of the various intermediates (Ray & Long, 1976c) plus values of the steady-state catalytic constant in the forward and reverse reactions [630 and 200 s^{-1}; data of Ray and Roscelli (1964), after making a correction for both a difference in extinction coefficient of the enzyme (Ray et al., 1983) and a temperature difference (25 instead of 30 °C)]. The required operation (see Appendix 2) is more or less the inverse of that used for approximating the range of values for k_{cat}^f from the half-reactions of the Cd^{2+} enzyme (Appendix 1). This analysis shows that for the Mg^{2+} enzyme

$$340 \leq k_{-1}^{app} \leq 400 \quad (6)$$

and

$$960 \leq k_{-6}^{app} \leq 1660 \quad (7)$$

Thus, the data restrict the range of allowed values for k_{-1}^{app} more than for k_{-6}^{app} (see eq 3). Both EEB_1 and EEB_6 for the Mg^{2+} enzyme, calculated as above from k_{-1}^{app} and k_{-6}^{app}, also are shown in Figure 1 (right), where stippling is used to indicate the range of values consistent with the above approximations. As opposed to the Cd^{2+} enzyme, where $k_{-1}^{app} \approx k_{-6}^{app}$ (see above), the Mg^{2+} enzyme facilitates (PO_3^-) transfer from the 1-position of the bound bisphosphate to the dephosphoenzyme more efficiently than from the 6-position, as is indicated by the relative heights of the effective energy barriers in Figure 1. This difference will be further considered under Discussion.

Comparison of Rate Constants for Phosphate Transfer by the Mg(II) and Cd(II) Enzymes. The rate constant for the (PO_3^-)-transfer step involving the primary hydroxyl group (the 6-hydroxyl) of bound glucose phosphate when the transfer is in the thermodynamically favorable direction, E_P·M·Glc-1-P $\xrightarrow{k_{t1}^+}$ E_D·M·Glc-P_2, can be related to the net rate constant, k_{-1}^{app} (see eq 1), in the manner described in Appendix 3. A quantitative comparison of k_{t1}^+ for the Mg^{2+} and Cd^{2+} enzymes would require a knowledge of how E_P·M·Glc-6-P is partitioned between dissociation to E_P·M + Glc-6-P and return to E_D·M·Glc-P_2. Nevertheless, the analysis in Appendix 3 suggests that the (PO_3^-) transfer described by k_{t1}^+ may well differ for the Mg^{2+} and Cd^{2+} enzymes by no more than an order of magnitude and perhaps less.

Rate Constant for Dissociation of Glucose 1,6-Bisphosphate from Its Complex with the Cd(II) and Mg(II) Enzymes. The strategy used to determine the rate constant for dissociation of Glc-P_2 from E_D·Cd·Glc-P_2 is as follows. An equilibrium mixture of enzyme, glucose monophosphates, and Glc-P_2 was established with the monophosphate in *large excess* over Glc-P_2, but with sufficient Glc-P_2 present to saturate the enzyme and sufficient enzyme to rapidly equilibrate the monophosphates. After the addition of a trace of uniformly labeled [^{32}P]Glc-P_2 (50% acid-stable phosphate), the rate of approach to equilibrium (95% acid-stable phosphate) was measured. Under these conditions, the rate at which Glc-P_2 dissociates from the enzyme limits the introduction of additional label into the acid-stable (Glc-6-P) fraction. Plots of $\ln (P_t - P_0)$ versus t, where P refers to acid-stable phosphate and the subscripts refer to reaction times, were linear (except, in the case of the Cd^{2+} enzyme, for a small initial lag). Values for k_d were obtained from the slopes of such plots after multiplying by the reciprocal fraction ($1/f$) of the enzyme present as E_D·M·Glc-P_2: for the Cd^{2+} enzyme, $k_d = 0.11$ s^{-1} [$1/f = 1.18$; see the accompanying paper, Post et al. (1988)]; for the Mg^{2+} enzyme, $k_d = 10.7$ s^{-1} ($1/f = 1.85$; Ray & Long, 1976c).

In the case of the Cd^{2+} enzyme the value of k_d also was obtained via NMR magnetization transfer as described in the accompanying paper, but here conducted in the presence of an excess of Glc-P_2 and in the essential absence of free glucose monophosphates (see Experimental Procedures). Phosphorus resonances of free Glc-P_2 were independently irradiated for periods of 10 or 15 s, and the loss of magnetization from the 1- and 6-phosphates of bound Glc-P_2 was assessed by comparison with spectra obtained with an off-resonance irradiation time [i.e., a recycle time equal to $2.3-3.3T_1$; see the accompanying paper, Post et al. (1989)]. Because dissociation of the bound Glc-P_2 is slow relative to the catalytic rate (and thus slow relative to saturation transfer between the phosphates of bound Glc-P_2; see accompanying paper), the decrease in magnetization of both phosphates of bound bisphosphate was approximately equal, regardless of which phosphate of free Glc-P_2 was irradiated. The magnetization observed after long irradiation times, relative to that measured in the absence of irradiation, decreased to a plateau value of about 0.75. The rate constant for dissociation of the bisphosphate complex was approximated from the fractional steady-state magnetization and the known T_1 value (see Appendix 2 of the accompanying paper), although the small measured differences produced a substantial uncertainty in the results and calculated rate constants: average rate constant for dissociation of E_D·Cd·Glc-P_2 (±SE) from eight observations in four different experiments, 0.12 ± 0.05 s^{-1}.[5]

Rate Constant for Transfer of the Enzymic (PO_3^-) Group of the Li(I) Enzyme to Bound Glucose 1-Phosphate. The strategy for evaluating the rate constant k_{t1}^+ for transfer of the enzymic (PO_3^-) group of the Li^+ enzyme to bound Glc-1-P (cf. eq 4) to produce Glc-P_2 is as follows. ^{32}P-Labeled phosphoenzyme was treated with a saturating concentration

[4] In a simple system, such as

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \overset{k_2}{\rightarrow} C$$

a net rate constant describes the rate at which A is converted to C when the process is in the steady state. Here, the net rate constant for A → C is equal to $k_1k_2/(k_{-1} + k_2)$; see Cleland (1975).

[5] Because saturation transfer between the phosphate groups of bound Glc-P_2 is much more rapid than dissociation, the apparent rate constant for dissociation of Glc-P_2 assessed by irradiating only *one* of the phosphates of free Glc-P_2 is half that expected if both phosphates of free Glc-P_2 had been irradiated. It is the latter rate constant, viz., the constant that correlates with the molecular dissociation of Glc-P_2, as assessed above in the [^{32}P]phosphate exchange studies, that is reported here.

of Glc-1-P in the presence of saturating Li^+ and excess EDTA. A sufficient amount of Glc-P_2 was included in the reaction so that any [^{32}P]Glc-P_2 formed by the above (PO_3^-) transfer would have a very low probability of reacting further, viz., reacting with the dephosphoenzyme in processes analogous to the reverse of eq 4 and 5. Since the efficiency of (PO_3^-) transfer by the Li^+ enzyme is many orders of magnitude less than for transfer by the Mg^{2+} enzyme (see below), even minute traces of Mg^{2+} could render an assessment of (PO_3^-) transfer by the Li^+ enzyme impossible. But once Mg^{2+} is bound at the active site of phosphoglucomutase, it remains associated with the enzyme, on the average, for about 20 000 catalytic cycles at saturating Glc-1-P (since at 24 °C k_{cat} = 630 s^{-1} and k_d = 0.032 s^{-1}; Ray & Roscelli, 1966). Only the first of these cycles can involve [^{32}P]E_P, and the probability that the label transferred in this cycle will enter the bisphosphate pool approaches 0.02 [Viz., is equal to k_d/k_{-6}^{app} or 10.6 s^{-1} (see preceding section) divided by 630 (see eq 7)]. Hence, by measuring the rate at which the labeled enzymic phosphate group is transferred to the bisphosphate pool, one can measure the exceedingly slow (PO_3^-) transfer that characterizes the Li^+ enzyme. To accomplish this, the glucose monophosphates in quenched time aliquots were chromatograpically separated from the bisphosphate. (The labeled Glc-6-P also was quantified after hydrolysis of the acid-labile phosphate in the glucose monophosphate sample and precipitation of the released P_i; as expected, the radioactivity in the Glc-6-P fraction was less than 1% that in the Glc-P_2 fraction.) A plot (not shown) of ln f *versus* time from aliquots removed during the 72-h reaction was used to estimate the initial transfer rate, where f is the fraction of the labeled phosphate from [^{32}P]E_P that had entered the bisphosphate pool. (The recoverable catalytic activity after conversion of the enzyme from its Li^+ to its Mg^{2+} form decreased only by about 6% during the 72-h reaction.) From the rate of label transfer plus the concentration of enzyme used, the observed rate constant is about 4×10^{-6} s at 24 °C. Since such a slow rate is not likely limited by the rate of Glc-P_2 dissociation from the Li^+ enzyme (the corresponding rate constant for dissociation of Glc-P_2 from the Mg^{2+} enzyme is about 10 s^{-1}; see preceding section), the above value almost certainly defines k_{t1}^+ (eq 4) for the Li^+ enzyme. This value is compared with estimates of k_{t1}^+ for the Mg^{2+} and Cd^{2+} enzymes under Discussion. (In a parallel experiment under otherwise identical conditions, except for the radioactive label, the rate of Glc-6-P production, presumably by contaminating Mg^{2+} enzyme, was estimated as equivalent to that which would be produced if 7.5×10^{-5} of the enzyme were in the Mg^{2+} form throughout the experiment; this fractional abundance of the Mg^{2+} enzyme in turn would be produced by about 1.7×10^{-6} M free Mg^{2+} in the presence of 22 mM Li^+.)

Rate Constant for Transfer of the 6-Phosphate of Bound Glucose Bisphosphate to the Li(I) Form of the Dephosphoenzyme. Technical problems prevented the direct measurement of k_{t1}^- (eq 4) for the Li^+ enzyme. The value of this constant, about 5×10^{-7} s^{-1}, was obtained from those for k_{t1}^+ and the equilibrium constant (E_D·Li·Glc-P_2)/(E_P·Li·Glc-1-P) (9). Although the Li^+ enzyme is not sufficiently active to establish such an equilibrium in a reasonable time interval, the required equilibrium can be established by adding a trace of Mg^{2+} to the system, as was done previously (Ray & Long, 1976c) to establish the analogous equilibrium in the absence of (all but a trace of) a metal ion. (Although no more than 0.5% of the enzyme was present as its Mg^{2+} complex, this enzyme facilitated the formation of a mixture of complexes of the Li^+ enzyme involving Glc-1-P, Glc-P_2, and Glc-6-P; the composition of the mixture did not vary with time from 10 min to 2.3 h after addition of Glc-1-P.) The observed equilibrium constant, 9, differs only marginally from the value observed in the presence of Mg^{2+}, 4.2, or that for the metal-free system, 11, relative to the enormous rate differences observed here (see Discussion).

Discussion

The primary objective of this study and that in the accompanying paper (Post et al., 1989) is to define the reaction sequence for Cd^{2+}-activated phosphoglucomutase with sufficient accuracy to determine the origin of the 100-fold difference in k_{cat} between this form of the enzyme and the Mg^{2+}-activated form and, if possible, to furnish support for using the Cd^{2+} enzyme to model some aspects of the enzymic reaction involving Mg^{2+}. A more interesting though less readily attainable objective is to specify the role of the bivalent metal ion activator in the (PO_3^-)-transfer process.

With regard to the first objective, the accompanying paper provides minimal estimates of rate constants for (PO_3^-) transfer by the Cd^{2+}-activated enzyme by direct measurement, using ^{31}P saturation-transfer techniques. The comparison of (PO_3^-) transfer in the Cd^{2+}- and Mg^{2+}-activated systems, below, is based partly on these constants, partly on the relative thermodynamic stability of the various intermediate complexes of the enzyme (Ray & Long, 1976c) plus the value for k_{cat}^f/k_{cat}^r for the Mg^{2+} enzyme (Ray & Roscelli, 1964), and partly on data under Results. (A comparison of rate constants obtained in NMR studies at 100 mg/mL with constants obtained at enzyme concentrations approximately 10^{-6} as large also is made under Results; cf. Table I.)

At chemical equilibrium in the Mg^{2+}-activated system, the relative abundance of the three intermediate complexes, E_P·Mg·Glc-1-P, E_D·Mg·Glc-P_2, and E_P·Mg·Glc-6-P, is 13:54:33 (Ray & Long, 1976c); i.e., the central E_D·Mg·Glc-P_2 complex predominates, but not by much, as one might expect for an enzyme that has evolved close to "perfection" (Albery & Knowles, 1976). However, in the Cd^{2+} system the relative abundance of the corresponding complexes is about 3:91:6. As is illustrated by Gibbs energy profiles of the reaction intermediates for both enzyme forms (Figure 1), what is a relatively small depression, amounting to about −0.6 kcal/mol in a descending monotonic energy profile for the Mg^{2+} enzyme, becomes a more substantial "hole", amounting to about −1.8 kcal/mol in the Cd^{2+} enzyme system; see the vertical double-headed arrows in the figure. One might well view this hole as the Cd^{2+}-induced thermodynamic hole. Part of the reason why the Cd^{2+} enzyme is less active than the Mg^{2+} enzyme under steady-state conditions is that during a catalytic cycle the enzymic system will fall into and must extricate itself from this hole, regardless of the direction in which the reaction is conducted.[6]

A second problem with the Cd^{2+} enzyme is its reduced efficiency in the half-reaction involving Glc-6-P. This reduced efficiency can be described in terms of the height of the effective energy barrier (EEB) for E_D·Cd·Glc-P_2 $\xrightarrow{k_{-6}^{app}}$ E_P·Cd + Glc-6-P, where k_{-6}^{app} is the *net* rate constant[4] (Cleland, 1975) and EEB_6 is equal to −0.6 ln ($k_{-6}^{app}/10^{13}$) kcal/mol, i.e.,

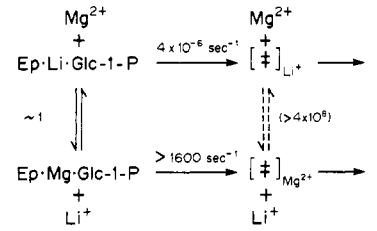
[6] Earlier observations indicate that the group transfer potential for the enzymic phosphate is too high for maximal catalytic efficiency when Cd^{2+} is the activator. Thus, $\Delta G°$ for the process E_P·M + Glc-1-P ⇌ E_D·M + Glc-P_2 becomes more negative by the substitution of Cd^{2+} for Mg^{2+} to about the same extent as $\Delta G°$ for E_P·M·Glc-1-P ⇌ E_D·M·Glc-P_2 (Ray & Long, 1976b).

is equal to $\Delta G^{\ddagger}$ for a one-step unimolecular process [cf. Glasstone et al. (1941)] with a numerically identical rate constant. Since k_{-6}^{app} depends on the rate constants for both (PO_3^-) transfer and product dissociation, the transition states for both processes contribute to EEB_6 (Ray, 1983). But regardless of the relative contributions of these steps (see below), a comparison of the effective energy barriers for the half-reactions of both forms of the enzyme shows that when Cd^{2+} replaces Mg^{2+}, the increase in EEB_6 is significantly greater than that in EEB_1. In fact, if k_{-6}^{app} for the half-reaction involving Glc-6-P were the same for the Cd^{2+} enzyme as it is for the Mg^{2+} enzyme, the value of $(k_{cat}^f)_{Cd}$ (Table I), which of necessity would include the energetics of the (PO_3^-)-transfer step for the half-reaction involving Glc-1-P, would be *at least* $0.3(k_{cat}^f)_{Mg}$ instead of about $0.01(k_{cat}^f)_{Mg}$. [The *minimum* value of k_{t1}^+ for E_P·Cd·Glc-1-P → E_D·Cd·Glc-P_2 at 25 °C is 230 s^{-1}, while $k_{-6}^{app} \geq (k_{cat}^f)_{Mg} = 630\ s^{-1}$.] It seems likely that the more severe problem in the half-reaction involving Glc-6-P is related to a more rigid steric requirement for (PO_3^-) transfer to and from a secondary as opposed to a primary hydroxyl group, coupled with the larger size of Cd^{2+} relative to Mg^{2+}.[7]

A comparison of rate constants for the actual bond-making/bond-breaking process facilitated by the Cd^{2+} and Mg^{2+} forms of the enzyme is more tenuous. In the first place, metal ion substitution alters the thermodynamic stability of "ground-state" species, although not to a major extent (see Figure 1).[8] Hence, we have chosen to make efficiency comparisons based on (PO_3^-) transfer in the thermodynamically favorable direction, i.e., on E_P·M·Glc-1-P $\xrightarrow{k_{t1}^+}$ E_D·M·Glc-P_2. Thus, the *minimum* values of k_{t1}^+ are 230 and 1600 s^{-1}, respectively, for the Cd^{2+} and Mg^{2+} enzymes. Although estimating the true ratio of k_{t1}^+ values for the Mg^{2+} and Cd^{2+} enzymes from net rate constants requires a knowledge about the partitioning of intermediates between (PO_3^-) transfer and product dissociation (cf. eq 23), it seems unlikely that $(k_{t1}^+)_{Mg}$ exceeds $(k_{t1}^+)_{Cd}$ by much more than an order of magnitude (see Appendix 3) and possibly not by this much.

In view of the above comparison, it seems reasonable to conclude that in its complex with the phosphoenzyme Mg^{2+} coordinates directly to the enzymic phosphate group, since direct coordination has been observed with $^{113}Cd^{2+}$ (Rhyu et al., 1983). Although the identity of the coordinating phosphate oxygen or oxygens has not been established, subsequent substrate binding probably produces at most minor alterations in the metal ion–phosphate interaction.[9] We thus view metal ion coordination in the E_P·Mg·Glc-1-P complex, just prior to (PO_3^-) transfer, as involving one or more oxy ligands of the enzymic phosphate group, either the bridging or nonbridging oxygens, or both.[10]

Scheme I: Relative Binding of Li^+ and Mg^{2+} in both Ground- and Transition-State Complexes for (PO_3^-) Transfer Facilitated by the Li^+ and Mg^{2+} Complexes of Phosphoglucomutase with Glc-1-P[a]



[a] An assessment of the relative rate constants for the Li^+ and Mg^{2+} forms of the enzyme is described under Results; that of the relative ground-state binding of Li^+ and Mg^{2+} is described in footnote 14.

Of the nonphysiological metal ion activators of phosphoglucomutase, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} (Ray, 1969), Cd^{2+} differs from the physiological activator, Mg^{2+}, to the greatest extent in terms of size (diameter = 2.28 versus 1.32 Å, respectively; Weast, 1987), ligand preference (Mg^{2+} is a "hard" and Cd^{2+} a "soft" metal ion; Pearson, 1963, 1965), and water substitution rates (about 10^5 s^{-1} for Mg^{2+} and about 3×10^8 s^{-1} for Cd^{2+}; Eigen & Hammes, 1962).[11] It is not obvious which of these properties or which combination of properties is responsible for the difference in the thermodynamics of (PO_3^-) transfer by the Mg^{2+} and Cd^{2+} complexes of the enzyme (Ray & Long, 1976c). But, as is suggested above, these differences apparently affect the efficiency of (PO_3^-) transfer from the enzyme to bound glucose 1-phosphate to only a relatively minor extent.

The above conclusion presents an interesting contrast with comparisons between the Mg^{2+} and Li^+ forms of phosphoglucomutase. Before making this comparison/contrast, it should be pointed out that a variety of published and unpublished evidence suggests that Mg^{2+} and Li^+ bind at the same site in phosphoglucomutase. For example, Li^+ binds competitively with Mg^{2+} and on binding elicits sizable changes in the UV absorbance of the enzyme similar to those produced by Mg^{2+} (Ray, 1978; Ray et al., 1978). In addition, the binding of glucose monophosphates (Ray et al., 1978) as well as glucose bisphosphate (W. J. Ray, Jr., unpublished results) is relatively unaffected by the substitution of Li^+ for Mg^{2+}. Both spectral changes (Ma & Ray, 1980) and changes in the ^{31}P NMR chemical shift of the enzymic phosphate group in the Mg^{2+} and Li^+ enzymes produced by the subsequent binding of Glc-6-P and a Glc-6-P analogue (W. J. Ray, Jr., unpublished results) also are quite similar.[12] In fact, Li^+ is much closer in size to Mg^{2+} (diameter = 1.36 versus 1.32 Å, respectively; Weast, 1978) than is Cd^{2+} (see above), and its

[7] A persistent problem in interpreting relative rates observed in these studies arises from an ambiguity in the structure of the E_D·Cd·Glc-P_2 complex observed in ^{31}P NMR studies [cf. the accompanying paper, Post et al. (1989)]: two such complexes should exist, and both should act as intermediates in the overall reaction (Ray et al., 1973; Ma & Ray, 1980). Thus, the observed complex might be a "single" species because of equilibrium effects, as is suggested by other studies (W. J. Ray, Jr., unpublished results), or might represent two complexes in rapid equilibrium. At present, we are unable to distinguish between these possibilities, although we have shown that interconversion of two such complexes cannot limit the catalytic process if these are present in similar amounts (C. B. Post, unpublished results).

[8] The equilibrium constant that relates the two expected E_D·M·Glc-P_2 complexes (see footnote 7) also may be altered by substituting Cd^{2+} for Mg^{2+}.

[9] The lack of a significant change in the ^{31}P–^{113}Cd NMR coupling constant produced on binding of 6-deoxyglucose 1-phosphate, a substrate analogue that induces large substrate-like changes in the near-UV spectrum of the enzyme (Ma & Ray, 1980) but which cannot act as (PO_3^-) acceptor, suggests that substrate binding does not appreciably alter the metal ion–phosphate interaction (W. J. Ray, Jr., unpublished results).

[10] Whether or not metal ion–phosphate coordination in E_P·Mg·Glc-1-P is ruptured in the (PO_3^-) transfer that produces E_D·Mg·Glc-P_2 still is a moot question [cf. Rhyu et al. (1983)]. However, a comparison of the equilibrium constant for E_P·Mg·Glc-1-P ⇌ E_D·Mg·Glc-P_2 (Ray & Long, 1976) with that for transfer involving free reactants and products (Peck et al., 1968) suggests that thermodynamically favorable interactions increase, not decrease, when (PO_3^-) transfer involves bound reactants and products.

[11] Differences in water substitution rate between ions of the same charge presumably reflect differences in ability to alter coordination geometry, i.e., to accommodate an elimination–addition or addition–elimination process [cf. Cotton and Wilkinson (1967)].

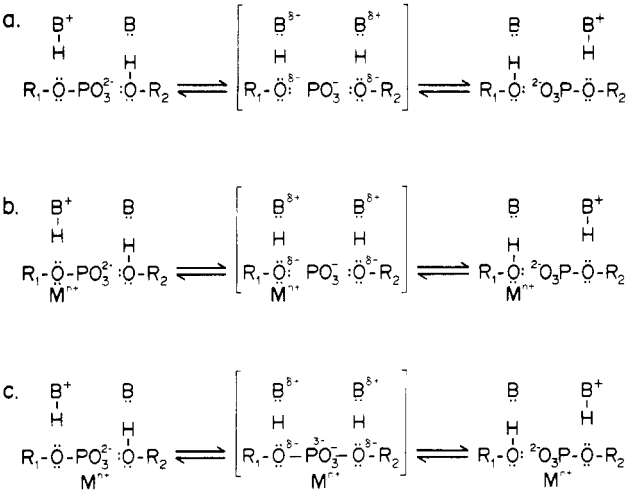
[12] In this study, 1-deoxyglucose 6-phosphate was bound to the Mg^{2+} enzyme [to prevent (PO_3^-) transfer], while Glc-6-P was bound to the Li^+ enzyme.

ligand preference (Li^+ is a hard metal ion; Pearson, 1963, 1965) also is closer to Mg^{2+} than to Cd^{2+}. In spite of these similarities we observe a greater than 10^8-fold difference between $(k_{t1}^+)_{Mg}$ and $(k_{t1}^+)_{Li}$ (Results), viz., a 10^8-fold difference in the ability of these forms of the phosphoenzyme to facilitate (PO_3^-) transfer to bound Glc-1-P. *By default*, this difference, or at least the major part of it, most likely arises from the unit difference in electrostatic charge. In addition, the small effect on the equilibrium E_P·M·Glc-1-P $\rightleftharpoons$ E_D·M·Glc-P_2, produced by replacing Mg^{2+} by Li^+ (see Results) shows that if electrostatic effects are important for (PO_3^-) transfer from the phosphoenzyme to bound substrate, they are approximately *equally important* for the reverse process. Possible restrictions on the role of the metal ion in the bond-breaking/bond-making process facilitated by phosphoglucomutase that might be proscribed on the basis of these observations are considered below.

Significance. Although similar in several of their properties (see above), Mg^{2+} binds to most chelons much more strongly than does Li^+, especially those with a net negative charge, e.g., about 10^6-fold more strongly to $EDTA^{4-}$. A reduced charge on the chelon not only reduces Mg^{2+} binding (by about 10^5-fold for the $EDTA^{3-}/EDTA^{4-}$ pair) but also reduces the ratio of binding constants for Mg^{2+} and Li^+, e.g., in the case of NTA^{3-} to about 10^3-fold (Martel & Smith, 1974). Such equilibrium binding effects could be related to an increased "transition-state binding" (Jencks, 1969, 1975; Wolfenden, 1976, 1987) of Mg^{2+} relative to Li^+ of at least (4×10^8)-fold[12] observed in the first (PO_3^-)-transfer step of the phosphoglucomutase reaction (parentheses, Scheme I). Thus, Mg^{2+} and Li^+ bind to E_P·Glc-1-P and E_D·Glc-P_2 equally, while the Gibbs energy difference between the transition states for (PO_3^-) transfer in the E_P·Mg·Glc-1-P and E_D·Li·Glc-1-P complexes is equivalent to a binding difference for these metal ions in their respective transition states of at least 11.9 kcal/mol.

Of course, part of the apparent transition-state-binding effect, above, could be produced by masked ground-state phenomena. For example, the binding of Mg^{2+} (but not Li^+) to the E_P·Glc-1-P complex might produce an energetically unfavorable conformational change that greatly facilitates (PO_3^-) transfer and also makes the ground-state binding of Mg^{2+} much weaker than otherwise [cf. Jencks (1975)]. Although we cannot state that masked ground-state changes do not contribute to the Li^+/Mg^{2+} rate effect, several lines of evidence make Mg^{2+}-induced ground-state effects seem less likely than a transition-state effect. Thus, Mg^{2+} not only binds to E_P, E_P·Glc-1-P, and E_D·Glc-P_2 with almost equal affinity,[13] but bound substrate produces only a minor effect on Mg^{2+} binding and dissociation rates. Moreover, bound Mg^{2+} produces only a minor effect on the standard chemical potential of the enzymic phosphate group in both E_P and E_P·Glc-1-P (as assessed in terms of equilibria such as E_P·M $\rightleftharpoons$ E_D·M + P_i; Ray & Long, 1976b), as well as on the chemical potential of the phosphates of the (bound) bisphosphate.[14] As a consequence, Mg^{2+} (as well as Li^+) also has only a relatively small effect on the equilibrium constant for (PO_3^-) transfer, viz., on E_P·Glc-1-P $\rightleftharpoons$ E_D·Glc-P_2 (Ray & Long, 1976).

Scheme II: Possible Representations of Bond Breaking and Bond Making in the Transition State for (PO_3^-) Transfer between Hydroxyl Groups Whose Anions Are Highly Basic and Possible Metal Involvements in Such Processes[a]



[a] In the process illustrated in (a), bond breaking in the transition state is essentially complete with little compensating bond formation (see Discussion). In (b), a metal ion is associated with the donor (leaving) group in the forward process (left to right) and with the acceptor (entering) group in the reverse process. In (c), a transition state with a pentacoordinate structure and a metal ion interacting with a peripheral oxygen or oxygens is represented.

But if ground-state differences are minimized, it is even more difficult to rationalize how a simple electrostatic effect such as positioning a metal ion close to a developing negative charge in the transition state could produce a rate effect of the observed magnitude—especially in view of what is known about bond breaking/bond making from solution studies of (PO_3^-) transfer and what has been suggested about the mechanism of the phosphoglucomutase reaction [on the basis of the efficiency of a sulfur analogue of the normal substrate as a (PO_3^-) acceptor (Knight et al., 1984)]: extensive bond breaking in the transition state with little compensating bond making. How a direct electrostatic effect might operate in the phosphoglucomutase reaction is further complicated, relative to most model reactions, by the fact that both donor *and* acceptor oxygens derive from very weakly acidic alcohols and that *two* proton-transfer steps, whose timing is obscure, must be considered in the overall process. In fact, because of the timing of proton transfer, the reaction may well be borderline [between S_N1 and S_N2; cf. Williams (1987)] and thus particularly difficult to deal with. Nevertheless, how the activating metal ion does *not* act in the phosphoglucomutase reaction can be considered (see below) in terms of two extreme cases so as to restrict the ways in which it might act. In both cases a dissociative or S_N1-like mechanism (Jencks, 1981; Williams, 1987; Cullis, 1987), with extensive P–O bond breaking and relatively little bond making in the transition state, as suggested above, is considered. Arguments are based on the conclusion (Rhyu et al., 1985) that the enzymic phosphate in E_P·Li and E_P·Cd, as well as the sugar phosphates

[13] The binding of Mg^{2+} to a specific complex in the equilibrium mixture of complexes E_P·Glc-1-P, E_D·Glc-P_2, and E_P·Glc-6-P cannot be measured directly. However, an approximation which shows that binding is similar for all three complexes can be obtained from the weighted average of the Mg^{2+} dissociation constants for each species at equilibrium [the "isotope exchange constant" for Mg^{2+} (45 μM; Ray et al., 1965)] and the equilibrium distribution of complexes in both the presence and absence of Mg^{2+} (Ray & Long, 1976c): K_d for E_P·Mg·Glc-1-P, 24 μM; for E_D·Mg·Glc-P_2, 66 μM; and for E_P·Mg·Glc-6-P, 20 μM. The relative binding of Mg^{2+} and Li^+ to E_P·Glc-1-P is taken as equal to the relative binding of Mg^{2+} and Li^+ to the equilibrium mixture of monophosphate and bisphosphate complexes of the enzyme, about 1:1 (Ray et al., 1978).

[14] For example, since Mg^{2+} binds to E_D·Glc-1-P almost as well as to E_D·Glc-P_2 [dissociation constants are 300 μM (calculated from constants in Ray et al. (1966)) and 66 μM (footnote 8), respectively], to a first approximation the equilibrium E_D·Glc-P_2 $\rightleftharpoons$ E_D·Glc-1-P + P_i, which defines the chemical potential of the 6-phosphate of bound Glc-P_2, also is relatively independent of Mg^{2+} binding.

in E_C·Cd·Glc-P_2, are dianionic because of the pH dependence of their ^{31}P NMR chemical shifts.

Model studies of simple organic phosphates show that $\partial \log k/\partial pK_{lg}$, or $-\beta_{lg}$, is in the neighborhood of 1 for (PO_3^-) transfer when the reactant is a dianionic phosphate with a *good* leaving group but that $-\beta_{lg}$ approaches zero when the leaving group is strongly basic. The decreased value of $-\beta_{lg}$ is reasonable since in such cases partial protonation of the incipient oxyanion is necessary to assist bond breaking, as is illustrated in Scheme IIa [cf. Hall and Williams (1986)]. Thus, in an enzymic reaction where the substrate binding step juxtaposes the incipient leaving group with a bound metal ion, *and the metal ion serves primarily to lower the effective pK_a of the leaving group*, as in Scheme IIb, a large metal ion induced rate effect is *not* expected when the leaving group has a high pK_a and bond breaking is assisted by partial protonation of that group, other factors being equal [cf. Herschlag and Jencks (1987)]. In such a case, a failure of the monovalent Li^+ to lower the pK_a of the leaving group as efficiently as would Mg^{2+} should not produce nearly as large a difference in (PO_3^-)-transfer rate as was observed.

An alternative possibility is that metal ion coordination lowers the effective pK_a of the leaving group sufficiently that bond breaking in an S_N1-like process does not require assistance from proton transfer and that the active-site serine leaves as a metal-coordinated anion. This possibility also seems unlikely, even though in such a case a large decrease in rate would be expected from a large increase in effective pK_a of the leaving group produced by employing a metal ion that is a less effective Lewis acid, e.g., substituting Li^+ for Mg^{2+}. But, when the leaving group departs as an anion, as in $ROPO_3^{2-} + H_2O \rightarrow RO^- + HPO_4^{2-} + H^+$, a change in the pK_a of the leaving group produces an even larger change in the equilibrium constant for (PO_3^-) transfer. For example, $\partial \log K_e/\partial pK_{lg}$ for the above process is −1.35 (Bourne & Williams, 1984) while $\partial \log k/\partial pK_{lg}$ is in the neighborhood of −1 (cf. Cullis, 1987).[15] Hence, *on the sole basis of differences in induced pK_a*, other factors being equal, replacing bound Mg^{2+} by Li^+ should effect the equilibrium constant for the transfer process to an even larger extent than its effect on the forward rate constant (k_{t1}^+). But while the observed rate effect for Li^+/Mg^{2+} replacement is large [(4×10^8)-fold], the equilibrium effect is negligible (about 2-fold).

On the other hand, if the P–O bond-breaking/bond-making process in the phosphoglucomutase reaction were much more nearly synchronized or S_N2-like than in most model reactions, substantial formation of the new P–O bond and much less extensive breaking of the old P–O bond would characterize the transition state. In such a case, charge density in general and charge density particularly at the peripheral oxygens should increase in the transition state. An increase in charge on the peripheral oxygens also might be coupled with increased electrostatic interactions in a manner analogous to the way in which the serine proteases use an oxyanion binding pocket (Kraut, 1977), although no evidence for a mechanism of this type has been obtained from model studies [cf. Hershlag and Jencks (1987)]. Whether such a mechanism can provide a reasonable rationale for the efficiency of (PO_3^-) transfer exhibited by the Mg^{2+} form of phosphoglucomutase, as well as the enormous differential rate effect observed when Li^+ is substituted for bound Mg^{2+}, must wait further studies in this system.

[15] The absolute value of β is expected to be greater for the overall equilibrium than for the forward rate constant since $\delta \log K_e/\delta pK_{lg} = -(\beta_{lg} + \beta_n)$, where β_n refers to the effect of the altered pK_a on the ability of the leaving group to act as the nucleophile in the *reverse* transfer process.

Appendix 1

Comparison of Saturation-Transfer Rates with the Steady-State Catalytic Constant: Cd(II) Enzyme. Minimal estimates for the rate constants k_{t1}^+ and k_{t6}^+ (eq 4 and 5), the measured values of k_{-1}^{app} and k_{-6}^{app} (eq 1) for saturation transfer, plus the equilibrium concentration of the various intermediates (Ray & Long, 1976b) can be used to restrict allowed values for the steady-state catalytic constants k_{cat}^f and k_{cat}^r in the forward and reverse reactions, respectively. The process is illustrated for $1/k_{cat}^f$, which can be expressed as a series of terms, each related to the contribution made by a transition state between E_P·Cd·Glc-1-P and E_P·Cd + Glc-6-P to the effective energy barrier for the overall process [cf. eq 39 and 40 of Ray (1983)]:

$$1/k_{cat}^f = 1/k_{t1}^+ + (1/k_{t6}^-)(1 + k_{t1}^-/k_{t1}^+) + (1/k_{d6})[1 + (k_{t6}^+/k_{t6}^-)(1 + k_{t1}^-/k_{t1}^+)] \quad (8)$$

(The rate constants in eq 8 are defined in eq 4 and 5.) To transform eq 8 into eq 16, various factors and terms are replaced by three types of parameters: C_1 and C_6 are equilibrium constants evaluated previously (Ray & Long, 1976c); k_{-1}^{app} and k_{-6}^{app} are rate constants (see eq 1) evaluated in the accompanying paper; $(1 + p_1)$ and $(1 + p_6)$ are steady-state partition coefficients whose values have not been established.

$$C_1 = k_{t1}^+/k_{t1}^- = (E_D\text{·Cd·Glc-}P_2)/(E_P\text{·Cd·Glc-1-P}) \quad (9)$$

$$C_6 = k_{t6}^+/k_{t6}^- = (E_D\text{·Cd·Glc-}P_2)/(E_P\text{·Cd·Glc-6-P}) \quad (10)$$

$$k_{t1}^+ = k_{-1}^{app}(1 + p_1)C_1 \quad (11)$$

$$k_{t6}^- = k_{-6}^{app}(1 + p_6) \quad (12)$$

$$k_{d6} = k_{-6}^{app}(1 + 1/p_6)C_6 \quad (13)$$

$$p_1 = k_{t1}^+/k_{d1} \quad (14)$$

$$p_6 = k_{t6}^+/k_{d6} \quad (15)$$

Substituting eq 9–15 into eq 8 gives

$$1/k_{cat}^f = C_1^{-1}[k_{-1}^{app}(1 + p_1)]^{-1} + (1 + C_1^{-1}) \times [k_{-6}^{app}(1 + p_6)]^{-1} + (1 + C_1^{-1} + C_6^{-1})[k_{-6}^{app}(1 + p_6^{-1})]^{-1} \quad (16)$$

By using the values below, eq 16 becomes

$$\frac{1}{k_{cat}^f} = \frac{1}{k_{-6}^{app}}\left[\frac{0.03}{1 + p_1} + \frac{1.04}{1 + p_6} + \frac{1.11}{1 + 1/p_6}\right] \quad (17)$$

where $C_1 = 28.3$, $C_6 = 14.2$, $k_{-1}^{app} = 12\ s^{-1}$, and $k_{-6}^{app} = 11\ s^{-1}$ [see the accompanying paper, Post et al. (1989)]. Thus, because of the relatively large equilibrium constant for E_P·Cd·Glc-1-P $\rightleftharpoons$ E_D·Cd·Glc-P_2, the transition state for the process in eq 9, above (first term on right-hand side of eq 17), makes a negligible contribution to the effective energy barrier for k_{cat}^f. Hence

$$k_{cat}^f \approx \frac{k_{-6}^{app}(1 + p_6)}{1.04 + 1.11p_6} \quad (18)$$

which gives rise to the restriction on k_{cat}^f shown in eq 3 under Results.

An analogous process for the reverse reaction provides the following relationship:

$$\frac{1}{k_{cat}^{r}} = \frac{1}{k_{-1}^{app}}\left[\frac{0.08}{1 + p_6} + \frac{1.07}{1 + p_1} + \frac{1.11}{1 + 1/p_1}\right] \quad (19)$$

Considerations analogous to those above produce the relationship

$$10.8\ s^{-1} \leq k_{cat}^{r} \leq 11.2\ s^{-1}$$

The range of values for the steady-state parameters in initial velocity studies, k_{cat}^{f} and k_{cat}^{r}, calculated in this manner are in good agreement with the more directly calculated value for the steady-state rate constant describing saturation transfer at chemical equilibrium, since

$$k_{tr}^{f} = \tfrac{1}{2}(1/k_{cat}^{f} + 1/k_{cat}^{r})^{-1} \quad (20)$$

Thus, the range of values for k_{tr}^{f} calculated with the above values of k_{cat}^{f} and k_{cat}^{r} is 2.5–2.6 s^{-1}, while the value calculated more directly from saturation-transfer measurements (which eliminate any dependency of k_{tr}^{f} on assumed values of p_1 and p_6; see eq 2) is 2.4 s^{-1}.

Appendix 2

Comparison of Net Rate Constants for Phosphate Transfer by the Mg^{2+} Enzyme. Values of the net rate constants for the processes in eq 21 were approximated in the following way.

$$E_P{\cdot}Mg + Glc\text{-}1\text{-}P \xleftarrow{k_{-1}^{app}} E_D{\cdot}Mg{\cdot}Glc\text{-}P_2 \xrightarrow{k_{-6}^{app}} E_P{\cdot}Mg\ Glc\text{-}6\text{-}P \quad (21)$$

Equation 16 for $1/k_{cat}^{f}$ and the analogous equation for $1/k_{cat}^{r}$ comprise two simultaneous equations with four unknowns: k_{-1}^{app}, k_{-6}^{app}, p_1, and p_6. Hence, an expression for k_{-6}^{app} can be obtained in which the only unknowns are p_1 and p_6 and similarly for k_{-1}^{app}. In the latter case the expression takes the form

$$k_{-1}^{app} = \frac{a(p_1 + b)}{(p_1 + 1)(p_6 + c)} + \frac{d(p_1 + e)}{(p_1 + 1)} \quad (22)$$

Although k_{t1}^{+} and k_{d1}, which determine p_1, can assume a wide range of values and thus produce a wide variation in p (see eq 14), the values of k_{-1}^{app}, $k_{t1}^{-}/(1 + p_1)$, produced by such a variation are much more severely restricted, as are the values of the corresponding equation for k_{-6}^{app}.

In theory, values of both p_1 and p_6 can range from very small to very large. But in the phosphoglucomutase reaction the two half-reactions are more or less mirror images of each other, and it seems reasonable that the partition of $E_P{\cdot}Mg{\cdot}Glc\text{-}1\text{-}P$ between (PO_3^-) transfer and dissociation, p_1, should not be vastly different from the analogous partition of $E_P{\cdot}Mg{\cdot}Glc\text{-}6\text{-}P$, p_6, especially since the equilibrium binding of Glc-1-P and α-Glc-6-P, as well as k_{cat}^{f} and k_{cat}^{r}, differ only by a fewfold. Hence, in a survey of possible values of k_{-1}^{app} and k_{-6}^{app}, we assume that $0.1 \leq p_1/p_6 \leq 10$, but consider individual values of p_1 and p_6 from very small to very large. With this restriction a comprehensive survey of possible values shows that 340 $s^{-1} \leq k_{-1}^{app} \leq 400\ s^{-1}$ ($p_1 = 0.17$; $p_6 = 1.7$) and 960 $s^{-1} \leq k_{-6}^{app} \leq 1660\ s^{-1}$ ($p_1 = 3$; $p_6 = 0.3$).

Appendix 3

Comparison of Rate Constants for Phosphate Transfer by the Mg(II) and Cd(II) Enzymes. An expression for the rate constant k_{t1}^{+} for transfer of the (PO_3^-) group of the enzyme to the 6-position of bound Glc-1-P (eq 5) for both metal ion forms (subscript M) of the enzyme can be obtained from eq 9 and 11 plus known values of C_1 for Mg^{2+} and Cd^{2+} forms of the enzyme (see Appendix 1). The ratio of these expressions is

$$\frac{(k_{t1}^{+})_{Mg}}{(k_{t1}^{+})_{Cd}} \approx 7\frac{1 + (p_1)_{Mg}}{1 + (p_1)_{Cd}} \quad (23)$$

The (PO_3^-)-transfer rate for Mg^{2+} will be no more than an order of magnitude larger than for Cd^{2+} if any of the following relationships hold.

(a) The partition of $E_P{\cdot}M{\cdot}Glc\text{-}1\text{-}P$ between (PO_3^-) transfer (k_{t1}^{+}) and dissociation of Glc-1-P (k_{d1}) is small for both Mg^{2+} and Cd^{2+}; i.e., in the process, $E_D{\cdot}Mg{\cdot}Glc\text{-}P_2 \rightarrow E_P{\cdot}Mg$ + Glc-1-P, (PO_3^-) transfer is solely rate limiting. This is possible, although it would require a value of about $1 \times 10^9\ M^{-1}\ s^{-1}$ for $(k_{a1})_{Mg}$, the bimolecular rate constant for binding of Glc-1-P.

(b) The values of both $(p_1)_{Mg}$ and $(p_1)_{Cd}$ are large and essentially the same; i.e., product dissociation in the above process is solely rate limiting and $(k_{d1})_{Mg} \approx (k_{d1})_{Cd}$. The latter possibility is in accord with, but certainly not required by, the observation that the *equilibrium* binding of Glc-1-P to $E_P{\cdot}M$ is essentially independent of the identity of M^{2+}, not only for Mg^{2+} and Cd^{2+} but also for Zn^{2+}, Co^{2+}, and Mn^{2+} (Ray & Long, 1976c).

(c) The value of $(p_1)_{Mg}$ is larger than $(p_1)_{Cd}$; i.e., product dissociation is more nearly rate limiting for the less efficient metal ion form of the enzyme. Although one normally expects product dissociation to be more nearly rate limiting for a good substrate than for a poor one, such a pattern does not necessarily hold when reduced efficiency is produced by a change in the identity of the activating metal ion. For example, in the present system the rate constant for dissociation of $Glc\text{-}P_2$ from $E_D{\cdot}M{\cdot}Glc\text{-}P_2$ is decreased by about 0.01 by the substitution of Cd^{2+} for Mg^{2+} (see Results) without a significant decrease in the *equilibrium* binding constant of $Glc\text{-}P_2$ (Ray & Long, 1976b); i.e., the association rate is similarly affected. Similarly, $E_P{\cdot}M{\cdot}Glc\text{-}1\text{-}P$ may well dissociate more slowly when M = Cd than when M = Mg, so that k_d dominates the expression for $(k_{t1}^{+})_{Cd}$ but not that for $(k_{t1}^{+})_{Mg}$.

Only if product dissociation from the Mg^{2+} enzyme is more nearly rate limiting than from the Cd^{2+} enzyme, viz., if $(p_1)_{Mg} > (p_1)_{Cd}$, would (PO_3^-)-transfer efficiency involving the 6-hydroxyl group of bound Glc-1-P be more than an order of magnitude greater for the Mg^{2+} then the Cd^{2+} enzyme.

Registry No. Glc-1-P, 59-56-3; Glc-6-P, 15209-11-7; Cd^{2+}, 22537-48-0; Mg^{2+}, 22537-22-0; Li^{+}, 17341-24-1; phosphoglucomutase, 9001-81-4.

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Rat Brain Has the $\alpha 3$ Form of the (Na^+,K^+)ATPase†

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ABSTRACT: Multiple forms of the catalytic subunit of the (Na^+,K^+)ATPase have been identified in rat brain. While two of them ($\alpha 1$ and $\alpha 2$) have been well characterized, the third form ($\alpha 3$) of these catalytic subunits only recently has been described by cDNA cloning; the corresponding polypeptide has not been isolated. In this paper it is shown that rat brain contains the $\alpha 3$ chain. The catalytic subunits of the (Na^+,K^+)ATPase from rat brain axolemma were purified by SDS–PAGE and subjected to formic acid cleavage. Amino acid sequence analysis of the resulting fragments revealed that axolemma has the $\alpha 3$ form of the catalytic subunit. In addition, $\alpha 3$-specific antiserum was raised in rabbits immunized with a synthetic peptide. Immunoblotting with this antiserum revealed that the $\alpha 3$ form of the (Na^+,K^+)ATPase is present also in whole brain microsomes. In SDS–PAGE, the mobilities of the three catalytic subunits of brain (Na^+,K^+)ATPase follow the order $\alpha 1 > \alpha 2 > \alpha 3$. Determination of the ouabain-inhibitable ATPase activity indicates that if the $\alpha 3$ form of the (Na^+,K^+)ATPase is able to hydrolyze ATP, it is present in a form of the enzyme with a high affinity for this cardiac glycoside and is similar to the $\alpha 2$ form in this respect.

(Na^+,K^+)ATPase [sodium and potassium ion activated ATP phosphohydrolase (EC 3.6.1.3)] is the enzyme in the plasma membrane of animal cells that establishes the sodium and potassium ion gradients across the plasma membrane at the expense of ATP. The enzyme is composed of two subunits, a large catalytic subunit of M_r 112000 (M_r 100000 by SDS–PAGE) and a smaller glycoprotein subunit of M_r 50000 of unknown function in a 1:1 stoichiometry (Cantley, 1986; Jorgensen, 1982). Biochemical studies revealed that there are two forms of the large subunit, which are called $\alpha 1$ and $\alpha 2$ chains (Sweadner, 1979; Schellenberg et al., 1981; Matsuda et al., 1984; Lytton et al., 1985). [In this paper, the α, $\alpha+$,

†This work was supported by NIH Grant HL 08893 to G.G.

0006-2960/89/0428-0569$01.50/0