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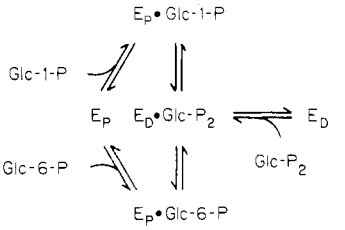
Enzyme-Bound Intermediates in the Conversion of Glucose 1-Phosphate to Glucose 6-Phosphate by Phosphoglucomutase. Phosphorus NMR Studies†

Gyung Ihm Rhyu, William J. Ray, Jr., and John L. Markley*

ABSTRACT: The interactions between metal ions and the phospho form of rabbit muscle phosphoglucomutase (EC 2.7.5.1) have been studied by ^{31}P NMR. In the metal-free enzyme, the width at half-height of the ^{31}P signal is 10 ± 1 Hz at 81 MHz. In enzyme-Cd^{2+} complexes, the presence of spin-spin coupling with $^{113}Cd^{2+}$ ($J_{^{113}Cd\text{-}O\text{-}^{31}P}$ = 16 Hz) and the absence of such splitting with $^{114}Cd^{2+}$ indicate that Cd^{2+} binds directly to the enzymic phosphate. The absence of detectable splitting on transfer of the phosphate group to the acceptor hydroxyl group of bound glucose 1-phosphate, or glucose 6-phosphate (to give the $^{113}Cd^{2+}$ complex of the dephosphoenzyme and glucose 1,6-bisphosphate), indicates that this transfer eliminates the direct metal ion-phosphate interaction. The enzyme-catalyzed reaction is slowed sufficiently by the addition of Li^+ to allow studies of three discrete intermediate complexes by NMR techniques: glucose 1-phosphate bound to the phosphoenzyme, glucose 1,6-bisphosphate bound to the dephosphoenzyme (only one complex of this type was observed), and glucose 6-phosphate bound to the phosphoenzyme. Complete assignments of the phosphorus resonances of these intermediates have been made by labeling the phosphate ester group of either the enzyme or the sugar with ^{17}O and by NMR polarization transfer studies. The effect of bound metal ions on these resonances also was determined. A ^{31}P NMR titration study of the Li^+ complex of the dephosphoenzyme with glucose 1,6-bisphosphate and a ^{31}P NMR polarization transfer experiment indicate that β-glucose 1,6-bisphosphate binds to the enzyme less tightly than α-glucose 1,6-bisphosphate. The relative mobilities and solvent accessibility of the phosphate ester groups in the free phosphoenzyme and the above complexes have been investigated by measurements of ^{31}P NMR line widths as a function of magnetic field strength, nuclear Overhauser effects, and spin-lattice relaxation times in ^1H_2O and ^2H_2O. The serine phosphate in the free phosphoenzyme is highly accessible to solvent molecules. Binding of Li^+ does not affect this solvent accessibility. In a ternary complex (phosphoenzyme, glucose 6-phosphate, metal ion), the enzymic phosphate becomes much less accessible and possibly inaccessible to solvent. The phosphate ester group of the substrate also is partially immobilized, but not to as great an extent as the enzymic phosphate. An analysis was conducted of contributions to the line width of the ^{31}P NMR signal of the phosphoenzyme provided by various relaxation mechanisms, including relaxation induced by ^{17}O substitution.

Phosphoglucomutase catalyzes the interconversion of glucose 1-phosphate and glucose 6-phosphate via a type of ping-pong reaction sequence in which glucose 1,6-bisphosphate is considered as both the first product and second substrate (Ray & Peck, 1972). The active form of the enzyme is phosphorylated at Ser-116 (Ray et al., 1983). The sequence of phosphate transfer steps that occur during a catalytic cycle is shown below.[1]



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Since there is only one attachment site for the phosphate group in the phosphoenzyme, both phosphate groups in the E_D·Glc-P_2 complex must be capable of transfer to the same position on the dephosphoenzyme—by means of either a "minimal motion" or an "exchange" mechanism (Ray et al., 1973). The enzyme requires Mg^{2+} for maximal activity; but other metal ions can be substituted for Mg^{2+}, although these yield altered activities and different equilibrium ratios of intermediates (Ray & Peck, 1972; Ray & Long, 1976). Because the various activating metal ions dissociate much more slowly than does the product, the enzyme behaves in some respects like a metalloenzyme. For studies involving one or only a few cycles, it is appropriate to replace E_P in the above scheme by E_P·M, where M is an activating bivalent metal ion (Ray & Peck, 1972). On the other hand, in studies involving much longer time intervals, the enzyme must be treated as a metal-activated enzyme.

Earlier NMR studies of phosphoglucomutase dealt with relaxation effects produced by paramagnetic metal ion activators: relaxation of water protons by Mn^{2+} (Ray & Mildvan, 1970); relaxation of the $-PO_3^{2-}$ groups of substrate analogues by Mn^{2+} (Ray & Mildvan, 1973); and relaxation of the serine phosphate of the phosphoenzyme by Ni^{2+} (Ray et al., 1977). The phosphate binding experiments with the Mn^{2+} form of the enzyme (Ray & Mildvan, 1973) suggested that in the dephosphoenzyme a strong phosphate binding site is located 10–11 Å away from the metal ion and that a weak phosphate binding site is much closer, about 5 Å away. In the Ni^{2+}–phosphoenzyme experiment, an estimate for the metal to phosphorus distance of 4–5 Å was obtained. Thus, the strong phosphate binding site clearly lies outside the coordination sphere of the metal ion while the weak phosphate binding site of the dephosphoenzyme, which appears to be the attachment site for the enzymic phosphate in the phosphoenzyme, is much closer to the metal ion. For a second-sphere complex with a water molecule intervening between the phosphate and metal ion, the phosphorus–metal distance is expected to be about 4.5–6 Å, while for direct coordination the E_P–metal distance should be about 3 Å. On the basis of the Mn^{2+} and Ni^{2+} binding experiments, the above authors concluded that the metal–phosphate interaction in E_P·M involves a second-sphere complex. Because serious problems can arise in attempts to assess metal to phosphorus distances in first- and second-sphere complexes by the use of paramagnetic metal ions, we have reinvestigated this interaction in complexes involving nonparamagnetic metal ions, again by using ^{31}P NMR spectroscopy. The results indicate that although Cd^{2+} binds to E_P in the first coordination sphere, the metal–E_P interaction is altered when this phosphate is transferred to the bound substrate.

Experimental Procedures

Enzymes and Chemicals. The phospho and dephospho forms of phosphoglucomutase were prepared according to the procedure of Ma & Ray (1980) except that Tris-HCl was used instead of Tris–sulfate. Metal-free phosphoglucomutase was obtained by multiple dialysis steps, first against EDTA in 20 mM Tris-HCl buffer at pH 7.5 and later against the buffer alone. The specific activity of the enzyme was at least 730 units mg^{-1}. The phosphoenzyme contained less than 10% dephosphoenzyme; the dephosphoenzyme was free of phosphoenzyme unless indicated. Enzyme concentrations were determined spectrophotometrically by using $E_{278} = 0.70$ and $M_r = 62\,000$ (Ray et al., 1983). Glc-1-P and Glc-6-P were obtained from Sigma Chemical Co. and used without further purification. α,β-Glc-P_2 (predominately the α anomer) was synthesized from Glc-6-P by phosphorylating the tetraacetate with anhydrous phosphoric acid in a procedure similar to that used by Hanna & Mendicino (1970). Pure α-Glc-P_2 was prepared according to Ray & Roscelli (1964). Other chemicals were purchased from the following sources: DL-glyceraldehyde 3-phosphate (diethylacetal monobarium salt) from Sigma; $^6Li_2CO_3$, 96.3 atom % ^{113}CdO, and 98.55 atom % ^{114}CdO from Oak Ridge National Laboratory; 50%-enriched $H_2{}^{17}O$ from Monsanto Research Corp.; ultrapure Tris from Mann Co. Additional reagents were of the highest purity available.

Preparation of ^{17}O-Labeled Sugar Phosphates. ^{17}O-Labeled orthophosphate was prepared by a modification of the method of Risley & Van Etten (1978). A 25-μL pellet of $H_2{}^{17}O$ was made by freezing in liquid nitrogen. The pellet was added to 20–40 mg of PCl_5 in a small, stoppered test tube cooled in a dry ice bath. The tube was allowed to warm to room temperature to complete the reaction. The resulting solution was frozen in a dry ice bath. The test tube containing the frozen solution was placed in a vacuum thimble containing NaOH pellets. The thimble was evacuated and left overnight at 30 °C. Then 4 equiv of imidazole was added per mol of PCl_5 initially present.

^{17}O-Labeled glucose phosphates were prepared by a modification of the method of Ray & Koshland (1963). Unlabeled and ^{17}O-labeled GA-1,3-P_2 were synthesized from glyceraldehyde 3-phosphate by the procedure of Rose (1968) as modified by Ma & Ray (1980) and stored as frozen pellets in liquid nitrogen.

Phosphorylation of Phosphoglucomutase. To the concentrated dephosphoenzyme (60–100 mg mL^{-1}) was added 1 equiv of GA-1,3-P_2 ($\sim$ 1 mM) in glycylglycine buffer at pH 7.5 containing 0.12 M NaCl. After 1 h, the protein solution was dialyzed in a collodion thimble (Schleicher & Schuell) against 20 mM Tris–1 mM EDTA at pH 7.5 under pressure ($\sim$600 mmHg) to concentrate the partially phosphorylated enzyme solution. This procedure was repeated twice. After the third addition, the enzyme solution was transferred to dialysis tubing and dialyzed against 20 mM Tris buffer at pH 7.5, with or without 5 mM EDTA, depending on which metal ion was to be added subsequently. Between 83 and 90% of the enzyme was phosphorylated in this procedure. The activity of the enzyme was assayed before and after phosphorylation; an activity decrease of less than 10% was observed.

NMR Spectroscopy. ^{31}P NMR spectra were obtained in the Fourier-transform mode by using Nicolet 4.7 T, 7.1 T, and 11.1 T spectrometers, operating at 80.99 MHz, 121.47 MHz, and 190.23 MHz, respectively. A Varian FT-80 spectrometer was used for the line-width measurement at 32.4 MHz. Sample solutions contained 10% ^2H_2O for the ^2H field-frequency lock except as noted for some NOE and T_1 studies. For all spectra, 60° pulses were used (the 90° pulse was 30 μs), and coherent broad-band proton decoupling was applied

[1] Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, α,β-D-glucose 6-phosphate; E_P and E_D, phospho and dephospho forms, respectively, of rabbit muscle phosphoglucomutase; Glc-P_2, D-glucose 1,6-bisphosphate; M, metal ion; GA-1,3-P_2, D-1,3-bis(phosphoglyceric acid); EDTA, ethylenediaminetetraacetic acid; CDTA, *trans*-cyclohexyldiaminetetraacetic acid; NOE, nuclear Overhauser effect ($1 + \eta$); DD, dipole–dipole; CSA, chemical shift anisotropy; ppm, parts per million; $[^{17}O]E_P$, mixture of $[^{17}O_3,{}^{16}O_1]E_P$, $[^{17}O_2,{}^{16}O_2]E_P$, $[^{17}O_1,{}^{16}O_3]E_P$, and $[^{16}O_4]E_P$; $[^{17}O]$Glc-1-P, mixture of $[^{17}O_4]$Glc-1-P, $[^{17}O_3,{}^{16}O_1]$Glc-1-P, $[^{17}O_2,{}^{16}O_2]$Glc-1-P, $[^{17}O_1,{}^{16}O_3]$Glc-1-P, and $[^{16}O_4]$Glc-1-P; $[^{17}O]$Glc-6-P, mixture of $[^{17}O_3,{}^{16}O_1]$Glc-6-P, $[^{17}O_2,{}^{16}O_2]$Glc-6-P, $[^{17}O_1,{}^{16}O_3]$Glc-6-P, and $[^{16}O_4]$Glc-6-P; OAc, acetate; Tris, tris(hydroxymethyl)aminomethane.

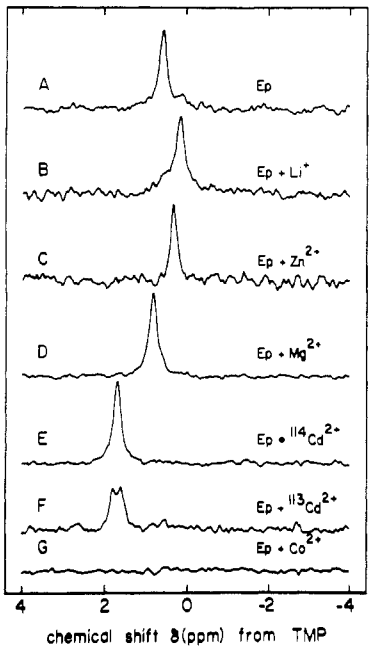

FIGURE 1: Effect of metal ion binding on the ^{31}P NMR spectrum of E_P in 40 mM Tris-HCl, pH 7.5, in 90% ^1H_2O: (A) 1.3 mM E_P, no metal ion; (B) 0.9 mM E_P, 1 mM CDTA, 10 mM ^7LiCl; (C) 0.85 mM E_P, 0.85 mM $Zn(OAc)_2$; (D) 0.9 mM E_P, 1.5 mM $Mg(NO_3)_2$; (E) 1.3 mM E_P, 1.3 mM $^{114}Cd(OAc)_2$; (F) 1.3 mM E_P, 1.3 mM $^{113}Cd(OAc)_2$; (G) 1.3 mM E_P, 1.3 mM $Co(OAc)_2$.

with high power (1.5 W) during acquisition and low power (0.5 W) not during acquisition. The pulse repetition time was 3.4 s. The spectral width was 10 KHz, and the line-broadening factor used in exponential apodizations was 5 Hz; 8K data points were used to digitize the spectra. NMR measurements were made at 20 ± 1 °C by using a sample volume of 2.5–2.8 mL contained in a 20-mm sphere inserted into a sample tube (20-mm outside diameter) filled with water; 1024 or 4096 transients were accumulated. The chemical shifts are given relative to trimethyl phosphate (the actual internal reference was trimethylphosphine oxide, which was assigned a chemical shift of 50.092 ppm). Spin–lattice (T_1) relaxation times were obtained by the inversion–recovery (Vold et al., 1968) and saturation–recovery (Markley et al., 1971) methods. Dynamic nuclear Overhauser effects were measured by the gated proton-decoupling technique (Freeman et al., 1972). Polarization transfer by selective peak inversion (Dahlquist et al., 1975) was accomplished by a 180 ° soft pulse (18 ms) followed by a 60° nonselective pulse (20 μs) with a short delay time (100 μs) between pulses. Other experimental details are given in the figure legends.

Results and Discussion

Effect of Metal Ion Binding on the ^{31}P NMR Spectrum of Phosphoglucomutase. ^{31}P NMR spectra of E_P were obtained in the absence (Figure 1A) and presence (Figure 1B–G) of various metal ions. The signal for the metal-free enzyme at pH 7.5 occurs at 0.6 ppm and has a line width of about 10 Hz at 81 MHz.[2] Binding of 1 equiv of Co^{2+} (Figure 1G) causes the disappearance of the ^{31}P NMR signal as is expected from previous studies (Ray et al., 1977). Splitting of the ^{31}P NMR peak by ^{113}Cd but not by ^{114}Cd (Figure 1E,F) clearly indicates that Cd^{2+} binds directly to the enzymic phosphate group. The spin–spin coupling (16 Hz) disappears, or decreases to an insignificant value relative to the line width, on addition of a substrate, either Glc-1-P or Glc-6-P (spectra not shown). Since the predominant species under these conditions is the E_D·Cd·Glc-P_2 complex (Ray & Long, 1976), the phosphate ester group may be forced out of the first coordination sphere of bound Cd^{2+} in this complex as was suggested earlier on the basis of the lack of a metal ion specific difference in the binding of the bisphosphate to E_D·M (Ray & Long, 1976); if not, distortion of the $^{113}Cd^{2+}$ ion could lead to its rapid relaxation and loss of the coupling with the ^{31}P nucleus. However, the latter possibility is ruled out by ^{113}Cd NMR studies which show that the line width of ^{113}Cd is roughly the same in E_P·^{113}Cd and E_D·^{113}Cd·Glc-P_2 (G. I. Rhyu, unpublished results). Whether the phosphate transfer step, per se, or a structural change accompanying binding of the substrate interrupts the Cd^{2+}–phosphate ester interaction is not known.

Binding of the quadrupolar nuclei, $^7Li^+$ (Figure 1B) or $^6Li^+$ (spectrum not shown), changes the chemical shift of the ^{31}P resonance of E_P but does not broaden it.[3] The lack of broadening could be explained by rapid relaxation of the lithium nuclei, binding of Li^+ at a site different from the Cd^{2+} binding site and more distant from the phosphate, or the smaller ionic radius of Li^+ (0.68 Å) compared to that of Cd^{2+} (0.95 Å) which may prevent it from coordinating directly to the enzymic phosphate. The present data do not permit us to distinguish among these possibilities.

The rate of interconversion of free E_P and E_P·M is slow on the NMR time scale as is shown by the following observations: (1) In the presence of less than a stoichiometric amount of the bivalent metal ions Mg^{2+} or Cd^{2+}, separate signals are obtained by ^{31}P NMR for both the metal-bound and metal-free forms of the enzyme. (2) In the presence of 0.5 equiv of Co^{2+}, the intensity of the ^{31}P NMR signal decreases by about 50% without changing the line width of the remaining free E_P (data not shown). (3) In a titration of E_P with $^7Li^+$, which binds much less tightly than bivalent metal ions (Ray et al., 1978), slight broadening of the signals from both E_P and E_P·Li is observed at an intermediate enzyme/metal ion ratio (data not shown). Previous studies have demonstrated that the rates of dissociation of bivalent metals (Mg^{2+}, Co^{2+}, and Zn^{2+}) from E_P·M are relatively slow (Ray, 1969).

Effect of ^{17}O Labeling and $^{113}Cd^{2+}$ Binding on the ^{31}P NMR Line Width. An ^{17}O directly bonded to ^{31}P can provide a rapid phosphorus relaxation mechanism which usually leads to extreme broadening of the signal from a mobile, ^{17}O-labeled phosphate. Decreasing the mobility of the phosphate group, e.g., by binding the group rigidly to a protein, partially decouples the rapid relaxation mechanism and results in sharpening of the ^{31}P signal (Tsai, 1982). To assess the effect of ^{17}O labeling on the ^{31}P resonance of phosphoglucomutase, identical samples of the dephosphoenzyme were phosphorylated by reaction with either labeled or unlabeled GA-1,3-P_2. In the former case, a mixture of isotopically labeled species is produced with ^{17}O statistically distributed among the nonbridging oxygens.[4] The line width and intensity data are summarized in Table I. The total intensity of the ^{31}P NMR peaks from the phosphoenzyme produced from 50% ^{17}O-la-

[2] The high-field shoulder on the E_P resonance is sensitive to pH and salt concentration.

[3] The low-field shoulder on the E_P·Li resonance and the high-field shoulder on the E_P·Mg resonance are caused by residual metal-free enzyme.

[4] This estimate is based on the concentration of ^{17}O initially present in the H_2O used to prepare GA-1,3-P_2, the known mechanism of the phosphate transfer process, and the assumption that no exchange of phosphate oxygens with the solvent occurred during the subsequent separation and storage of the product or during its incorporation into [^{17}O]E_P. The expected isotopic distribution is the following: $^{16}O_4$, 12.5%; $^{16}O_3$,$^{17}O_1$, 37.5%; $^{16}O_2$,$^{17}O_2$, 37.5%; and $^{16}O_2$,$^{17}O_3$, 12.5%.

Table I: Line Widths and Areas of the ^{31}P NMR Resonance of Phosphoglucomutase and the $^{113}Cd^{2+}$ Complexes at 81 MHz[a]

spectrum	line	species	line width (Hz)	% area
Figure 2C	1	E_P	12.5[d]	86
	2	*c*	34.6	14
Figure 2F	1	E_P	14.0	19
	2	$[^{17}O]E_P$	69.2	63
	3	*c*	22.8	4
	missing intensity		*e*	14[f]
Figure 1F	1	$E_P{\cdot}^{113}Cd$	11.3	51
	2	$E_P{\cdot}^{113}Cd$	11.3	49
b	1	$E_P{\cdot}^{113}Cd$	11.3	7.8
	2	$E_P{\cdot}^{113}Cd$	11.3	8.2
	3	$[^{17}O]E_P{\cdot}^{113}Cd$	85.6	84
	missing intensity		*e*	5[f]

[a] Samples were in 20 mM Tris-HCl, pH 7.5 at 20 °C. [b] Spectrum not shown. [c] Uncharacterized minor form of E_P. [d] In the majority of experiments, this peak had a line width of 10 ± 1 Hz. [e] Too broad to be detected. [f] Percentage determined by difference.

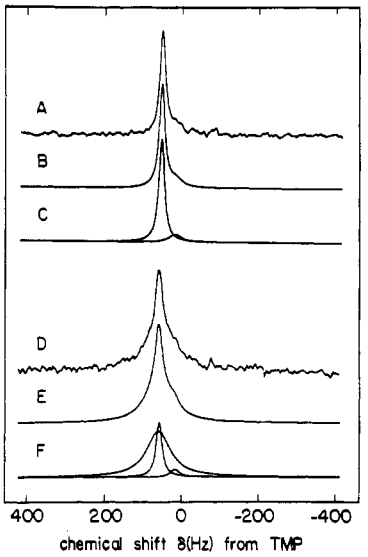

FIGURE 2: Analysis of ^{31}P NMR spectra of E_P and $[^{17}O]E_P$. (A) Experimental spectrum of E_P, 1.28 mM enzyme (1.09 mM E_P form) in 20 mM Tris-HCl, pH 7.5; (B) best-fit spectrum of E_P involving two peaks; (C) individual peaks in (B); (D) experimental spectrum of 1.09 mM $[^{17}O]E_P$ (total enzyme, 1.32 mM); (E) best-fit spectrum of $[^{17}O]E_P$ involving three peaks; (F) individual peaks in (E).

beled GA-1,3-P_2 is about 14% less than that for E_P produced from unlabeled GA-1,3-P_2. Since $[^{17}O_3,^{16}O_1]E_P$ is expected to account for about 13% of the phosphoenzyme in the former sample, its ^{31}P NMR signal probably is broadened beyond detection. The remaining intensity in the labeled system can be decomposed (Figure 2E) into a sharp peak (19% intensity relative to unlabeled E_P) and a broad peak (63% intensity). The sharp peak, whose line width is similar to that of unlabeled E_P (here, about 12.5 Hz), appears to arise from $[^{16}O_4]E_P$ (expected intensity about 13%). The broad component of the peak in Figure 2E (apparent width about 69 Hz) is expected to be a composite of broad peaks from the species with one or two ^{17}O-labeled oxygens (expected combined intensity 75%). The theoretical and experimental intensities probably are within experimental error in view of the difficulties in determining the base line and performing the curve fitting.

A smaller shoulder peak of about 5% intensity appears in the absence of metal ion (Figure 2B,C) but disappears in its presence. The origin of this shoulder is unknown, but it appears to be associated with high pH and high salt concentration and may represent a complex of the enzyme with either a monovalent cation or an anion.

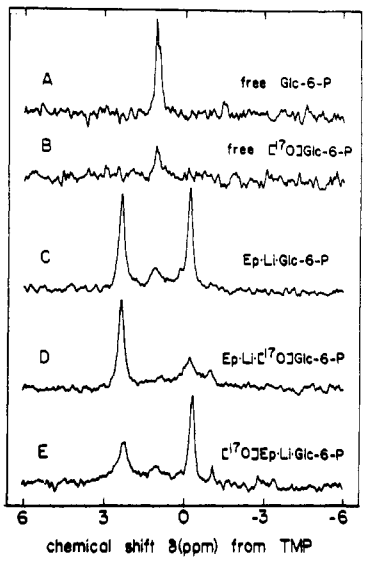

FIGURE 3: Assignment of the ^{31}P NMR peaks of E_P·Li·Glc-6-P to particular phosphates by ^{17}O labeling. (A) ^{31}P NMR spectrum of "free" Glc-6-P in ^2H_2O containing 1 mM EDTA and 0.4 mM Tris-HCl buffer, pH 7.5; (B) free $[^{17}O]$Glc-6-P under the same conditions; (C) E_P·Li·Glc-6-P—1.2 mM enzyme (1.1 mM E_P form) plus 1.2 mM Glc-6-P in 20 mM LiCl, 22 mM Tris-HCl, and 1 mM EDTA, pH 7.5; (D) same as in (C) but with $[^{17}O]$Glc-6-P; (E) $[^{17}O]E_P$·Li·Glc-6-P—1.23 mM enzyme (1.05 mM E_P form) plus 1.23 mM Glc-6-P in 20 mM LiCl, 20 mM Tris-HCl, and 5 mM EDTA, pH 7.5.

Binding of $^{113}Cd^{2+}$ splits the ^{31}P peak of E_P but does not change its line width (Figure 1F). When $^{113}Cd^{2+}$ is added to $[^{17}O]E_P$, the spectrum can be decomposed into two sharp lines (each about 11 Hz wide) which arise from $[^{16}O_4]E_P{\cdot}Cd$ and one broad line (about 86 Hz wide, Table I) which arises from ^{17}O-labeled $E_P{\cdot}Cd$ species. As expected, the width of the broad-component peak in the spectrum of $[^{17}O]E_P{\cdot}Cd$ is greater than that of $[^{17}O]E_P$ by 16 Hz, approximately the magnitude of the ^{113}Cd–O–^{31}P coupling constant.

Use of ^{17}O Labeling To Assign Phosphate Resonances and To Probe Phosphate Mobility. The metal-free enzyme is quite inactive (Ray et al., 1978). In the presence of 10 mM Li^+, the activity of phosphoglucomutase is less than 2×10^{-8} times that of the Mg^{2+} form of the enzyme. In fact, under such conditions, the three enzyme-bound intermediates analogous to those in eq 1 (E_P·Li·Glc-6-P, E_P·Li·Glc-1-P, and E_D·Li·Glc-P_2) can be maintained as separate entities at room temperature for several hours. Hence, ^{17}O labeling of enzyme and substrate phosphates can be used to assign the ^{31}P NMR peaks for the first two of the above complexes and to investigate the mobilities of the phosphate groups in the free enzyme and its complexes. The results are shown in Figures 3 and 4. Labeling of free Glc-1-P or Glc-6-P with one ^{17}O nucleus apparently is sufficient to broaden the ^{31}P NMR peak beyond detection; the residual intensity observed with the ^{17}O-labeled Glc-6-P sample can be accounted for by the unlabeled material present. The broadening caused by ^{17}O labeling allows the assignment of peaks in Figures 3 and 4 to specific phosphate groups. Two minor peaks in these spectra can be identified by their chemical shifts: 1.3 ppm (Figure 3C,E), free Glc-6-P (an excess of the sugar phosphate was used); −1.02 ppm (Figure 3D), E_P·Li·Glc-1-P produced during the experiment. The spectrum of $[^{17}O]E_P$·Li (not shown), which is essentially identical with that of $[^{17}O]E_P$ (Figure 2D), appears to broaden slightly upon addition of Glc-6-P (Figure 3E).

NMR Relaxation. To quantify the effect of ^{17}O labeling on the phosphates, NOE and T_1 relaxation measurements of

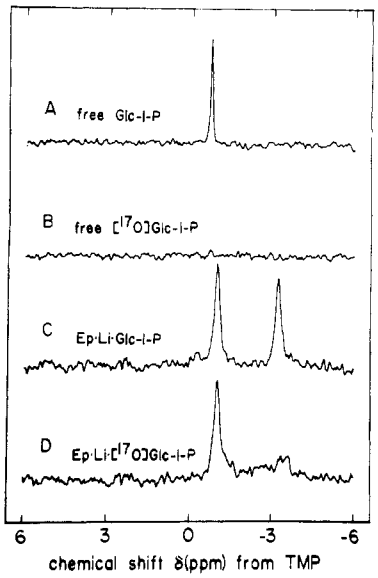

FIGURE 4: Assignment of the ^{31}P NMR peaks of E_P·Li·Glc-1-P to particular phosphates by ^{17}O labeling. (A) "Free" Glc-1-P in ^2H_2O containing 1 mM EDTA and 0.4 mM Tris-HCl buffer, pH 7.5; (B) free [^{17}O]Glc-1-P under the same conditions; (C) E_P·Li·Glc-1-P under the same conditions as in Figure 3C; (D) E_P·Li·[^{17}O]Glc-1-P under the same conditions as in Figure 3D.

Table II: NOE, T_1 Relaxation, and Line Width ($\Delta\nu_{1/2}$) Data for the Phosphates of Free and Complexed Phosphoglucomutase[a]

species[b]	NOE 90% ^1H_2O[c]	NOE 99% ^2H_2O[d]	T_1 (s) 90% ^1H_2O[c]	T_1 (s) 99% ^2H_2O[d]	$\Delta\nu_{1/2}$ (Hz) 90% ^1H_2O[c]
E_P	1.4	1.0	2.8 ± 0.1[f]	6.5 ± 0.6[f,j]	10 ± 1
			2.8 ± 0.1[g,h]		18 ± 1[h]
			3.2 ± 0.1[g,i]		40 ± 2[i]
E_P·Li[e]	1.4	1.0	2.6 ± 0.1[g]		10 ± 1
E_P·Li·Glc-6-P[e]	1.0	0.9	3.6 ± 0.1[g]		16 ± 1
E_P·Li·Glc-6-P[e]	1.3	1.3	6.5 ± 0.2[g]		11 ± 1
E_P·Cd	1.5		4.1 ± 0.2[g]		10 ± 1

[a] Samples were in 20 mM Tris-HCl, pH 7.5 at 20 °C. All measurements were at 81 MHz unless otherwise noted. [b] In complexes involving two phosphate ester groups, the group whose relaxation is tabulated is italicized. [c] The solution contained 10% ^2H_2O. [d] The protein was dialyzed against the buffer solution in 99.8% ^2H_2O. [e] In the presence of 5 mM EDTA and 20 mM LiCl. [f] T_1 was determined by the inversion-recovery method. [g] T_1 was determined by the saturation-recovery method. [h] Measured at 121 MHz. [i] Measured at 190 MHz. [j] The line width of E_P in ^2H_2O was 9 ± 1 Hz.

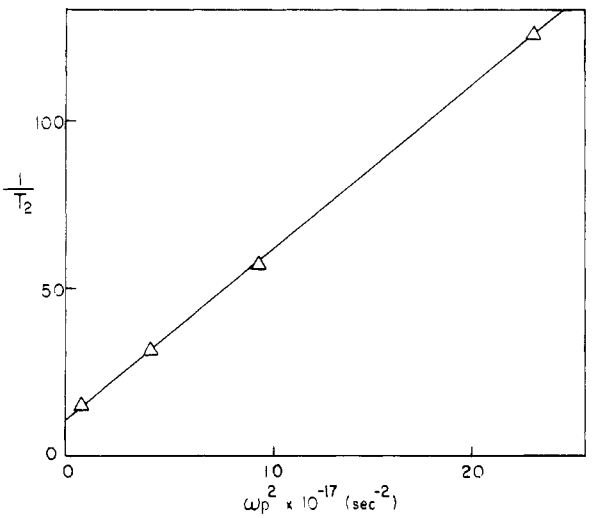

FIGURE 5: Plot of the spin–spin relaxation rate ($1/T_2$) as a function of the square of the magnetic field strength (ω_P^2) for the ^{31}P NMR resonance of E_P. The $1/T_2$ values were determined from the line width of E_P. The sample contained 1.3 mM E_P in 20 mM Tris-HCl in 90% ^1H_2O. The extrapolated intercept value is 3.5 Hz.

the enzyme and its complexes were conducted. The field dependence of the line width of E_P also was measured (Figure 5). The results are summarized in Tables II and III, together with evaluations of the expected dipole–dipole (DD) and chemical shift anisotropy (CSA) contributions to the spin–spin relaxation rate ($1/T_2$) and the spin–lattice relaxation rate ($1/T_1$) at 81 MHz (Table III). The equations used are provided in footnotes to Tables II and III. $T_{1,CSA}$ and τ_c, the correlation time for motions of the enzymic phosphate, were calculated by the iterative procedure described below. Measurements obtained in ^1H_2O and ^2H_2O solutions were compared, and the correlation times were extracted from the ratio $T_{1,CSA}/T_{2,CSA}$ by the method of Brauer & Sykes (1981). The value of $1/T_{2,CSA}(^1H_2O)$ from Figure 5 (20.4 s^{-1}) was used to calculate the corresponding value of $1/T_{2,CSA}(^2H_2O)$ (24.7 s^{-1}), and, as an initial estimate, $1/T_{1,CSA}(^2H_2O)$ was taken as equal to $1/T_{1,obsd}(^2H_2O)$. Together, these values allowed calculation of (1) a provisional estimate of $\tau_c(^2H_2O)$ (see above), (2) a provisional estimate of $1/T_{1,DD}(^2H_2O)$ from (1) plus the β-H···P distance in serine phosphate, and (3) an initial estimate of $T_{1,CSA}(^2H_2O)$ from $T_{1,obsd}(^2H_2O)$ plus (2). The values of $\tau_c(^2H_2O)$ and $1/T_{1,DD}(^2H_2O)$ converged after calculating a new value for $\tau_c(^2H_2O)$ and iterating the procedure twice. The convergent values were used to calculate $\tau_c(^1H_2O) = 2.8 \times 10^{-8}$ s and $1/T_{1,CSA}(^1H_2O) = 0.15\ s^{-1}$, together with $T_{1,DD}(^1H_2O) = 0.20\ s^{-1}$. By contrast, a much shorter corre-

Table III: Analysis of the Chemical Shift Anisotropy and Dipole-Dipole Contributions to the Experimental T_1 and T_2 Relaxation Rates of the ^{31}P Resonance of the Enzymic Phosphate of Phosphoglucomutase at 81 MHz[a]

solvent	$1/T_{2,obsd}$ (s^{-1})	$1/T_{2,DD}$ (s^{-1})	$1/T_{2,CSA}$ (s^{-1})	$1/T_{1,obsd}$ (s^{-1})	$1/T_{1,DD}$ (s^{-1})	$1/T_{1,CSA}$ (s^{-1})
90% ^1H_2O	31.4[b]	11.0[c]	20.4	0.35$_7$	0.20$_3$[f]	0.15$_4$[e]
99% ^2H_2O	28.3[b]	3.6[d]	24.7[e]	0.15$_4$	0.02$_7$[g]	0.12$_7$[g]

[a] The calculations assumed that $\omega^2\tau_c^2 >> 1$ (nonextreme narrowing limit). This assumption appears justified on the basis of the experimentally derived correlation time and the small contribution to $1/T_{2,DD}$ by the hydrogen-bonded protons (see Table IV). [b] Calculated from the line widths given in Table II. [c] Obtained from the intercept in Figure 5. [d] Obtained as the difference between $1/T_{2,obsd}$ and the estimated value of $1/T_{2,CSA}$. [e] Calculated on the basis that $1/T_{2,CSA}$ or $T_{1,CSA}$ is proportional to τ_c in the nonextreme narrowing limit and that τ_c is 1.21 times longer in ^2H_2O than in ^1H_2O because of the difference in viscosity. [f] Obtained as the difference between $1/T_{1,obsd}$ and the estimated value of $1/T_{1,CSA}$. [g] The value of τ_c was calculated from the ratio of $T_{1,CSA}/T_{2,CSA}$ by the equation of Brauer & Sykes (1981): $\tau = [^3/_2(T_{1,CSA}/T_{2,CSA})(1/\omega_0^2)]^{1/2}$. After two iterations, τ_c (99% ^2H_2O) converged from 3.1×10^{-8} to 3.4×10^{-8} s. The contribution to $1/T_{1,DD}$ from the β-protons of Ser-116 was calculated to be 0.027 s^{-1} (99% ^2H_2O) from τ_c and the H-C-O-P distance in footnote *a* of Table IV. The above value becomes 0.033 s^{-1} in 90% ^1H_2O.

lation time (5.4×10^{-11} s) was calculated from $1/T_{1,DD}(^1H_2O)$ by the method of McCain & Markley (1980). This correlation time probably reflects the residence time (τ_w) of water molecules in the first coordination sphere of the phosphate rather than the rotational correlation time of the phosphate (τ_c). This value may be compared with the somewhat smaller value ($\tau_w = 1.1 \times 10^{-11}$ s) reported for orthophosphate (McCain & Markley, 1980); it seems reasonable to expect that the residence time for a water coordinated to an enzyme phosphate would be longer than that for water coordinated to inorganic phosphate.

The large change in the NOE and T_1 values of E_P on going from ^1H_2O to ^2H_2O (Table II), in conjunctions with the effect produced by substrate binding, suggests that the phosphate group of E_P is accessible to the solvent. Binding of Li^+ does not affect this solvent accessibility. In the ternary complex with Glc-6-P, the enzymic phosphate becomes much less accessible and possibly inaccessible to solvent. The phosphate ester group of the substrate also is partially immobilized, but not to as great an extent as the enzymic phosphate. The lack of a substantial decrease in the NOE for the phosphoenzyme on the binding of Cd^{2+}, which is known to bind directly to the enzymic phosphate, indicates that a change or lack of change in the NOE of a phosphate group, per se, is not a reliable criterion for a change in the number of hydrogen-bonded water molecules.[5]

A comparison of $T_{1,obsd}$ values measured in ^1H_2O and in ^2H_2O (Table III) suggests that dipole–dipole interactions are responsible for about 57% of the T_1 relaxation. However, the T_1 value does not show a significant magnetic field dependence (first three entries, column 4, Table II). Since $1/T_{1,DD}$ decreases to about 13% of its original value on going from 90% ^1H_2O to 99% ^2H_2O (Table III), the major T_1 relaxation mechanism must involve exchangeable protons, probably those having a correlation time of 5.4×10^{-11} s (see above) which is in the extreme narrowing limit. Dipole–dipole contributions to T_1 relaxation are not expected to be field dependent at this limit. By using this correlation time (5.4×10^{-11} s) and assuming that six protons are hydrogen bonded to the phosphate of E_P, we calculated 1.6 as the expected NOE value in 90% ^1H_2O.[6] This value is in reasonable agreement with the experimental value of 1.4, considering experimental errors in measuring the NOE and T_1 values.

In calculating the dipole–dipole contributions to the line width of the enzymic phosphate (Table IV), we assumed that the dipolar relaxation of the ^{31}P nucleus in ^1H_2O can be accounted for by interactions with the two β-protons of Ser-116 ($\tau_c = 2.8 \times 10^{-8}$ s; see above), which would provide a contribution of 1.08 Hz to the line width, and by interactions with six protons hydrogen bonded to the nonbridging oxygens of the enzymic phosphate ($\tau_c = 5.4 \times 10^{-11}$ s; see footnote *g* of Table III), which would provide a contribution of 0.2 Hz. The sum of these, about 1.3 Hz, is substantially smaller than the value of 3.5 Hz for $1/T_{2,DD}(^1H_2O)$ obtained from the intercept of Figure 5. However, the calculated contribution to $1/T_2$ from the β-protons of Ser-116 is in excellent agreement with the value independently estimated for $1/T_{2,DD}(^2H_2O)$ (1.1 Hz; see Table III). Apparently, the increase in $1/T_{2,DD}$ in going from ^2H_2O to ^1H_2O (about 2.4 Hz, Table III) is significantly larger than the calculated contribution of six hydrogen-bonded water molecules (about 0.2 Hz). This result suggests that other exchangeable protons adjacent to the phosphate having much longer correlation times (more on the order of 2.8×10^{-8} s found for the serine phosphate) also contribute to $1/T_{2,DD}$ in ^1H_2O. In any case, the relatively small change observed in $1/T_{2,obsd}$ upon going from ^1H_2O to ^2H_2O (Table III) is consistent with the dominance of $1/T_2$ by $1/T_{2,CSA}$, and this internal consistency suggests that expanding the above treatment to T_2 values for the system involving $[^{17}O]E_P$ is reasonable.

Table IV: Calculated Dipolar Contributions to the Line Width of the ^{31}P Resonance of the Enzymic Phosphate of Phosphoglucomutase at 81 MHz

calcd correlation time (s)	atoms contributing to DD relaxation	contribution to line width (Hz)[a]
5.4×10^{-13} [b]	6 hydrogen-bonded protons	0.1_8
2.8×10^{-8} [c]	2 β-protons of Ser-116	1.0_8
	1 hydrogen-bonded proton	1.0_4
	3 ^{17}O nuclei	10.1
	CSA	6.5_0

[a] The bond distances used in the calculations were 1.5 Å for P-O (Sundaralingam & Putkey, 1970), 2.6 Å for the β-H···P of methylene phosphate (Sundaralingam & Putkey, 1970; Ray et al., 1977), and 2.33 Å for P-O···H (McCain & Markley, 1980). [b] Calculated from $1/T_{1,DD}$ (Table III) by the equation from McCain & Markley (1980): $1/T_{1,DD} = \hbar^2\gamma_H^2\gamma_P^2 n\tau_w b^{-6}$ where $\hbar$ is Planck's constant devided by 2π, γ_H and γ_P are the magnetogyric ratios of proton and phosphorus nuclei, respectively, n is the number of hydrogen-bonded water protons (5.4 in 90% ^1H_2O), and b is the average bond distance for P-O···H. In this calculation, the contribution of the β-protons of Ser-116 (0.033 s^{-1}) was subtracted from the total dipole–dipole contribution (0.203 s^{-1}). [c] See footnote *g* of Table III.

The calculations in Table IV indicate that the dipolar contribution of three ^{17}O nuclei to T_2 of the enzymic ^{31}P nucleus at 81 MHz should be only about 10 Hz (using $\tau_c = 2.8 \times 10^{-8}$ s). Experimentally, however, this resonance appears to be broadened beyond detection. Even the species having one to two ^{17}O atoms ($[^{17}O_1,^{16}O_3]E_P$ and $[^{17}O_2,^{16}O_2]E_P$) give rise to a composite peak whose width is about 57 Hz greater than that of unlabeled E_P. Since the observed line width is much greater than that calculated on the basis of dipolar interactions, it is clear that T_2 relaxation of ^{17}O-labeled E_P is dominated by scalar coupling.

The scalar contribution to the line width of the dipolar species (^{31}P) is given by

$$(\Delta\nu_{1/2})_s = \tfrac{4}{3}\pi I(I+1)T_q J^2 \qquad (2)$$

where I is the spin quantum number of the quadrupolar nucleus ($I = {}^5/_2$ for ^{17}O), T_q is the relaxation time of the quadrupolar nucleus (^{17}O), and J is the ^{31}P–^{17}O scalar coupling constant (Tsai et al., 1980). The ^{31}P···^{17}O scalar interaction can be altered by binding a metal ion to the phosphate as has been observed with small molecules. For example, Mg^{2+} binding to ^{17}O-labeled ATP reduces the broadening caused by the ^{31}P···^{17}O interaction. The reduced broadening is caused by a large decrease in T_q. A decrease in J could also contribute, but the coupling constant has not been determined (Tsai et al., 1980). The present results show that binding of Li^+ or Cd^{2+} to $[^{17}O]E_P$ does not affect the experimentally observed line width of the phosphate. Since Cd^{2+} binds directly to the enzymic serine phosphate, the metal ion binding either does not perturb T_q significantly or, if it does, leads to compensatory changes in T_q and J. However, quantitation of the ^{17}O-labeling experiments is complicated by the fact that the isotopic purity of the label was only 50%.

[5] A small increase in the water residence time in the first coordination sphere of the phosphate resulting from Cd^{2+} binding would cause an increase in $1/T_{1,DD}(^1H_2O)$ and a possible increase in the NOE.

[6] In 90% ^1H_2O, the six hydrogen bonds assumed would involve 5.4 ^1H nuclei.

Table V: Assigned Positions of ^{31}P NMR Peaks of Phosphoglucomutase, Substrates, and Complexes[a]

phosphate species[b]	chemical shift (ppm)[c] for no metal ion	Li^+	Zn^{2+}	Mg^{2+}	Cd^{2+}	Co^{2+}
E_P·M	0.6_0	0.1_7	0.3_3	0.8_2	1.6_8	*e*
E_P·M·Glc-6-P	2.8_8	2.3_4	2.1_3	*d*		
E_P·M·Glc-6-*P*	-0.4_5	-0.2_4	-0.3_1	*d*		
E_P·M·Glc-1-P	-1.5_5	-1.0_2				
E_P·M·Glc-1-*P*	-2.9_8	-3.2_4				
E_D·M·Glc-1,*6*-P_2	-0.6_1	-1.6_1	0.7_2	*d*	5.1_9	
E_D·M·Glc-*1*,6-P_2	-3.1_4	-3.0_9	-2.8_3	*d*	-2.7_6	
Glc-6-P		1.3_4				
Glc-1-P		0.7_8				
Glc-1,*6*-P_2 (α/β)		$1.2_4/1.3_0$				
Glc-*1*,6-P_2 (α/β)		$-1.0_5/-0.8_5$				

[a] Samples were in 20 mM Tris-HCl, pH 7.5 at 20 °C; 5 or 10 mM EDTA was added for the experiment involving Li^+. [b] In complexes containing two phosphates, the one for which chemical shifts are tabulated is italicized. [c] Chemical shifts are in ppm from internal trimethyl phosphate. [d] Peaks could not be resolved because of chemical exchange. [e] Peak too broad to be observed.

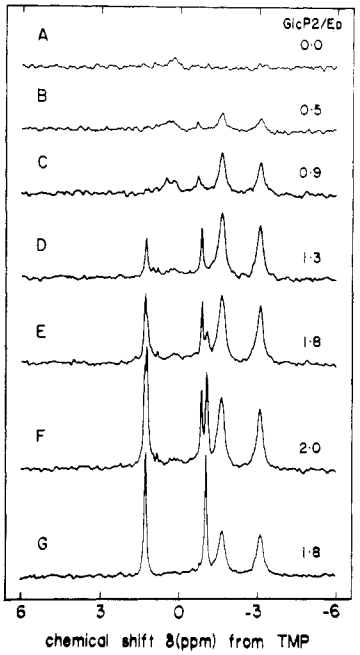

FIGURE 6: Titration of E_D·Li with Glc-P_2. All solutions were at pH 7.5 and contained 20 mM Tris-HCl, 5 mM EDTA, 20 mM LiCl, and 90% ^1H_2O. (A) ^{31}P NMR spectrum of 1.3 mM E_D; (B–F) spectra after addition of the number of equivalents of Glc-P_2 (86% α form, 14% β form) indicated in the figure; (G) 1.3 mM E_D plus 1.8 equiv of anomerically pure α-Glc-P_2.

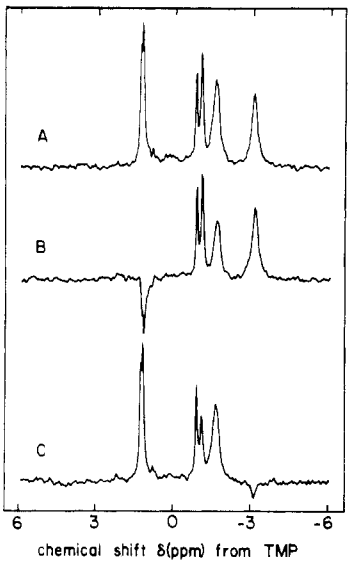

FIGURE 7: ^{31}P NMR polarization transfer studies (by selective peak inversion) of the binding of Glc-P_2 to E_D·Li. (A) Normal spectrum, [Glc-P_2]/[E_D] = 2.0, other conditions as in Figure 6; (B) selective inversion of the 6-phosphate peak of Glc-P_2 showing the decrease in the intensity of the 1-phosphate peak of E_D·Li·Glc-P_2 at −1.6 ppm; (C) selective inversion of the E_D·Li·Glc-P_2 peak at −3.1 ppm showing the decrease in intensity of the 1-phosphate peak of α-Glc-P_2. NMR conditions: 180° soft pulse (18 ms), 60° nonselective pulse (20 μs), delay time between pulses of 100 μs, delay between pairs of pulses of 3.4 s.

Binding of Glucose 1,6-Bisphosphate to the Dephosphoenzyme. Spectra illustrating the titration of E_D·Li with Glc-P_2 are shown in Figure 6. The small intensity at 0.6 ppm (Figure 6A) arises from residual phosphoenzyme after the dephosphorylation procedure. Two peaks at −1.61 and −3.09 ppm appear with the first addition of Glc-P_2 and grow to a limiting intensity as more Glc-P_2 is added. These are assigned to the bound substrate (E_D·Li·Glc-P_2). Additional peaks at 1.30 and −0.85 ppm appear later and grow continuously with the addition of Glc-P_2. Separate peaks are observed for the α- and β-anomers of Glc-P_2, which were present in a ratio of 86/14 in the sample of Glc-P_2 used here. The chemical shift difference between these anomers is more pronounced for the 1-phosphate than for the 6-phosphate; the peak at higher field corresponds to the 1-phosphate of the α-anomer. Preferential depletion of the signal of the α-anomer in the presence of the dephosphoenzyme (Figure 6E,F) clearly indicates that the dephosphoenzyme binds the α-anomer more strongly than the β-anomer; such preferential binding is expected since the α-sugar phosphate is the normal substrate of phosphoglucomutase (Ray & Peck, 1972). These results subsequently were confirmed by the titration of E_D·Li with α-Glc-P_2 free of β-Glc-P_2 (Figure 6G).

At the end point of the above titration, the ratio of [Glc-P_2] to [E_D] was about 1.09:1 (data not shown), which is close to the expected ratio of 1:1 (Ray & Peck, 1972). The equilibrium constant for the dissociation of E_D·Li·Glc-P_2 to E_D·Li and Glc-P_2 is estimated to be in the range of 10–35 μM, i.e., much larger than the dissociation constant for the corresponding complex with Mg^{2+} in place of Li^+ (Ray & Long, 1976).

Individual assignments of the ^{31}P peaks for the E_D·Li·Glc-P_2 complex also were made by polarization transfer experiments using selective peak inversion (Figure 7). When the 6-phosphate peak of α-Glc-P_2 at 1.24 ppm is selectivity inverted (Figure 7B), the peak at −1.61 ppm loses some intensity; conversely, when the peak at −3.09 ppm is inverted (Figure 7C), the 1-phosphate peak of α-Glc-P_2 is diminished. Hence, the peak at −1.61 ppm is assigned to the 6-phosphate, and the peak at −3.09 ppm is assigned to the 1-phosphate in this complex. The selective inversion spectra confirm again that

the α form of Glc-P_2 binds to the enzyme more tightly than the β form.

^{31}P NMR chemical shifts of various species are summarized in Table V. Assignments not discussed previously were made by noting the chemical shifts and intensities of the peaks, by comparing these with those from peaks assigned by ^{17}O labeling or polarization transfer studies, and by referring to the equilibrium distributions of the ternary complexes of phosphoglucomutase determined by Ray & Long (1976).

Implications for Phosphoglucomutase Reaction Mechanism. Although there is only a single attachment site for the enzymic phosphate of phosphoglucomutase (Ray & Roscelli, 1964; Ray et al., 1983), it is possible that internal rotation places the attached phosphate group in different positions prior to binding of Glc-1-P, as opposed to Glc-6-P. In fact, Britton & Clarke (1968) have detected differences in the enzymic forms to which these two phosphates bind, although these differences were averaged in time intervals on the order of 0.1 μs. Such a time period is too short to allow a resolution of possible environmental differences in the chemical shift of the enzymic phosphate group by means of ^{31}P NMR spectroscopy. On the other hand, binding of Glc-1-P or Glc-6-P gives rise to a substantial differences in the chemical shift of the enzymic phosphate group, in either the presence or the absence of bound metal ion. Thus, Glc-6-P produces an upfield shift while Glc-1-P produces a shift in the opposite direction (Table V). It is a moot point whether this difference is induced *during* the binding step or merely reflects *existing* differences between alternative forms of the free phosphoenzyme that are averaged out by rapid interconversion in the absence of bound substrate. In any case, as judged by observations with the inactive E_P·Glc-1-P and E_P·Glc-6-P complexes, as well as the corresponding complexes involving bound Li^+, the transfer of alternative phosphate groups from bound Glc-P_2 to Ser-116 of the dephosphoenzyme does not produce identical phospho forms of the enzyme—although this lack of identity could involve relatively minor differences, e.g., differences involving the C–O–P bond angle.

Only a single form of the intermediate E_D·Glc-P_2 complex was detected in the present study, whether in the absence of metal ion, in the inactive complex involving Li^+, or in the reactive complex involving Cd^{2+}. [The Cd^{2+} form of the enzyme is 1% as active as the Mg^{2+} form (Ray & Long, 1976).] The NMR signals from the E_D·M·Glc-P_2 complexes where M is varied are unlikely to represent time-averaged signals from the two different forms that have been proposed as intermediates in the enzymic reaction: one in which the 1-phosphate is correctly positioned for transfer to Ser-116 and the other in which the 6-phosphate is so positioned (Ray & Roscelli, 1966; Ma & Ray, 1980). In such a case, the NMR signals from both phosphates should be affected both by metal ion binding and by the identity of the bound metal ion. Experimentally, the 1-phosphate in the E_D·M·Glc-P_2 complex produces a signal at -2.9 ± 0.2 ppm irrespective of M, while the chemical shift of the 6-phosphate in these complexes varies from -1.6 to $+5.2$ ppm in the series M = Li^+, no metal ion, Zn^{2+}, or Cd^{2+} (Table V). Apparently, in the E_D·M·Glc-P_2 complex that is observed, the 6-phosphate group is close to the bound metal ion and the nearby acceptor hydroxyl group of Ser-116, in accord with conclusions drawn earlier from spectral studies (Ma & Ray, 1980). In what way the diphosphate complex with the 1-phosphate in position for transfer to Ser-116 differs from the observed complex is not clear. It is hoped that this can be deduced from X-ray diffraction studies.[7]

Conclusions

In the present study, Cd^{2+} isotopes have been used to elucidate the interaction between the metal ion activator and the enzymic phosphate group in the phosphoglucomutase reaction. The J coupling between $^{113}Cd^{2+}$ and the enzymic phosphate provides unequivocal evidence for inner-sphere metal–phosphate coordination in the E_P·Cd complex. The only other example of ^{113}Cd–O–^{31}P coupling in an enzymic system is the 30-Hz coupling observed in $^{113}Cd^{2+}$–alkaline phosphatase (Otvos et al., 1979). The proximity of the metal ion to the serine phosphate at the active site of phosphoglucomutase had been inferred previously from paramagnetic broadening of the phosphate resonance induced by Co^{2+} or Mn^{2+} (Ray & Mildvan, 1970, 1973; Ray et al., 1977). However, difficulties can arise from exchange effects that may obscure the true metal-ion–phosphate distance (Otvos et al., 1979). In addition, this method usually allows only a lower limit to be placed on the distance so that first- or second-sphere coordination of phosphate may not be distinguished. Thus, the detection of J coupling in the diamagnetic enzyme may offer the only unequivocal NMR evidence for direct metal–phosphate interaction. ^{31}P NMR chemical shifts in phosphate analogues are known to be sensitive both to the deshielding effect of a directly coordinated diamagnetic metal ion and to relatively small O–P–O bond angle distortions (Gorenstein, 1975; Otvos et al., (1979). The effects are difficult to disentangle since, at least in the case of Cd^{2+}, metal binding causes a larger change in the chemical shift of the 6-phosphate peak of E_D·Glc-P_2 ($\Delta\delta$ = -0.6 to $+5.2$ ppm) to which Cd^{2+} apparently does not bind than in the chemical shift of the serine phosphate peak of E_P ($\Delta\delta$ = $+0.6$ to $+1.7$ ppm) to which Cd^{2+} does bind. The phosphate group of E_P is readily accessible to water molecules as indicated by the large effect of ^2H_2O on the NOE (Table I). The solvent accessibility of this phosphate is lost upon substrate binding, whereas the 6-phosphate group of the substrate still has some free rotation and is accessible to solvent (see Table I).

Comparison of the present results with those obtained for alkaline phosphatase demonstrates the difficulty of formulating generalizations about Cd^{2+}–phosphate interactions. The chemical shift of the serine phosphate in both phosphoglucomutase and alkaline phosphatase is displaced downfield by a relatively small amount, 1.1 and 1.8 ppm, respectively, on binding of Cd^{2+}, although in the present case a direct Cd–O–P interaction is produced while in the other (alkaline phosphatase) no direct coordination occurs (Gettins & Coleman, 1982). In addition, in both cases, cleavage of the phosphoserine bond without removing the phosphate group from the active site produces a substantially larger change in chemical shift: +5.8 ppm in the phosphoglucomutase system and +11 ppm in alkaline phosphatase. However, in the phosphoglucomutase system, this cleavage appears to eliminate direct Cd–O–P interaction while in the alkaline phosphatase system a direct interaction of this type is produced.

Phosphoglucomutase appears be the third example of a metal-activated phosphate transferase in which the transfer step produces/eliminates coordination between the bound metal ion and the group that is transferred during reactant/product interconversion. the other example, besides alkaline phosphatase, is creatine kinase (Reed et al., 1978). The possible significance of a change in coordination during phosphate transfer is clouded in all three cases by the use of complexes in which one component of the complex differs from

[7] A 3.5-Å map recently has been constructed.

that required for efficient catalysis.

Acknowledgments

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Registry No. Phosphoglucomutase, 9001-81-4; Glc-1-P, 59-56-3; Glc-6-P, 56-73-5; Glc-P_2, 10139-18-1; Co, 7440-48-4; Cd, 7440-43-9; Li, 7439-93-2; Mg, 7439-95-4; Zn, 7440-66-6.

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