Multinuclear Magnetic Resonance Studies of Metal Ion Binding Sites of Phosphoglucomutase†

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ABSTRACT: Metal binding at the activating site of rabbit muscle phosphoglucomutase has been studied by ^{31}P, ^{7}Li, and ^{113}Cd NMR spectroscopy. A ^{7}Li NMR signal of the binary Li^+ complex of the phosphoenzyme was not observed probably because of rapid transverse relaxation of the bound ion due to chemical exchange with free Li^+. The phosphoenzyme–Li^+–glucose 6-phosphate ternary complex is more stable, kinetically, and yields a well-resolved peak from bound Li^+ at −0.24 ppm from LiCl with a line width of 5 Hz and a T_1 relaxation time of 0.51 ± 0.07 s at 78 MHz. When glucose 1-phosphate was bound, instead, the chemical shift of bound $^{7}Li^+$ was −0.13 ppm; and in the Li^+ complex of the dephosphoenzyme and glucose bisphosphate a partially broadened $^{7}Li^+$ peak appeared at −0.08 ppm. Thus, the bound metal ion has a somewhat different environment in each of these three ternary complexes. The ^{113}Cd NMR signal of the binary Cd^{2+} complex of the phosphoenzyme appears at 22 ppm relative to $Cd(ClO_4)_2$ with a line width of 20 Hz at 44.4 MHz. Binding of substrate and formation of the Cd^{2+} complex of the dephosphoenzyme and glucose bisphosphate broaden the ^{113}Cd NMR signal to 70 Hz and shift it to 75 ppm. The 53 ppm downfield shift upon the addition of substrate along with ^{1}H NMR data suggests that one oxygen ligand to Cd^{2+} in the binary complex is replaced by a nitrogen ligand at some intermediate point in the enzymic reaction. In addition to the binding of Li^+ and Cd^{2+} at the activating site for metal ions, weaker binding of either metal ion at an ancillary site was observed in the presence of bound substrate. Binding of either Li^+ or Cd^{2+} at the weak site of the Cd^{2+}–dephosphoenzyme–bisphosphate complex results in similar chemical shift changes in the ^{31}P NMR peaks of the bisphosphate, presumably by virtue of a conformational change in the enzyme. No effect of ancillary binding was observed on the enzymic phosphate in the absence of bound substrate. Signals from Li^+ or Cd^{2+} bound at the weak site were not observed in the ^{7}Li NMR or ^{113}Cd NMR studies, apparently because of rapid transverse relaxation resulting from a chemical exchange process. Although the binding of the monovalent Li^+ at the activating site for divalent metal ions may be peculiar to phosphoglucomutase, the present studies suggest that the use of Li^+ as a probe of the binding site for bivalent metal ions in other enzymic systems might prove fruitful.

In the first step of the phosphoglucomutase reaction the enzymic phosphate group is transferred to bound glucose phosphate to produce a dephosphoenzyme–glucose bisphosphate complex:[1]

$$E_P\text{·Glc-1-P} \rightleftharpoons E_D\text{·Glc-P}_2 \rightleftharpoons E_P\text{·Glc-6-P} \quad (1)$$

In a similar subsequent step, a phosphate group of the bisphosphate is returned to the enzyme so that there are three different enzyme–substrate complexes (central complexes) in the overall reaction, as is indicated above (Ray & Peck, 1972). When Li^+, instead of the natural activator, Mg^{2+}, is bound at the activating site for metal ions, the phosphate-transfer steps that interconvert the central complexes are slowed sufficiently so that each can be examined separately (Ma & Ray, 1980). A previous paper (Rhyu et al., 1984) reported ^{31}P NMR chemical shifts for both phosphates in the Li^+ forms of all three central complexes, as well as that of the binary Li^+ complex of the phosphoenzyme. In the present sutdy, the metal ion binding site in the central complexes is examined by ^{7}Li NMR spectroscopy. This study apparently is the first in which the NMR signal of enzyme-bound Li^+ has been observed directly, although ^{7}Li relaxation measurements of bulk Li^+ have been used to determine the distance between an enzyme-bound (paramagnetic) bivalent metal ion and the binding site for a monovalent metal ion (Grisham & Hutton, 1978; Raushel & Villafranca, 1980; Villafranca & Raushel, 1983).

When a bivalent metal ion is bound at the activation site of phosphoglucomutase, an equilibrium among the central complexes is established quite rapidly. But in the case of Cd^{2+}, a poor activator, this equilibrium at neutral pH is so heavily in favor of the dephosphoenzyme–bisphosphate form of the central complexes (Ray & Long, 1976) that this intermediate can be examined directly. In the present study the environment of bound Cd^{2+} in the binary E_P·Cd complex, where direct coordination with the enzymic phosphate has been demonstrated by means of ^{113}Cd–^{31}P spin–spin coupling (Rhyu et al., 1984), is compared with that present in the E_D·Cd·Glc-P_2 complex, i.e., after substrate binding and the first phosphate

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[1] Abbreviations: E_P and E_D, the phospho and dephospho forms of rabbit muscle phosphoglucomutase; M, metal ion; Glc-P_2, D-glucose 1,6-bisphosphate; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, α,β-D-glucose 6-phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; ppm, parts per million; NOE, nuclear Overhauser effect; OAc, acetate.

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transfer step, by use of both ^{31}P and ^{113}Cd NMR spectroscopy. As was suggested in the previous study, the coordination of Cd^{2+} bound at the activation site apparently is altered by this process.

Experimental Procedures

Enzyme and Chemicals. The phospho and dephospho forms of phosphoglucomutase and glucose phosphates were prepared as in Rhyu et al., (1984). $^{7}LiCl$ was obtained from Alfa Products, 95.3 atom % ^{113}Cd metal from Prochem, and 98.55 atom % ^{114}CdO from Oak Ridge National Laboratory. The ^{113}Cd metal and ^{114}CdO were converted to the acetate before use. Additional reagents were of the highest purity available.

NMR Spectroscopy. ^{31}P NMR spectra (80.99 MHz) were obtained in the Fourier-transform mode with a Nicolet NT-200 4.7 T spectrometer. Coherent broad-band proton decoupling was applied with high power (1.5 W) during acquisition and low power (0.5 W) during the pulse-repetition delay of 3 or 4.2 s. Pulses of 60° were used (the 90° pulse was 30 μs). The spectral width was 10 kHz, and the line-broadening factor resulting from exponential apodizations was 5 Hz. A total of 8K data points was used to digitize the spectra. NMR measurements were made at 20 ± 1 °C with 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. A total of 4096 transients was accumulated. The chemical shifts are reported relative to trimethyl phosphate (the actual internal reference was trimethylphosphine oxide, which was assigned a chemical shift of 50.092 ppm). These chemical shifts can be converted to 85% H_3PO_4 as the external reference by adding 2.7 ppm.

Spin–lattice (T_1) relaxation times were obtained by the saturation–recovery method (Markley et al., 1971). Dynamic nuclear Overhauser effects were measured by the gated proton-decoupling technique (Freeman et al., 1972).

^{7}Li NMR spectra (77.75 MHz) and ^{113}Cd NMR spectra (44.37 MHz) were obtained in the Fourier-transform mode with a Varian XL-200 4.7 T spectrometer equipped with a broad-band, wide-bore probe. NMR measurements were made at ambient temperature (21–25 °C) with a sample volume of 2 mL contained in a 10-mm tube with a vortex plug inserted. Proton decoupling was not employed. For ^{7}Li NMR spectroscopy, 90° pulses (21 μs) were used, and the pulse repetition time was 6 s (to allow for partial relaxation of free Li^+); the spectral width was 2 kHz, and 4K data points were used to digitize the spectra consisting of 1500 transients. The chemical shifts are given relative to 0.1 M LiCl in $^{2}H_2O$ for ^{7}Li NMR as an external reference. For ^{113}Cd NMR spectroscopy, 60° pulses were used (the 90° pulse was 24 μs), and the pulse repetition time was 0.9 s; the spectral width was 10 kHz, and 8K data points were used to digitize the spectra consisting of 64 000 or 65 536 transients. The chemical shifts are given relative to 0.1 M $Cd(ClO_4)_2$ in $^{2}H_2O$ as an external reference. Other experimental details are given in the figure legends.

Results

^{31}P NMR Study. Figure 1A shows the ^{31}P NMR spectrum of a solution containing the dephosphoenzyme in the presence of a 30% molar excess of glucose 1,6-bisphosphate and an 8% molar excess of Cd^{2+}. All the peaks in this spectrum have been assigned previously (Rhyu et al., 1984) except those resulting from the species E_D·Cd·Cd·Glc-P_2, which are assigned below in this work. The spectrum contains two pairs of broad peaks with different intensities. The major broad peaks at 5.2 and −2.8 ppm are assigned to the sugar phosphates of the ternary complex E_D·Cd·Glc-P_2 (Rhyu et al., 1984). The minor broad

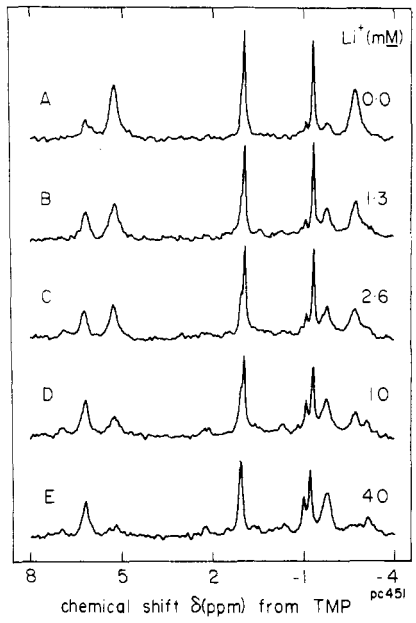

FIGURE 1: Titration of E_D·Cd·Glc-P_2 with LiCl followed by ^{31}P NMR spectroscopy at 81 MHz. All solutions were at pH 7.5 and contained initally 1.3 mM E_P, 1.4 mM $^{114}Cd(OAc)_2$, 1.3 mM Glc-1-P, 0.39 mM α-Glc-P_2, 20 mM Tris-Cl, and 10% $^{2}H_2O$. In the presence of Cd^{2+}, the E_P and Glc-1-P mixture is converted to the E_D·Cd·GlcP_2 complex. (A) No LiCl; (B–E) after addition of the amount of LiCl indicated in the figure.

peaks at 6.2 and −1.8 ppm, which are observed only at Cd^{2+} concentrations in excess of the enzyme concentration, are assigned to the sugar phosphate present in a quaternary complex, E_D·Cd·Cd·Glc-P_2. When E_D·Cd·GlcP_2 is titrated with Li^+, the intensities of the peaks at 5.2 and −2.8 ppm decrease while those of the peaks at 6.2 and −1.8 ppm increase and eventually become predominant peaks (Figure 1B–E). Binding of Cd^{2+} at the activating site, the tighter metal binding site, increases the chemical shift of the 6-phosphate by about 5.8 ppm whereas binding of Cd^{2+} to the the second metal ion binding site further increases the chemical shift of this phosphate by only 1.0 ppm (Rhyu et al., 1984). The sharp peaks at 1.0 and −1.3 ppm are from excess α-Glc-P_2. Over a period of hours, α-Glc-P_2 in this solution is degraded to Glc-6-P and inorganic phosphate either by a contaminating phosphatase or by a very low intrinsic phosphatase activity of phosphoglucomutase, itself. The peak at 1.1 ppm is attributed to Glc-6-P and the shoulder at −1.1 ppm to inorganic phosphate.

Figure 2 shows the change in the ^{31}P NMR spectrum when the essentially inactive E_P·Li·Glc-6-P complex (Figure 2A) is titrated with Cd^{2+} in the presence of 40 mM LiCl. The addition of 0.5 equiv of Cd^{2+} (Figure 2B) displaces the much more weakly bound Li^+ from the activating site of the corresponding amount of enzyme and establishes an equilibrium among E_P·Li·Glc-6-P (2.2 and −0.4 ppm), E_D·Li·Glc-P_2 (−1.7 and −3.2 ppm), E_D·Cd·Glc-P_2 (5.2 and −2.8 ppm), and species represented by two additional peaks, E_D·Cd·Li·Glc-P_2, E_D·Cd·Cd·Glc-P_2, or both (6.2 and −1.8 ppm).[2] [E_P·Li·Glc-1-P is not detected because of its low equilibrium concentration (Ray & Long, 1976)]. At 1.1 equiv of Cd^{2+}, the equilibrium

[2] The assignment of peaks from ternary complexes involving Li^+ are described in Rhyu et al. (1984). However, on the basis of the arguments presented below, these complexes actually may contain two bound Li^+ ions (they are produced in the presence of excess Li^+). Since we have no way of independently verifying this possibility, for the present we continue to represent them as having a single Li^+.

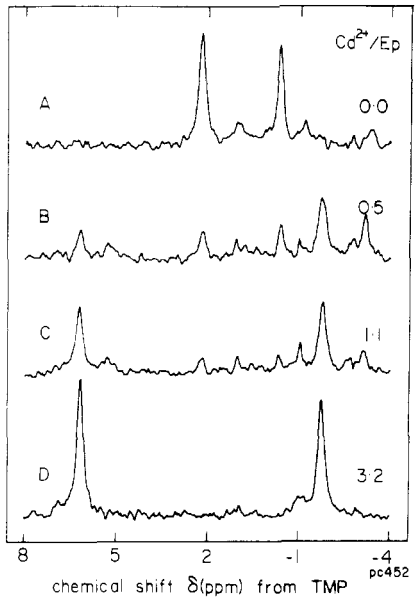

FIGURE 2: Titration of E_P·Li·Glc-6-P with $^{114}Cd(OAc)_2$ followed by ^{31}P NMR spectroscopy at 81 MHz. All solutions contained 1.3 mM E_P, 40 mM LiCl, 1.3 mM Glc-6-P, 20 mM Tris-Cl, and 10% ^2H_2O. The pH was 7.5. (A) Spectrum of E_P·Li·Glc-6-P; (B–D) spectra obtained after addition of the number of equivalents of Cd^{2+} indicated in the figure.

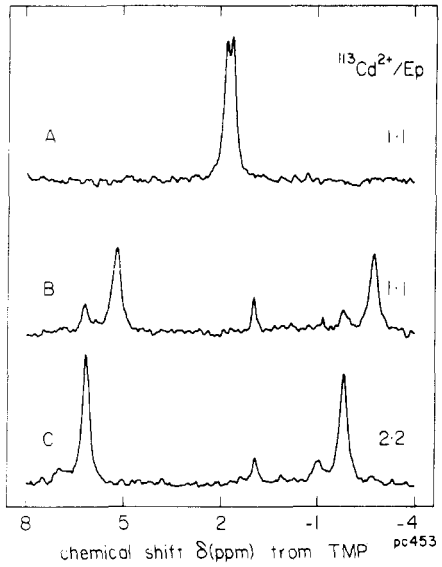

FIGURE 3: Binding of $^{113}Cd^{2+}$ and substrate to phosphoglucomutase as followed by ^{31}P NMR spectroscopy at 81 MHz. All solutions contained 20 mM Tris-Cl and 10% ^2H_2O. The pH was 7.5. (A) E_P·^{113}Cd [the solution contained 1.3 mM E_+ and 1.4 mM $^{113}Cd(OAc)_2$]; (B) after further addition of 1 equiv of glucose 1-phosphate (the primary species present is E_D·Cd·Glc-P_2); (C) after addition of a second equivalent (relative to the enzyme) of $^{113}Cd(OAc)_2$.

shifts to those additional species containing two metal ions, but some residual E_P·Li·Glc-6-P and E_D·Cd·Glc-P_2 remain (Figure 2C). Finally, in the presence of 3.2 equiv of Cd^{2+}, only the peaks from E_D·Cd·Cd·Glc-P_2 at 6.2 and −1.8 ppm are detected (Figure 2D). A similar titration was also followed by ^{7}Li NMR spectroscopy (see below).

The different ^{31}P NMR chemical shifts of the mono- and dicadmium complexes of E_D·Glc-P_2 can be observed by treating E_P·Cd (Figure 3A) with 1.0 equiv of glucose 1-phosphate (Figure 3B) followed by addition of a second equivalent of Cd^{2+} (Figure 3C). The spectra show that coupling between ^{113}Cd and ^{31}P of the serine phosphate at the active site disappears on the addition of substrate (Rhyu et al., 1984). The

Table I: ^{31}P NMR NOE and T_1 Relaxation Data for the Phosphates of Phosphoglucomutase Complexes[a]

species[b]	NOE	T_1 (s)
E_P·Cd	1.5	4.1 ± 0.2
E_D·Cd·Glc-*1*,6-P_2	1.2	5.9 ± 0.3
E_D·Cd·Glc-1,*6*-P_2	1.3	6.2 ± 0.2
E_D·Cd·Cd·Glc-*1*,6-P_2	1.4	4.8 ± 0.3
E_D·Cd·Cd·Glc-1,*6*-P_2	1.4	4.4 ± 0.2
E_D·Li·Glc-*1*,6-P_2	1.4	6.6 ± 0.5
E_D·Li·Glc-1,*6*-P_2	1.3	4.4 ± 0.2
E_P·Li·Glc-6-P	1.0	3.6 ± 0.1
E_P·Li·Glc-*6*-P	1.3	6.5 ± 0.2

[a] Samples were in 20 mM Tris-Cl containing 10% ^2H_2O. The pH was 7.5 and the temperature 20 °C. All ^{31}P NMR measurements were at 81 MHz. [b] In complexes involving two phosphate ester groups, the group whose relaxation is tabulated is italicized.

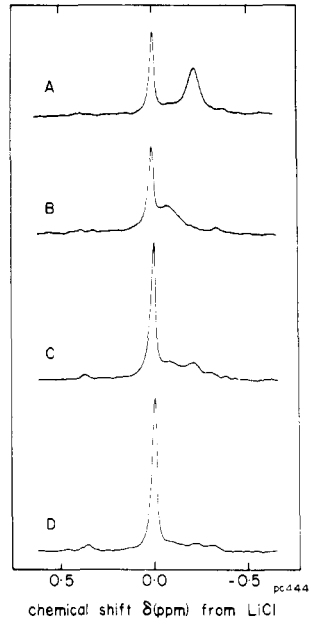

FIGURE 4: ^{7}Li NMR study at 78 MHz of Li^+ bound to phosphoglucomutase. All solutions contained 1.3 mM enzyme, 2.0 mM sugar phosphate, 1.3 mM LiCl, 20 mM Tris-Cl, and 10% ^2H_2O. The pH was 7.5. (A) E_P·Li·Glc-6-P; (B) E_D·Li·Glc-P_2; (C) spectrum obtained after addition of 0.5 equiv of $^{113}Cd(OAc)_2$ to E_D·Li·Glc-P_2; (D) spectrum obtained after the amount of $^{113}Cd(OAc)_2$ added to E_D·Li·Glc-P_2 was increased to 1.0 equiv.

line width of the 6-phosphate, which is located closer to the metal ion than the 1-phosphate at least in the predominant E_D·Cd·Glc-P_2 complex (Rhyu et al., 1984), was 19 ± 1 Hz (Figure 3B) in the case of ^{113}Cd and 18 ± 1 Hz in the case of ^{114}Cd (Figure 1A). Since the substitution of $^{114}Cd^{2+}$ by $^{113}Cd^{2+}$ does not affect the line width of the ^{31}P NMR signals in E_D·Cd·Glc-P_2 beyond the range of experimental error, the metal ion does not seem to interact directly with either of the phosphate groups in this complex. (The data do not rule out a ^{113}Cd–^{31}P coupling constant equal to or less than 5 Hz.) Comparison of the spectrum in Figure 3C with those in Figures 2C and D indicates that the chemical shifts of Glc-P_2 are the same in E_D·Cd·Cd·Glc-P_2 and E_D·Cd·Li·Glc-P_2, i.e., that binding of either Li^+ or Cd^{2+} at the ancillary site of E_D·Cd·Glc-P_2 alters the ^{31}P NMR signal of both phosphate groups in the same way. In the absence of substrate, addition of a second equivalent of Cd^{2+} to E_P·Cd does not affect the ^{31}P NMR chemical shift of the serine phosphate (data not shown).

The NOE and T_1 relaxation parameters for the above complexes of phosphoglucomutase (Table I) were used to interpret the relative intensities of the peaks in the above spectra. Without additional information needed to determine

the ^{31}P relaxation mechanism in these species, these data (Table I) cannot be interpreted in terms of molecular dynamics.

^{7}Li NMR Study. The tight binding of Li^+ to phosphoglucomutase–substrate complexes permits the observation of the NMR signal from the bound ion (Figure 4). The ^{7}Li chemical shift depends on the nature of the substrate bound: −0.24 ppm for E_P·Li·Glc-6-P, −0.13 ppm for E_P·Li·Glc-1-P, and −0.08 ppm for E_D·Li·Glc-P_2. The sharp peak in each spectrum in Figure 4 is produced by free $^{7}Li^+$. The peak for $^{7}Li^+$ in E_P·Li·Glc-6-P (Figure 4A) has a line width of 5 Hz and a T_1 of 0.51 ± 0.07 s compared with a reported value of 15.1 s for free $^{7}Li^+$ (Raushel & Villafranca, 1980).

The ^{7}Li NMR spectrum of E_P·Li showed only a single sharp peak at the chemical shift of free Li^+ (spectrum not shown). Under the condition of the experiment (10 mM Li^+, 1.3 mM E_P), only about 6% of the Li^+ is expected to be in the E_P·Li form (K_D for E_P·Li = ~10 mM; Ray et al, 1978). Failure to observe a signal from E_P·Li could be a result of any of several factors: coincidence of the chemical shifts of E_P·Li and free Li^+, fast exchange between free and enzyme-bound forms of ^{7}Li, broadening caused by exchange effects, or rapid quadrupolar relaxation of ^{7}Li in E_P·Li. The last possibility, however, appears remote since others have found relatively small quadrapolar effects on ^{7}Li relaxation (Villafranca & Raushel, 1980; Woessner et al., 1968).

Li^+ binds tightly to the enzyme in the presence of bound glucose phosphates. Thus, the estimated equilibrium constant for dissociation of Li^+ from the E_P·Li·Glc-6-P complex (about 0.8 mM) was calculated from the intensities of free and bound forms of Li^+ in the ^{7}Li NMR spectrum (Figure 4A) plus a knowledge of the total amount of Li^+ and E_P in solution. In this calculation the intensity of the free Li^+ peak was corrected for the loss in intensity expected as a result of the limited delay time used in signal accumulation. Moreover, from the results in Figure 4B, the dissociation constant for the E_D·Li·Glc-P_2 complex must be similar to that for the E_P·Li·Glc-6-P complex, and the same conclusion can be drawn for the E_P·Li·Glc-1-P complex (not shown). Thus, ^{7}Li in these complexes binds much less tighlty under the conditions of the NMR experiments than previously estimated on the basis of Li^+ inhibition of the initial velocity reaction (the estimated dissociation constant of Li^+ from the central complexes was about 10 μM; Ray et al., 1978). Since the rate of onset of Li^+ inhibition under initial velocity conditions is well correlated with the rate of dissociation of Mg^{2+} from the central complexes (W. J. Ray, Jr., unpublished results), competitive binding of Mg^{2+} and Li^+ appears to involve the metal ion activating site as opposed to an ancillary site or sites. At present, we have no rationale for the difference in apparent binding constants under these different conditions. The large discrepancy cannot be explained by an error in the concentration of enzyme or Li^+ used in the NMR experiments.

^{7}Li NMR also can be used to follow the displacement of bound Li^+ from E_P·Li·Glc-6-P or E_D·Li·Glc-P_2 by added Cd^{2+}. The ^{7}Li NMR signal from E_D·Li·Glc-P_2 appears as a broad shoulder on the sharper peak from free Li^+ (Figure 4B). In the presence of 0.5 equiv of Cd^{2+}, ^{7}Li resonances are detected from both E_P·Li·Glc-6-P and E_D·Li·Glc-P_2 (Figure 4C); the same species were detected under similar conditions by ^{31}P NMR (Figure 2B). The spectrum obtained after the addition of 1.0 equiv of Cd^{2+} to E_D·Li·Glc-P_2 shows no enzyme-bound Li^+, as is expected, since Cd^{2+} binds at the active site much more tightly than does Li^+ [compare Ray & Long (1976) with Ray et al. (1978)]; a similar spectrum (not shown) is obtained after addition of 1.0 equiv of Cd^{2+} to E_P·Li·Glc-6-P.

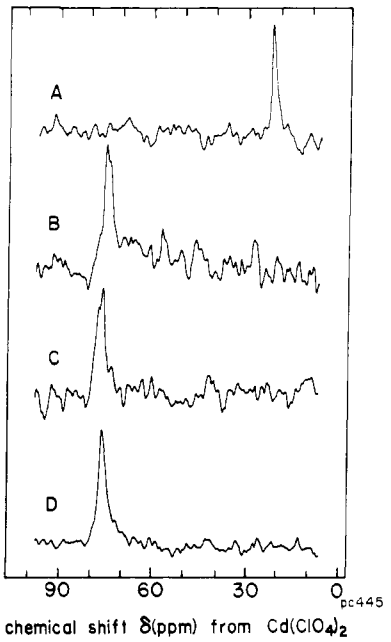

FIGURE 5: ^{113}Cd NMR spectra obtained at 44.4 MHz of Cd^{2+} bound to phosphoglucomutase. All solutions contained 20 mM Tris-Cl in 10% $^{2}H_2O$. The pH was 7.5. (A) E_P·Cd [the solution contained 1.3 mM E_P and 1.3 mM $^{113}Cd(OAc)_2$]; (B) E_D·Cd·Glc-P_2 [the solution initially contained 1.5 mM E_P, 1.5 mM Glc-6-P, and 1.5 mM $^{113}Cd(OAc)_2$]; (C) E_D·Cd·Li·Glc-P_2 [the solution initially contained 1.5 mM E_P, 1.5 mM Glc-6-P, 1.5 mM $^{113}Cd(OAc)_2$, and 40 mM LiCl]; (D) E_D·Cd·Cd·Glc-P_2 [the solution initially contained 1.5 mM E_P, 1.5 mM Glc-6-P, 3.0 mM $^{113}Cd(OAc)_2$, and 0.45 mM Glc-P_2].

^{113}Cd NMR Study. The ^{113}Cd NMR spectrum of E_P·Cd (Figure 5A) shows a single line at 22 ppm with a line width of 20 Hz. The 16-Hz ^{113}Cd–^{31}P coupling observed in the ^{31}P NMR peak of this species (Figure 3A) is not detected in the ^{113}Cd NMR spectrum because of a poor signal to noise ratio and the large broadening factor used in exponential apodization (20 Hz). E_P·Cd·Glc-P_2 (Figure 5B) shows a resonance at 75 ppm with a line width of 70 Hz. Addition of 1.0 equiv of Li^+ (Figure 5C) or a second equivalent of Cd^{2+} (Figure 5D) to E_D·Cd·Glc-P_2 did not cause a shift of the peak at 75 ppm beyond the experimental error. Although it is known from the ^{31}P and ^{7}Li NMR studies presented above that a second metal ion binds to form E_D·Cd·M·Glc-P_2, the second Cd^{2+} is not observed by ^{113}Cd NMR spectroscopy. In the absence of substrate, addition of a second equivalent of Cd^{2+} results neither in a ^{113}Cd chemical shift change of the E_P·Cd peak nor in the generation of a new resonance from bound or free Cd^{2+}. Therefore, it appears that the second equivalent of Cd^{2+} added to either E_D·Cd·Glc-P_2 or E_P·Cd is relaxed rapidly by an intermediate exchange process (Armitage & Otvos, 1982) created either by central metal exchange or by facile ligand exchange. Alternatively, exchange broadening of the ^{113}Cd NMR signal from the second equivalent of $^{113}Cd^{2+}$ added to E_P could be the result of nonspecific binding, and the specific binding site is formed only when substrate is bound. In either case, binding of a second metal ion does not affect the ^{31}P NMR chemical shift of the enzymic phosphate or the chemical shift of $^{113}Cd^{2+}$ bound to the tight site. The second equivalent of Cd^{2+} is not critical to the activity of the enzyme since, in common with other metal ions (Ray, 1969), the addition of 1 equiv of Cd^{2+} to inactive, metal-free enzyme produces maximal activity (W. J. Ray, Jr., unpublished results) and since the enzyme produced by the addition of excess Cd^{2+} is maximally active for many minutes in the presence of excess

Table II: Summary of ^{31}P, ^{7}Li, and ^{113}Cd NMR Chemical Shifts of Metal Liganded Forms of Phosphoglucomutase[a]

species[b]	chemical shift		
	^{31}P	^{7}Li	^{113}Cd
Ep·Cd	1.7		22
E_D·Cd·Glc-1,6-P_2	−2.8/5.2		75
E_D·Cd·Cd·Glc-1,6-P_2	−1.8/6.2		75
E_D·Cd·Li·Glc-1,6-P_2	−1.8/6.2		75
E_D·Li·Glc-1,6-P_2	−3.2/−1.0	−0.08	
E_P·Li·Glc-1-P	−1.0/−3.2	−0.13	
E_P·Li·Glc-6-P	2.2/−0.4	−0.24	

[a] Samples were in 20 mM Tris-Cl–10% $^{2}H_2O$, pH 7.5. [b] In the complexes involving two phosphate ester groups, the chemical shifts are given in the order of the phosphorus atoms in the abbreviation for the species (column 1).

EDTA, i.e., under conditions where a loosely bound metal ion would rapidly dissociate (Ray, 1969). The chemical shifts of the various metal liganded forms of phosphoglucomutase studied are summarized in Table II.

Discussion

The ability to slow the rate of the catalytic transfer of phosphate groups between the enzyme and its bound substrate to approximately 10^{-8} that of the normal transfer rate by what appears to be a structurally conservative change—substitution of Li^+ for Mg^{2+} at the activation site for metal ions—allows structural studies to be conducted, separately, on each of the three central complexes specified in eq 1. A previous paper describes ^{31}P NMR studies of the two phosphate groups in these complexes (Rhyu et al., 1984). The significant differences in chemical shifts for these phosphates that are produced by substrate binding indicate that the process of transferring phosphate groups to and from the enzyme does not involve the same environment for the phosphates of Glc-1-P and Glc-6-P nor is the environment of the enzymic phosphate group the same in the presence of bound Glc-1-P (reactant) and bound Glc-6-P (product). The present study extends these findings by showing that the environment of the bound $^{7}Li^+$ also is different in each of these complexes.

Previous studies in other enzymic systems have utilized the effect of bound Li^+ on the NMR signal from bulk $^{7}Li^+$ to deduce properties of the Li^+ binding site. Such studies depend on rapid exchange between the two states and usually involve Li^+ binding at the site for monovalent cations, where the bound form of Li^+ is less likely to be observed. To our knowledge, the present study is the first in which the bound state of $^{7}Li^+$ has been directly observed. A major problem in using Li^+ to study metal binding sites in diamagnetic systems, where bound Li^+ must be observed, is lifetime broadening of the resonance. In fact, we were unable to observe Li^+ bound in the binary E_P·Li complex, presumably because of relaxation by chemical exchange. Only in the presence of bound substrate, which substantially slows the rates of dissociation for bivalent metal ion activators (Ray, 1969), was bound $^{7}Li^+$ observed directly.

The small chemical shifts of bound $^{7}Li^+$ (relative to aqueous LiCl) observed in the three enzyme–substrate complexes stand in contrast with the much larger chemical shifts that are observed for bound Cd^{2+} [relative to aqueous $Cd(ClO_4)_2$; see below]. In fact, even when chemical shifts are observed for bound Li^+ these may well prove too insensitive to environment to be of much diagnostic use, except for internal comparisons. But significant differences in the chemical shift of bound $^{7}Li^+$ in the E_P·Li·Glc-1-P, E_D·Li·Glc-P_2, and E_P·Li·Glc-6-P complexes (Figure 4 and Table II) do provide further support for the suggestion that an altered coordination may accompany the phosphate transfer steps that interconvert these complexes (Rhyu et al., 1984; see also below). thus, on going from E_P·Li·Glc-1-P to E_D·Li·Glc-P_2 and to E_P·Li·Glc-6-P, the chemical shift of Li^+ changes from −0.13 to −0.08 and finally to −0.24 ppm. In the first of these complexes the primary 6-hydroxy group is positioned to serve as the phosphate acceptor group, whereas in the third complex the secondary 1-hydroxyl group is so positioned. Apparently, this difference is sufficient not only to significantly alter the environment of the enzymic phosphate, as noted above, but also the environment of Li^+ bound at the metal ion activating site. Obviously, the environments of the enzymic phosphate group and Li^+ bound to this site are interrelated, although at present we have no firm evidence for direct coordination between the metal ion and phosphate groups in the case of Li^+.

Presumably, the phosphate group will be bound at the same general site on the enzyme after transfer either to the 1-hydroxyl group of Glc-6-P or to the 6-hydroxyl group of Glc-1-P (Ray et al., 1976). However, the environment of Li^+ in the E_D·Li·Glc-P_2 complex is different from that in either of the monophosphate complexes, which shows that even if the general site is the same, some of the properties have been altered. In addition, the ^{7}Li NMR studies fail to provide evidence for two different binding modes for the bisphosphate in its complex with the dephosphoenzyme, in contrast with what has been inferred from earlier work, although a model in which one binding mode predominates (Ma & Ray, 1980) is not ruled out.

The ^{113}Cd–^{31}P coupling in E_P·Cd provides unequivocal evidence for direct coordination of Cd^{2+} by the enzymic phosphate group (Rhyu et al., 1984) but leaves unanswered the question of whether smaller metal ions such as Mg^{2+} and Li^+ are similarly coordinated (the ionic radii for Cd^{2+}, Mg^{2+}, and Li^+ are 0.97, 0.66, and 0.68 Å, respectively; Weast 1980). Also inconclusive, relative to coordination identities, are experiments showing competitive binding by different pairs of metal ions, to which now can be added the Cd^{2+}/Li pair (see below). In fact, differences in the effect of pH on competitive binding of Mg^{2+}, Mn^{2+}, and Zn^{2+} indicate that the mutually exclusive nature of the binding demonstrated in competitive experiments should not be lightly interpreted in terms of identical arrays of coordinating ligands (Ray, 1969), although one or more mutual ligands is expected. Since bound $^{7}Li^+$ or $^{6}Li^+$ was found to have no effect on the line width of the ^{31}P NMR signal of the enzymic phosphate in E_P·Li (Rhyu et al., 1984), this experiment was inconclusive with regard to possible Li^+–phosphate coordination. The large paramagnetic effect on the enzymic phosphate produced by bound Ni^{2+} and Co^{2+}, however, certainly indicates a close proximity of the activating metal ion to the enzymic phosphate, if not direct coordination (Ray et al., 1977).

The elimination of observable splitting in the ^{31}P NMR spectrum of E_D·Cd·Glc-P_2 (i.e., after binding of glucose monophosphate to E_P·Cd followed by the first phosphate transfer step) also is somewhat equivocal but suggests that in this complex bound Cd^{2+} no longer is directly coordinated with either phosphate group of the bisphosphate (Rhyu et al., 1984). The lack of a significant difference in the ^{31}P NMR line widths for the enzymic phosphate when $^{113}Cd^{2+}$ is replaced by $^{114}Cd^{2+}$ indicates that if any residual ^{113}Cd–^{31}P coupling remains, the coupling constant is not more than about 5 Hz.

Binding of a second Cd^{2+} to the enzyme was not detected by ^{113}Cd NMR, probably as the result of broadening caused by exchange between the free and bound forms of Cd^{2+} as expected for a weak binding site, although excessive line broadening by exchange of a metal ligand cannot be ruled out.

The ^{113}Cd NMR resonance of $^{113}Cd^{2+}$ in E_D·Cd·Glc-P_2 is 53 ppm down field from its resonance in E_P·Cd. From data available at present, such a change in chemical shift is in the direction expected for the replacement of an oxygen ligand by a nitrogen ligand (Armitage & Otvos, 1982). Recent ^1H NMR pH titration studies of phosphoglucomutase show that one histidine side chain is liganded to Cd^{2+} in E_D·Cd·Glc-P_2 but not in E_P·Cd (Rhyu et al., 1985). Hence, in thise case, the downfield shift of ^{113}Cd in E_D·Cd·Glc-P_2 could be explained by replacement of one oxygen ligand in E_P·Cd by an imidazole nitrogen ligand in E_D·Cd·Glc-P_2.

It is clear from the existence of a second pair of ^{31}P NMR peaks that a second metal (either Cd^{2+} or Li^+) can bind to the enzyme to form a quaternary E_D·Cd·M·Glc-P_2 complex. Since added Cd^{2+} displaces bound Li^+ in E_P·Li·Glc-6-P or E_D·Li·Glc-P_2 (Figure 4), Cd^{2+} and Li^+ appear to bind at the same site in E_D·M·Glc-P_2. In the presence of 1.0 equiv each of Cd^{2+} and Li^+, Cd^{2+} binds at the activating site, and Li^+ binds at the weak ancillary site, which is also the binding site of the second Cd^{2+} in E_D·Cd·M·Glc-P_2. The second Cd^{2+} binds more tightly than Li^+ to the ancillary site and displaces it (compare Figures 1D and 3C).

The ^{31}P NMR chemical shifts of the sugar phosphates in E_P·Li·Glc-6-P or E_D·Li·Glc-P_2 are not affected appreciably by the addition of 1 equiv of Cd^{2+} (Figure 2B), although peak intensities are drastically reduced because of competitive binding with Li^+. But the addition of a second equivalent of either Cd^{2+} or Li^+ produces an equal change in the chemical shifts of both phosphates of glucose 1,6-bisphosphate in the E_D·Cd·Glc-P_2 complex. As is shown in Figures 1E, 2C,D, and 3C, the change in chemical shift is the same whether the second metal ion is Li^+ or Cd^{2+}. Because the same change is produced by quite different metal ions and involves both phosphate groups of the bisphosphate, the second metal ion probably does not interact directly with either phosphate. Instead, it seems more likely that binding of Li^+ or Cd^{2+} to the weak, ancillary metal ion binding site causes a conformational rearrangement that produces the same effect on the ^{31}P NMR peaks of both phosphate groups. But the catalytic activity of phosphoglucomutase is not significantly altered by metal ion binding at such ancillary sites, as is shown by following activity regain during titration of the metal-free enzyme with tight-binding metal ions [Mn^{2+}, Co^{2+}, Zn^{2+} (Ray, 1969), or Cd^{2+} (W. J. Ray, Jr., unpublished results)] and by the linearity in double-reciprocal plots of activity and metal ion concentration [from $0.6K_m$ to $50K_m$ (Ray & Roscelli, 1966; W. J. Ray, Jr., unpublished results)] for the more loosely bound physiological activator, Mg^{2+}.

As a probe of activating sites on enzymes, Li^+ usually has been used for monovalent cation binding sites. The results reported here, as well as inhibition studies with glucose bisphosphate synthase (Wong & Rose, 1976) and *myo*-inositol-1-phosphatase (Hallcher & Sherman, 1980), suggest that Li^+ also may be a more useful probe of enzymic binding sites for bivalent metal ions than has been suspected previously.

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