4746 *Biochemistry* **1985**, *24*, 4746–4753

Active-Site Serine Phosphate and Histidine Residues of Phosphoglucomutase: pH Titration Studies Monitored by ^1H and ^{31}P NMR Spectroscopy[†]

Gyung Ihm Rhyu,[‡] William J. Ray, Jr.,[§] and John L. Markley*[,‡]
Departments of Chemistry and Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received December 4, 1984

ABSTRACT: ^1H and ^{31}P NMR pH titrations were conducted to monitor changes in the environment and protonation state of the histidine residues and phosphoserine group of rabbit muscle phosphoglucomutase on binding of metal ions at the activating site and of substrate (glucose phosphate) at the catalytic site. Imidazole C_ϵ–H signals from 8 of the 10 histidines present in the free enzyme were observed in ^1H NMR spectra obtained by a spin-echo pulse sequence at 470 MHz; their pH* (uncorrected pH meter reading of a ^2H_2O solution measured with a glass electrode standardized with H_2O buffer) titration properties (in 99% ^2H_2O) were determined. Three of these histidine residues, which have pK_a values ranging from 6.5 to 7.9, exhibited an atypical pH-dependent perturbation of their chemical shifts with a pH_{mid} of 5.8 and a Hill coefficient of about 2. Since none of the observed histidines has a pK_a near 5.8, it appears that these three histidines interact with a cluster consisting of two or more groups which become protonated cooperatively at this pH*. Binding of Cd^{2+} at the activating site of the enzyme abolishes the pH-dependent transition of these histidines; hence, the putative anion cluster may constitute the metal ion binding site, or part of it. Two separate ^{31}P NMR peaks from phosphoserine-116 of the phosphoenzyme were observed between pH 6 and 9. Apparently, the metal-free enzyme exists as a pH-dependent mixture of conformers that provide two different environments, I and II, for the enzymic phosphate group; the transition of the phosphate group between these two environments is slow on the NMR time scale. The pH-dependent transitions arise from prototropic equilibria that may involve neighboring groups rather than the enzymic phosphate itself. The fractional populations of these species as a function of pH specify a pH_{mid} of 6.10 and a Hill coefficient of 1.50 for the low-pH transition from environment I to II; the high-pH transition, which was less well characterized, has a much smaller Hill coefficient. Binding of Cd^{2+} at the active site of the enzyme abolishes the transitions at both low and high pH. These observations are in accord with the direct interaction of the enzymic phosphate group with bound Cd^{2+} [Rhyu, G. I., Ray, W. J., Jr., & Markley, J. L. (1984) *Biochemistry 23*, 252–260]. Formation of the ternary complex of dephosphoenzyme, Cd^{2+}, and glucose bisphosphate (transfer of the enzymic phosphate to bound glucose phosphate) eliminates the interaction between the metal ion and what originally was the enzymic phosphate group [Rhyu, G. I., Ray, W. J., Jr., & Markley, J. L. (1985) *Biochemistry 24*, 2536-2541], although it does not normalize the pK_a of the phosphate group, which remains substantially below 5. Thus, the phosphate group that is transferred in the catalytic step is a dianion both before and after transfer, viz., both when it is attached to the enzyme and after it has become part of the bound bisphosphate. In addition, the above transformation appears to decrease the pK_a of a single histidine residue to well below 5, and it seems likely that the side chain of this histidine replaces the enzymic phosphate group when the latter group leaves the coordination sphere of the bound metal ion upon formation of the dephosphoenzyme–bisphosphate complex. This interpretation is in accord with the observation that the chemical shift of the C_ϵ–H nucleus of the histidine with the low pK_a in the dephosphoenzyme–glucose bisphosphate complex is dependent of the identity of the bound metal ion, viz., is 7.98 ppm for the complex involving Cd^{2+} and 8.39 ppm for the complex involving Li^+.

Phosphoglucomutase is a protein of M_r 61 600 whose sequence of 561 amino acid residues is known (Ray et al., 1983). The catalytically active enzyme is phosphorylated at serine-116 and requires a bivalent metal ion as an activator for phosphate transfer steps involving this group. When a bivalent metal ion (M)[1] is bound at the activating site and a substrate is bound at the catalytic site of the enzyme, an equilibrium is established among the intermediate species, E_P·M·Glc-1-P, E_D·M·Glc-P_2, and E_P·M·Glc-6-P, which in the case of Cd^{2+} is heavily in favor of E_D·Cd·Glc-P_2. Li^+ can also bind at the activating site for metal ions (Rhyu et al., 1985), but bound Li^+ blocks the phosphate transfer steps that interconvert these intermediates (Ray et al., 1978). Recently, the interaction beteween metal ions and the two phosphate groups in the intermediate complexes was studied by ^{31}P NMR spectroscopy (Rhyu et al.,

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[†] This work was supported in part by Grants GM19907 (J.L.M.) and GM08963 (W.J.R.) from the National Institutes of Health. The Purdue University Biochemical Magnetic Resonance Laboratory has financial support from Grant RR01077 from the Biotechnology Resources Program of the Division of Research Resources, National Institutes of Health.

[*] Address correspondence to this author at the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, WI 53706.

[‡] Department of Chemistry.

[§] Department of Biological Sciences.

[1] Abbreviations: E_P and E_D, 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; P_i, inorganic orthophosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3*-d_4; TMP, trimethyl phosphate; OAc, acetate; ppm, parts per million; NOE, nuclear Overhauser effect; T_1, spin–lattice relaxation time; pH*, uncorrected pH meter reading of a ^2H_2O solution measured with a glass electrode standardized with H_2O buffer.

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1984). The 16-Hz splitting of the ^{31}P NMR signal of the phosphoenzyme produced by bound ^{113}Cd^{2+} showed that the metal ion is bound within the coordination sphere of the enzymic phosphate group. However, reaction of $E_P{\cdot}^{113}Cd$ with glucose-1-P to produce the $E_D{\cdot}^{113}Cd{\cdot}Glc\text{-}P_2$ complex eliminated (or greatly reduced) this ^{113}Cd-induced splitting, presumably by removing the phosphate group from the coordination sphere of bound ^{113}Cd^{2+}. A subsequent study of possible changes in environment of the metal ion produced during or as the result of the above phosphate transfer step was conducted by means of ^{7}Li and ^{113}Cd NMR spectroscopy (Rhyu et al., 1985). The results showed that the environment of the metal ion is indeed altered by this process and that the absence of ^{113}Cd-induced splitting in the $E_D{\cdot}^{113}Cd{\cdot}Glc\text{-}P_2$ complex does not arise because of broadening or fragmentation of the ^{113}Cd NMR resonance. Instead, the 50 ppm change in the ^{113}Cd chemical shift is consistent with the introduction of a nitrogen ligand into the coordination sphere of the metal ion to replace the departing phosphate group.

The present high-resolution NMR study was undertaken to further examine the effect of metal ion binding on the enzymic phosphate group and to determine whether metal ion binding or the production of the $E_D{\cdot}M{\cdot}Glc\text{-}P_2$ complex alters the environments of any of the 10 histidine side chains of the enzyme. The results indicate that the bound metal ion tends to fix the environment of the enzymic phosphate group in the binary enzyme–metal ion complex and further suggest that the side chain of one of the histidine residues may replace the phosphate group when it is removed from the coordination sphere of the metal ion as the result of forming the dephosphoenzyme–glucose bisphosphate complex.

EXPERIMENTAL PROCEDURES

Enzyme and Chemicals. Deuterium oxide (99.8% isotopically pure) was purchased from Bio-Rad Laboratories. ^{7}LiCl was obtained from Alfa Products, and 95.3 atom % ^{113}Cd metal was obtained from Prochem. ^{113}Cd metal was converted to the acetate before use. Additional reagents were of the highest purity available. Sugar phosphates and the metal-free phospho and dephospho forms of phosphoglucomutase were prepared as in Rhyu et al. (1984). Metal-free enzyme is completely inactive; it regains total activity on the addition of 1 equiv of metal ion (Ray, 1969).

To minimize the water peak in ^{1}H NMR spectra, deuterated solvents were used. The protein solution in 20 mM Tris, pH 7.5, in H_2O was dialyzed against 4 volumes of the analogous buffer in ^2H_2O for 2.5 h with three changes of outside solution; pressure was applied to the contents of the dialysis tube to maintain an approximately constant protein concentration. After the fourth dialysis step, the final protein concentration was adjusted by dilution with an aliquot of the outside solution. Protein solutions used for ^{1}H NMR studies carried out below pH* 6.25 were prepared by dialysis overnight at 4 °C against 0.1 M acetate buffer, pH 6.0 in H_2O, containing 0.1 mM EDTA. Dialysis was continued for 6 h with four changes against 20 mM acetate, pH 6.0 in H_2O, and then for 2.5 h with four more changes against the same buffer in ^2H_2O. Unless otherwise indicated, all protein solutions contained 0.1 M KCl. The final enzyme concentration was 1.3 mM (80.6 mg/mL). Protein solutions were titrated as described previously (Markley & Porubcan, 1976).

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 as described elsewhere (Rhyu et al., 1984). ^{31}P NMR chemical shifts are reported with respect to trimethyl phosphate. These chemical shifts can be converted to 85% H_3PO_4 as the external reference by adding 2.7 ppm. ^{1}H NMR spectra (470 MHz) were obtained in the Fourier-transform mode with a Nicolet NT-470 11.1 T spectrometer. The residual water resonance in the spectrum was saturated by irradiation with a single radio-frequency pulse which was gated off during acquisition. A spin-echo pulse sequence, $90°_x$–$(\tau–180°_x–\tau)_2$–ACQ (Meiboom & Gill, 1958), was used to minimize the background of signals from undeuterated NH groups (Campbell et al., 1975). An average of 1024 transients were accumulated by digitization of 8192 data points. The spectral width was 6000 Hz the line broadening factor used in exponential apodization was 1 Hz, and the pulse repetition time was 3.7 s. Chemical shifts are reported with respect to internal sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP).

The histidine peaks have not been assigned to specific residues; they have been labeled with small letters in an arbitrary manner. The number associated with each letter refers to the figure in which the labeling is defined. The titration curves were constructed by tracing individual peaks from the highest pH* and/or lowest pH* value. When the connectivity of the points through the mid-pH* range could not be traced unambiguously on the basis of chemical shift data alone, the line widths of the peaks and their intensities, and/or the Hill coefficients of the resulting curves were also taken into consideration. The crossover points that have been deduced are indicated in the titration curves. The data were treated by a nonlinear least-squares analysis program (Markley, 1973) that fitted the data to a modified form of the Hill equation.

RESULTS

^{1}H NMR Study of the pH Titration of Phosphoglucomutase. (A) Phosphoenzyme. Spectra of E_P were obtained at 25 pH* values between 4.82 and 8.75. Out of the total of 10 histidine residues in the enzyme (Ray et al., 1983), ^{1}H NMR signals were observed from at least 8 imidazole C_ϵ–H groups, and their pH titration properties have been studied. Spectra obtained at 20 °C at five selected pH* values are shown in Figure 1. (The sharp peak at 4.86 ppm in the spectra obtained at pH* values 5.38 and 4.82 is from formate, a contaminant of the acetic acid.) Values of parameters assigned to the various transitions on the basis of a simple ionization function and an adjustable Hill coefficient are summarized in Table I. Fitted pH titration curves of histidine residues are shown in Figure 2. Histidine peaks 1a and 1b (Figure 1) are shifted downfield from other histidine resonances and have higher pK_a* values (7.88 and 8.55). Peaks 1b and 1f disappear near their pK_a, probably because of intermediate exchange broadening (Sudmeier et al., 1980). Peak 1f′ may represent either the deprotonated form of peak 1f or one of the two histidine residues otherwise unaccounted for, if its signal is narrow at high pH. These two possibilities cannot be distinguished with the present data. Peaks 1a, 1f, and 1h each exhibit a second transition with pH^*_{mid} values of 5.80, 5.89, and 5.80, respectively. The Hill coefficient for the second transition is 2.1 ± 0.2 for each of the three histidines (Table I). Since none of them exhibits a pK_a* near 5.8, the second transition likely arises from a conformational change induced by protonation of two or more groups that titrate cooperatively with a pH^*_{mid} near 5.8. Since the chemical shift perturbation produced by this change yields a continuous curve as a function of pH*, the lifetime (τ_i) of each conformational state (at the pH_{mid} of 5.8) must be less than the reciprocal of the chemical shift difference in hertz ($\tau_i < 1$ ms).

(B) Cd^{2+}–Enzyme. ^{1}H NMR spectra of $E_P{\cdot}Cd$ obtained at four selected pH* values are shown in Figure 3. The 16-point, computer-fitted pH titration curves of the seven

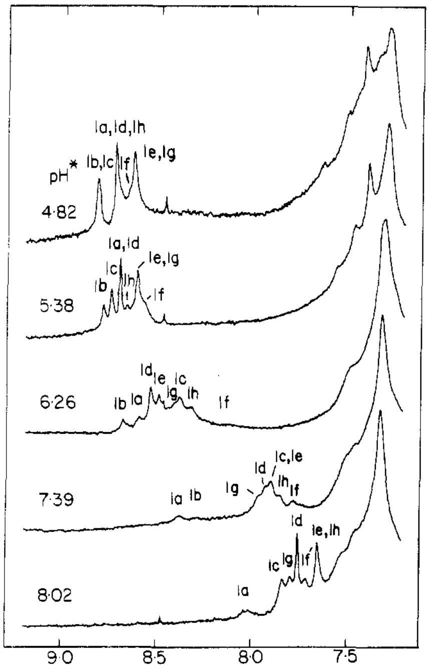

FIGURE 1: ^{1}H NMR spectra at 470 MHz (histidine C_ϵ–H region) of E_P at selected pH* values. The protein concentration was 1.3 mM. Solutions containing 1 M Tris base and 0.1 M acetic acid or 1 M acetic acid and 0.1 M Tris base were used as the basic or acidic titrants, respectively.

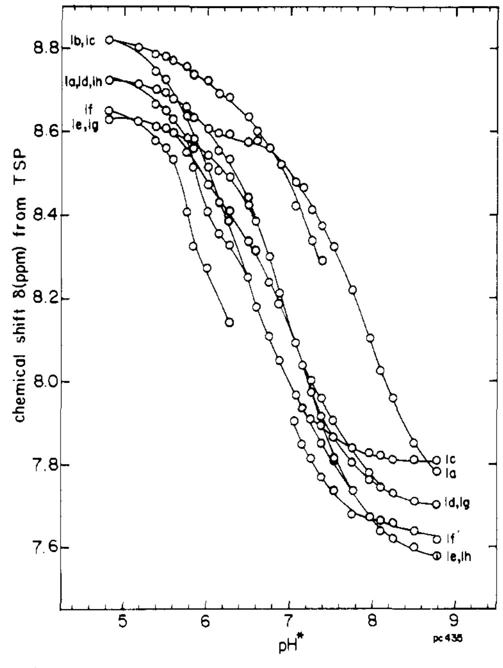

FIGURE 2: ^{1}H NMR (470 MHz) pH titration curves of the histidine resonances of E_P. Conditions are described in the legend of Figure 1.

Table I: ^{1}H NMR pH Titration Parameters for Histidine Residues of E_P[a]

histidine peak[b]	pK_a or pH$_{mid}$[c]	Hill coefficient	low-pH plateau	high-pH plateau
1a	5.80 ± 0.03[c]	1.85 ± 0.27	8.72 ± 0.01	8.57 ± 0.01
	7.88 ± 0.03	0.93 ± 0.05	8.63 ± 0.01	7.65 ± 0.03
1b	8.55 ± 0.01	0.53 ± 0.06	8.85 ± 0.01	5.94 ± 1.75
1c	6.35 ± 0.01	0.99 ± 0.02	8.85 ± 0.01	7.80 ± 0.00
1d	6.88 ± 0.01	1.06 ± 0.02	8.72 ± 0.01	7.69 ± 0.01
1e	7.06 ± 0.01	1.07 ± 0.02	8.63 ± 0.00	7.56 ± 0.01
1f	5.80 ± 0.03[c]	2.29 ± 0.32	8.65 ± 0.02	8.10 ± 0.03
	d			
1f′	*d*			<7.65
1g	6.91 ± 0.02	0.76 ± 0.03	8.66 ± 0.01	7.65 ± 0.01
1h	5.89 ± 0.05[c]	2.13 ± 0.44	8.71 ± 0.01	8.26 ± 0.05
	6.81 ± 0.07	0.71 ± 0.06	8.65 ± 0.07	7.53 ± 0.02

[a] For conditions, see the legend to Figure 1. Error estimates are from the curve-fitting routine and represent 1 standard deviation. [b] The labels refer to the peak designated in Figure 1. [c] The number represents the pH$_{mid}$ value for the spectroscopic pH transition, as opposed to the pK_a value for the titration of the indicated histidine residues. [d] The pK_a could not be determined because of broadening and disappearance of the histidine peak at a pH close to its pK_a value.

Table II: ^{1}H NMR pH Titration Parameters for Histidine Residues of E_P·Cd[a]

histidine peak[b]	pK_a	Hill coefficient	low-pH plateau	high-pH plateau
3a	8.01 ± 0.05	0.75 ± 0.03	8.66 ± 0.01	7.66 ± 0.04
3b	7.28 ± 0.02	0.82 ± 0.03	8.77 ± 0.01	7.72 ± 0.02
3c	6.35 ± 0.01	1.13 ± 0.03	8.81 ± 0.01	7.81 ± 0.01
3d	6.80 ± 0.01	1.09 ± 0.03	8.72 ± 0.01	7.70 ± 0.01
3e	6.91 ± 0.01	1.06 ± 0.03	8.62 ± 0.01	7.57 ± 0.01
3f	6.01 ± 0.13	0.93 ± 0.22	8.79 ± 0.06	7.73 ± 0.17
3g	6.51 ± 0.03	0.82 ± 0.04	8.71 ± 0.01	7.69 ± 0.01

[a] For conditions, see the legend to Figure 3. Error estimates are from the curve-fitting routine and represent 1 standard deviation. [b] The labels refer to peaks designated in Figure 3.

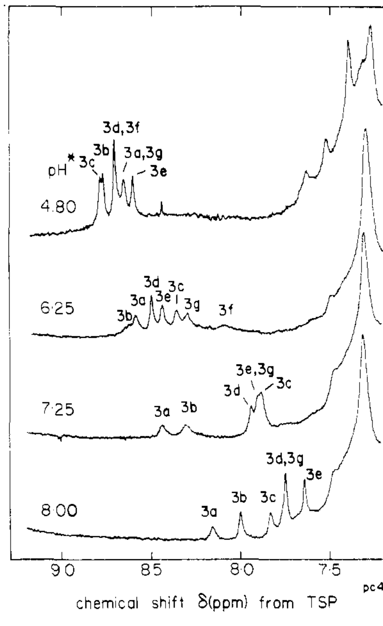

FIGURE 3: ^{1}H NMR spectra at 470 MHz (histidine C_ϵ–H region) of E_P·Cd at selected pH* values. The concentrations of protein and Cd(OAc)$_2$ were 1.3 mM; titrants are described in the legend of Figure 1.

observed histidines in E_P·Cd obtained between pH* 8.75 and 4.80 are shown in Figure 4. The two histidines with the highest pK_a* values (histidine peaks 3a and 3b in Figure 3 and Table II) yielded sharper peaks in E_P·Cd than in E_P (histidine peaks 1a and 1b in Figure 1 and Table I). No second pH transition was observed in the titration curve of any of the seven observable histidine residues in E_P·Cd.

(*C*) *Cd^{2+}–Enzyme in the Presence of Substrate.* ^{1}H NMR spectra of E_P·Cd in the presence of substrate obtained at four selected pH* values are shown in Figure 5. Fitted, 17-point pH titration curves of the eight histidine residues between pH* 8.75 and 5.0 are shown in Figure 6. The parameters obtained are given in Table III. Peak 5g (Figure 5) does not titrate between pH 6 and 8.2 and broadens out and disappears at low pH. In this pH range, the predominant species is E_D·Cd·Glc-P_2 (Ray & Long, 1976), and in this complex, Cd^{2+} appears to

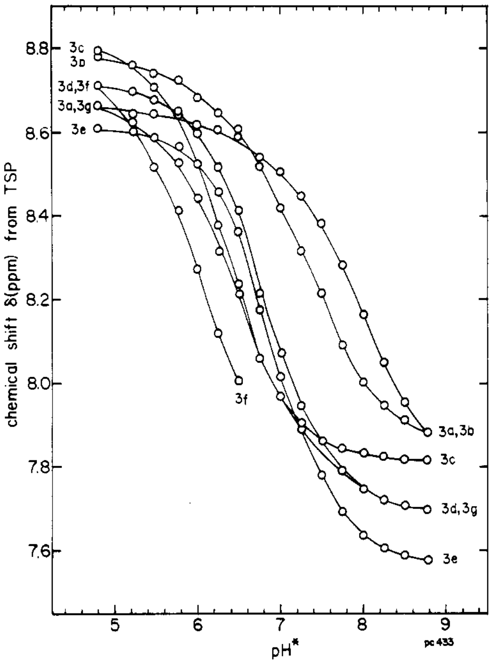

FIGURE 4: ^{1}H NMR (470 MHz) pH titration curves of the histidine resonances of E_P•Cd. The conditions are described in the legend of Figure 3.

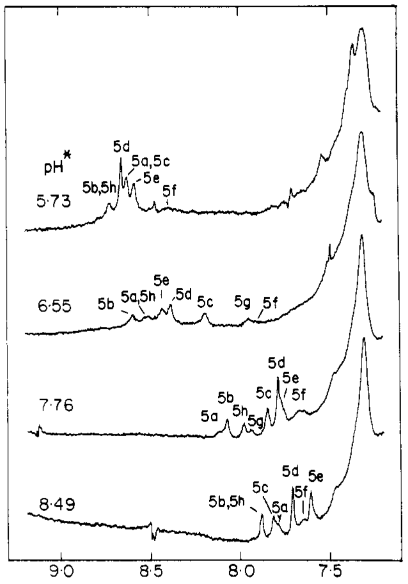

FIGURE 5: ^{1}H NMR spectra at 470 MHz (histidine C_ϵ–H region) of Cd^{2+}–enzyme in the presence of substrate at selected pH* values. The concentrations of $Cd(OAc)_2$ and Glc-1-P were 1.3 mM. At neutral pH, the major enzyme species present under these conditions is E_D•Cd•Glc-P_2. Titrants are described in the legend of Figure 1.

bind to one of the histidine residues of the enzyme, i.e., to the histidine which does not titrate (see Discussion). On the other hand, a similar titration study of the same complex monitored by ^{31}P NMR showed that at low pH* (pH* <6) the equilibrium shifts from E_D•Cd•Glc-P_2 to other species (spectra not shown). The chemical exchange that interconverts these species may well cause the extensive broadening of histidine resonance 5g that was observed at low pH*.

(*D*) *Li^+–Enzyme in the Presence of Substrate.* Spectra of E_D•Li•Glc-P_2 obtained at four selected pH* values are shown in Figure 7. The 17-point, fitted pH titration curves of the seven histidine that were observed in E_D•Li•Glc-P_2 between pH* 8.75 and 4.75 are shown in Figure 8; the titration parameters obtained are given in Table IV. The seven resolved

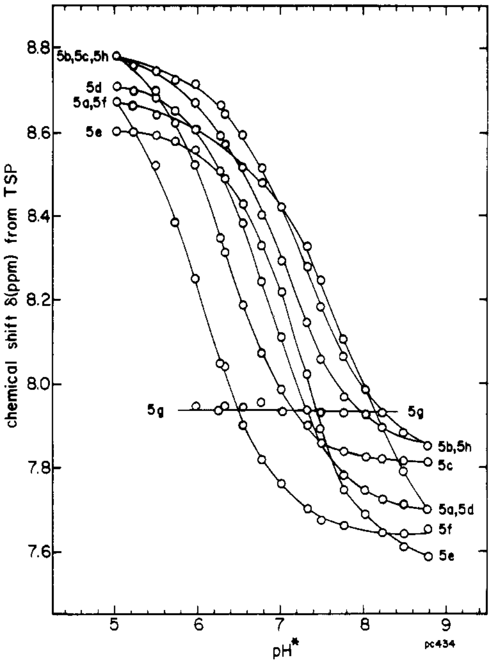

FIGURE 6: ^{1}H NMR (470 MHz) pH titration curves of histidine resonances of Cd^{2+}–enzyme in the presence of substrate. The conditions are described in the legend of Figure 5.

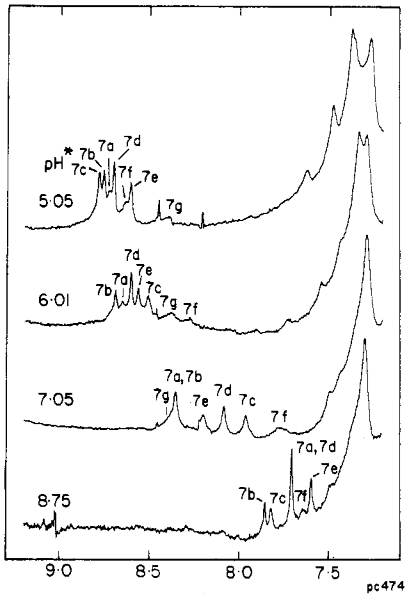

FIGURE 7: ^{1}H NMR spectra at 470 MHz (histidine C_ϵ–H region) of E_D•Li•Glc-P_2. The concentrations were as follows: protein, 1.3 mM; LiCl, 20 mM; EDTA, 5 mM; Glc-P_2, 3.9 mM.

Table III: ^{1}H NMR pH Titration Parameters for Histidine Residues of E_D•Cd•Glc-P_2[a]

histidine peak[b]	pK_a	Hill coefficient	low-pH plateau	high-pH plateau
5a	7.88 ± 0.05	0.66 ± 0.03	8.68 ± 0.01	7.44 ± 0.05
5b	7.29 ± 0.01	0.87 ± 0.02	8.78 ± 0.01	7.81 ± 0.01
5c	6.33 ± 0.01	1.01 ± 0.01	8.83 ± 0.01	7.81 ± 0.00
5d	6.84 ± 0.01	1.06 ± 0.01	8.72 ± 0.00	7.69 ± 0.00
5e	7.20 ± 0.01	1.09 ± 0.03	8.60 ± 0.01	7.57 ± 0.01
5f	6.04 ± 0.03	0.95 ± 0.05	8.78 ± 0.03	7.63 ± 0.01
5g	<5			
5h	6.97 ± 0.02	0.90 ± 0.04	8.79 ± 0.01	7.82 ± 0.02

[a] For conditions, see the legend to Figure 5. Error estimates are from the curve-fitting routine and represent 1 standard deviation. [b] The labels refer to peaks designated in Figure 5.

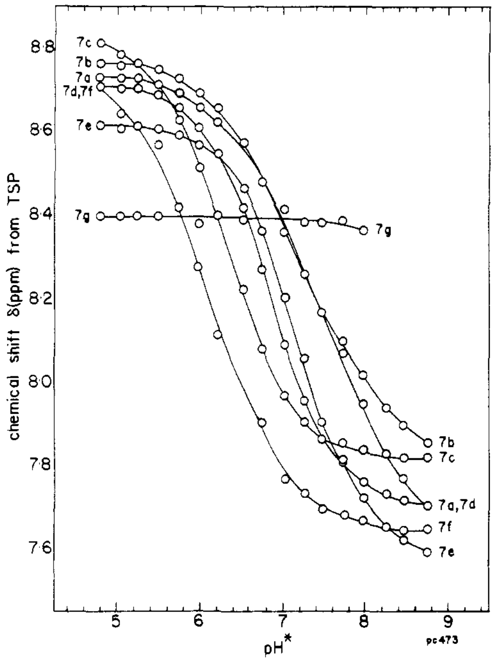

FIGURE 8: ^1H NMR (470 MHz) pH titration curves of the histidine resonances of E_D·Li·Glc-P_2. The conditions are described in the legend of Figure 7.

Table IV: ^1H NMR pH Titration Parameters for Histidine Residues of E_D·Li·Glc-P_2[a]

histidine peak[b]	pK_a	Hill coefficient	low-pH plateau	high-pH plateau
7a	7.49 ± 0.03	0.71 ± 0.03	8.75 ± 0.01	7.57 ± 0.03
7b	7.19 ± 0.02	0.79 ± 0.03	8.78 ± 0.01	7.81 ± 0.01
7c	6.33 ± 0.03	1.08 ± 0.06	8.84 ± 0.02	7.81 ± 0.01
7d	6.84 ± 0.01	1.12 ± 0.02	8.71 ± 0.00	7.70 ± 0.00
7e	7.18 ± 0.02	1.10 ± 0.03	8.62 ± 0.00	7.58 ± 0.01
7f	6.12 ± 0.03	0.95 ± 0.06	8.76 ± 0.02	7.64 ± 0.01
7g	<5			

[a] For conditions, see the legend to Figure 7. Error estimates are from the curve-fitting routine and represent 1 standard deviation. [b] The labels refer to peaks designated in Figure 7.

histidine residues in E_D·Li·Glc-P_2 behave somewhat similarly to seven of the eight resolved histidines in E_D·Cd·Glc-P_2; residue 5h, however, in E_D·Cd·Glc-P_2 (Figure 5) does not have an observable counterpart in spectra of E_D·Li·Glc-P_2 (Figure 7). It is particularly significant that residue 7g (Figure 7) does not titrate in the pH* range studied. This histidine residue seems to correspond to the residue that gives rise to peak 5g in spectra of E_D·Cd·Glc-P_2 (Figure 5) and may well interact directly with Li^+ in E_D·Li·Glc-P_2. The small pH inflection in the chemical shift of this histidine residue at high pH* may arise from an increase in the exchange rate between bound and free forms of Glc-P_2. A decreased binding of Glc-P_2 also would lead to dissociation of Li^+ from E_D·Li. In fact, a similar titration study of this complex monitored by ^{31}P NMR did show exchange broadening of signals from E_D·Li·Glc-P_2 at high pH.

^{31}P NMR Study of the pH Titration of Phosphoglucomutase. (A) Phosphoenzyme. The ^{31}P NMR spectrum of the phosphoenzyme (E_P) obtained at pH 7.42 in 0.1 M KCl contains one major peak labeled "I" and a minor shoulder on the high-field side labeled "II" (Figure 9A). Under these conditions, the intensity of peak II increases with decreasing or increasing pH from about pH 7.4. By contrast, in the absence of KCl, peak II appears only at low pH where the enzymic phosphate group exhibits a titration pattern (Figure 9B) similar to that observed in the presence of added KCl (Figure 9A). These results indicate that the process that gives rise to peak II at high pH is strongly dependent on ionic strength or specific ion effects.

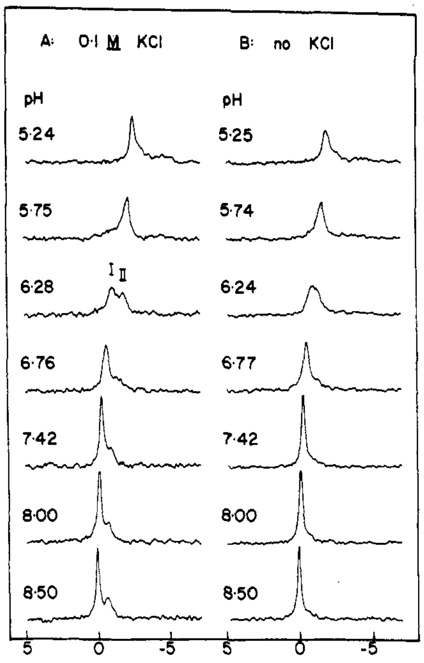

FIGURE 9: ^{31}P NMR spectra at 80.99 MHz of E_P illustrating their ionic strength and pH dependence. The protein concentration was 1.3 mM in 20 mM Tris-HCl at pH 7.5 initially. Titrant solutions (see the legend of Figure 1) were prepared in 10% ^2H_2O. The pH values are given in the figure. (A) Spectra obtained in the presence of 0.1 M KCl; (B) spectra obtained in the absence of KCl.

The data in Figure 9 can be explained by assuming that the phosphoenzyme exists as conformers which provide two different magnetic environments for the enzymic phosphate group. One environment (characterized by peak II) predominates at acidic pH, and the other (characterized by peak I) predominates at neutral pH. The enzymic phosphate group exchanges between these magnetic environments at a rate that is slow on the NMR time scale since two separate peaks are resolved. Because the chemical shift difference between the two peaks is 40 Hz at neutral pH, the lifetime of the phosphate group in either environment must be in excess of 4 ms. As noted above, at high pH (pH > 7.5), added KCl shifts the equilibrium so that the phosphate group moves to a magnetic environment similar to that observed at low pH. To present the pH dependence for the equilibrium of the phosphate group between environments I and II, the fraction of the total area associated with peak I, $I/(I + II)$ measured from Figure 9A (0.1 M KCl), is plotted as a function of pH in Figure 10. Under these conditions, the pH_{mid} for the low-pH transition of the phosphate group between environments I and II is about 6.1, and the Hill coefficient is in the range of 1.5. In the absence of KCl, similar values are obtained, about 6.0 and 1.4, respectively (plot not shown). This behavior might be expeected for the protonation of basic residues such as histidine; however, none of the histidines whose peaks were observed has a pK_a value close to 6, and none exhibits a Hill coefficient substantially greater than 1. Presumably, this transition is associated instead with the protonation of carboxyl groups whose pK_a values have an unusually small ionic strength dependence. The high-pH process that converts magnetic environment II back to magnetic environment I is less well characterized but involves a much smaller Hill coefficient of about 0.45 and a pH_{mid} of about 8.8 (0.1 M KCl). At a lower

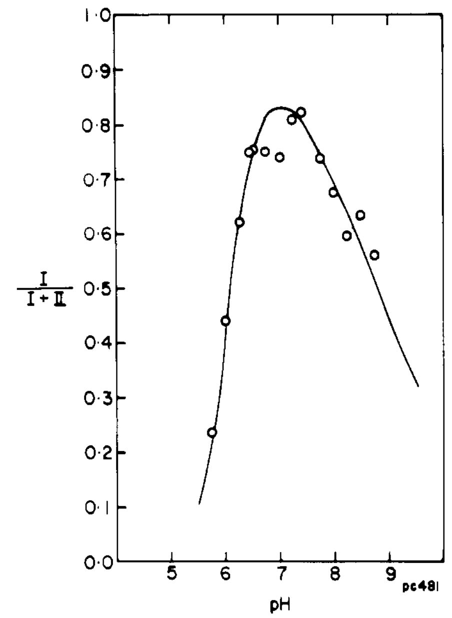

FIGURE 10: Plot of the fraction of the ^{31}P NMR signal associated with peak I, $I/(I + II)$, measured from Figure 9A, as a function of pH. The solid curve in the figure is a plot of the function $I/(I + II) = [1 + (H^+/K_1)^{1.5} + (K_2/H^+)^{0.45}]^{-1}$ where $pK_1 = 6.1$ and $pK_2 = 8.8$.

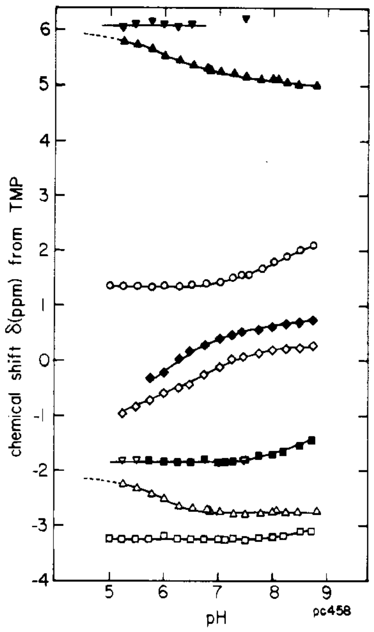

FIGURE 11: ^{31}P (80.99 MHz) NMR pH titration curves of phosphoglucomutase and its complexes: (♦) E_P, conformer I; (◇) E_P, conformer II; (○) E_P·Cd; (△) E_D·Cd·Glc-P_2; (▽) E_D·Cd·Cd·Glc-P_2; (□) E_D·Li·Glc-P_2. The points at pH 7.5 in the titration curve of E_D·Cd·Cd·Glc-P_2 were obtained in the absence of KCl. For the bisphosphate complexes, the closed symbols indicate the 6-phosphate group, and the open symbols indicate the 1-phosphate group.

ionic strength, this pH_{mid} apparently shifts to substantially larger values, since the high-pH shift no longer can be detected (plot not shown; however, see Figure 9B).

The experimental data yield separate pH titration curves for the two slowly interconverting conformational forms of E_P in Figure 11 (♦, environment I; ◇, environment II). The chemical shifts of the phosphate group in these two environments have a roughly parallel pH dependence as could result from direct protonation of the phosphate group itself or protonation of one or more basic groups close to the phosphate. If the latter case is true, the results imply that the pK_a of the enzymic phosphate group lies outside the experimentally accessible pH range. In either case, the data do not provide a reliable value for the pK_a of the group(s).

(*B*) *Cd^{2+}–Enzyme.* In the pH titration study of E_P·Cd by ^{31}P NMR, only one signal was observed for the enzymic phosphate group in the pH range 4.96–8.75 (spectra not shown). However, at higher pH, Cd^{2+} partially dissociates from E_P·Cd, and the ^{31}P NMR signal of free E_P appears. Both magnetic environments for the phosphate group of free E_P (I and II) are detected at pH 8.75 (spectra not shown). The pH dependence of the ^{31}P NMR peak of phosphoserine-116 in the E_P·Cd complex is shown in Figure 11. A transition is not observed in the normal pH range for phosphates, about pH 6, and no indication of a low-pH transition is seen even down to pH 5. This is not surprising since cadmium binds directly to the phosphate in the E_P·Cd complex (Rhyu et al., 1985); the bound metal ion should cause a substantial decrease in the pK_a of the phosphate group. Thus, assuming that the effect of ionization on chemical shift would be observable at least 1 pH unit above the midpoint, the pK_a for the serine phosphate monoanion in E_P·Cd must be less than 4. However, in the high-pH region, a definite pH-induced transition is observed with a pH_{mid} value of about 8.0. If the second pK_a of the phosphoserine is lower than 4, then the high-pH transition probably represents a magnetic perturbation produced by titration of an adjacent amino acid side chain, e.g., as was observed in the pH titration study of bovine (diisopropylphosphoryl)chymotrypsin A followed by ^{31}P NMR (Porubcan et al., 1979).[2] For E_P·Cd, the pH_{mid} of 8.04 is comparable to the pK_a of histidine resonance 3a (8.01; Table II) which is thought to be located close to the metal ion binding site (see Discussion).

(*C*) *Cd^{2+}–Enzyme in the Presence of Substrate.* ^{31}P NMR spectra of the Cd^{2+}–enzyme in the presence of substrate were obtained between pH 5.0 and 8.75. In this titration study, excess Glc-P_2 (1.3 equiv) was added initially to compensate for the slow hydrolysis of glucose bisphosphate to Glc-6-P and P_i in the presence of massive amounts of the Cd^{2+}–enzyme (Rhyu et al., 1985). However, at low pH, other, more rapid, reversible changes also occur in this system (spectra not shown). Thus, the intensities of the E_D·Cd·Glc-P_2 peaks decrease, and resonances from E_P·Glc-6-P (1.68 and −1.49 ppm at pH 5.24) and E_D·Cd·Cd·Glc-P_2 [second Cd^{2+} bound to the ancillary site: 6.09 and −1.8 ppm at pH 5.24; cf. Rhyu et al. (1985)] are observed. When the pH subsequently is readjusted to neutrality, these peaks disappear, and the intensities of the E_D·Cd·Glc-P_2 peaks increase toward their original values. These observations suggest that the equilibrium between E_D·Cd·Glc-P_2 and E_P·Cd·Glc-6-P, which is heavily in favor of E_D·Cd·Glc-P_2 at neutral pH (Ray & Long, 1976), is pH dependent and is more favorable toward E_P·Cd·Glc-6-P at low pH. In addition, the binding of Cd^{2+} to E_P·Glc-6-P apparently becomes weaker than its binding at the ancillary site of E_D·Cd·Glc-P_2, leading to a disproportionation that produces E_D·Cd·Cd·Glc-P_2 and E_P·Glc-6-P.

Although the equilibria noted above make chemical shift measurements more difficult by reducing intensities, they do not confuse the pH dependence of individual species since their lifetimes are long on the NMR time scale. Thus, the effect of pH on the chemical shift of two peaks shown in Figure 11 (△, 1-phosphate; ▲, 6-phosphate) represents only those changes that involve the E_D·Cd·Glc-P_2 complex. These peaks exhibit a pH dependence that is different from that of other phos-

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[2] In this case, the effect was assigned to titration of histidine-57 adjacent to the phosphorylated serine-195 at the active site.

phates in Figure 11: both move downfield as the pH is lowered, and both move in a parallel manner. It seems unlikely that the $\mathrm{p}K_a$ values and Hill coefficients for proton dissociation of both of these phosphate groups are essentially the same. Rather, the observed changes seem more likely to arise either directly or indirectly from prototropic reaction of nearby residues. Thus, the binding of $\mathrm{Cd^{2+}}$ to an ancillary site in the $\mathrm{E_D\cdot Cd\cdot Glc\text{-}P_2}$ complex shifts the resonances of *both* phosphate groups downfield to approximately the *same extent* (Rhyu et al., 1985), viz., in the same direction and to approximately the same extent as the shifts in $\mathrm{E_D\cdot Cd\cdot Glc\text{-}P_2}$ produced by changing the pH from 7 to 5. By contrast, the chemical shifts of both phosphates in the $\mathrm{E_D\cdot Cd\cdot Cd\cdot Glc\text{-}P_2}$ complex are independent of pH from 6.8 to 5.2 (Figure 11: ▽, 1-phosphate; ▼, 6-phosphate).

(*D*) *$\mathrm{Li^+}$–Enzyme in the Presence of Substrate.* $^{31}\mathrm{P}$ NMR spectra of $\mathrm{E_D\cdot Li\cdot Glc\text{-}P_2}$ were obtained at pH values between 5.0 and 8.75, and the chemical shifts vs. pH are shown in Figure 11 (□, 1-phosphate; ■, 6-phosphate). Because bound $\mathrm{Li^+}$ blocks phosphate transfer, a complex involving $\mathrm{E_P}$ and Glc-6-P was not observed at low pH in contrast with the results obtained with bound $\mathrm{Cd^{2+}}$ (see above). Although the phosphate groups in this complex differ substantially from those in the $\mathrm{E_D\cdot Cd\cdot Cd\cdot Glc\text{-}P_2}$ complex, especially the 6-phosphate, the chemical shifts in neither complex change with pH in the range 5–8. This behavior contrasts with that of the phosphates in the related $\mathrm{E_D\cdot Cd\cdot Glc\text{-}P_2}$ complex in which the chemical shifts of both phosphates move upfield with increasing pH (see previous section). This comparison suggests that the "$\mathrm{E_D\cdot Li\cdot Glc\text{-}P_2}$" complex, which always is studied in the presence of excess $\mathrm{Li^+}$, actually is $\mathrm{E_D\cdot Li\cdot Li\cdot Glc\text{-}P_2}$, i.e., involves $\mathrm{Li^+}$ bound at the ancillary metal ion site as well, as was suggested earlier by competitive binding studies involving $\mathrm{Cd^{2+}}$ and $\mathrm{Li^+}$ (Rhyu et al., 1985). Hence, we conclude that neither of the two phosphates in the various $\mathrm{E_D\cdot M\cdot X\cdot Glc\text{-}P_2}$ complexes, i.e., either with or without ancillary metal ion binding, is protonated at pH values as low as 5.

DISCUSSION

It is frequently difficult to resolve $^1\mathrm{H}$ NMR peaks from histidine residues in a protein as large as phosphoglucomutase (Markley, 1975), especially as here when 10 such residues are present. Those that were not observed (two to three in the present case) may represent buried histidines that have broad signals or abnormal $\mathrm{p}K_a$ values. In fact, a preliminary analysis of the electron density map (2.7-Å nominal resolution) of crystalline phosphoglucomutase ($\mathrm{Mg^{2+}}$ complex) does suggest that three histidine side chains are well shielded from the solvent (W. J. Ray, Jr., unpublished results). Shielding from solvent may produce extensive lifetime broadening of the peak if the rates of protonation/deprotonation are slowed. Alternatively, a histidine peak might be missed if its titration curve were superimposed on that of another histidine residue; intensities in spin-echo spectra are difficult to quantify because they depend on the original line width as well as its area.

The pH-induced change in chemical shift of the $^{31}\mathrm{P}$ nucleus of the free phosphoenzyme is complicated by the existence of conformers in which the phosphate group exhibits different chemical shifts. Whether these are interconverted by direct protonation of the enzymic phosphate group or other residues that affect conformational structure cannot be determined with the present data. However, another phosphoserine enzyme, glycogen phosphorylase *a*, also appears to exist in at least two conformational states on the basis of the frequency dependence (Vogel et al., 1982) and the pH dependence (Vogel & Bridger, 1983) of the $^{31}\mathrm{P}$ NMR line width. In addition, Hoerl et al. (1979) previously reported a split resonance for the thiophosphoserine residue of glycogen phosphorylase *a* which they assigned to two different conformational states of the enzyme in slow chemical exchange.

In the present case, the $^1\mathrm{H}$ NMR and $^{31}\mathrm{P}$ NMR data demonstrate the existence of pH-dependent conformational states of the apoenzyme, $\mathrm{E_P}$, that are reversibly interconverted by cooperative proton binding [Hill coefficient of 2.1 from the $^1\mathrm{H}$ NMR data (Figure 2) and 1.5 from the $^{31}\mathrm{P}$ NMR data (Figure 10)] and have a $\mathrm{pH_{mid}}$ in the range of about 5.8–6.1. However, differences between the lifetimes of the conformational states [less than 1 ms from the $^1\mathrm{H}$ NMR data (separate signals not resolved) but greater than 4 ms from the $^{31}\mathrm{P}$ NMR data (two different signals resolved)] show that two different sets of groups in the free enzyme control the interconversion of at least four conformational states (at pH values close to 6). However, the binding of $\mathrm{Cd^{2+}}$ (1 mol/mol of enzyme) eliminates evidence for three of these conformational states, at least in the pH range from 8 to 5. Thus, $\mathrm{Cd^{2+}}$ binding appears to produce a much less variable (pH-dependent) environment for the group whose chemical attachment is to be altered in the subsequent catalytic process. Such a "fixation" is in accord with what might be expected when such a group becomes liganded to a bound metal ion. Presumably, the binding of other metal ion activators in place of $\mathrm{Cd^{2+}}$ would affect the enzyme similarly.

The effect of pH on the $^{31}\mathrm{P}$ NMR signal of the enzymic phosphate in $\mathrm{E_P\cdot Cd}$ does not provide an unequivocal definition of the ionic status of this group. Thus, its chemical shift, while essentially constant from pH 5 to about 7, moves downfield at pH values above about pH 7 (○, Figure 11). These changes could be the result either of a phosphate group whose $\mathrm{p}K_a$ is abnormally high (8 or above) or of one whose $\mathrm{p}K_a$ is abnormally low (substantially less than 5) and whose chemical shift above about pH 7 moves downfield, not as a result of deprotonating this group but as a consequence of deprotonating a nearby group or groups. We strongly favor the latter possibility since it seems more likely that a phosphate group coordinated to a bivalent metal ion would exhibit a substantially decreased, rather than a substantially increased, $\mathrm{p}K_a$.

Some inferences about the ionic status of the phosphate group after its transfer to the 6-position of bound Glc-1-P can be made when the effects of protonation at an ancillary metal ion binding site (whose function is not known) are taken into consideration. Quite unexpectedly, conversion of $\mathrm{E_P\cdot Cd}$ to $\mathrm{E_D\cdot Cd\cdot Glc\text{-}P_2}$ by reaction with Glc-1-P introduces a pH sensitivity into *both* phosphate groups of this complex at low pH (△ and ▲, Figure 11) that was not present in the phosphate group of the $\mathrm{E_P\cdot Cd}$ complex (○, Figure 11).[3] However, this new pH sensitivity can be eliminated by binding a second $\mathrm{Cd^{2+}}$ at an ancillary site (▽ and ▼, Figure 11) and thus is unlikely to arise from a direct protonation of the bound phosphate groups (see Results).[4] Thus, neither phosphate in either the $\mathrm{E_D\cdot Cd\cdot Cd\cdot Glc\text{-}P_2}$ or the $\mathrm{E_D\cdot Cd\cdot Glc\text{-}P_2}$ complex (metal ion either present at the ancillary site or absent) is likely to be

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[3] In the bisphosphate complex, the phosphate originally attached to serine-116 of the enzyme now is attached to the bound sugar phosphate, but it almost certainly is close to its original attachment site, to which it must return in the reverse process.

[4] This is the second piece of evidence that indicates an alteration of the ancillary site and the active site upon substrate binding; earlier experiments (Rhyu et al., 1985) had shown that binding of a second $\mathrm{Cd^{2+}}$ to $\mathrm{E_D\cdot Cd\cdot Glc\text{-}P_2}$ produces a substantial increase in the chemical shifts of both phosphate groups in this complex—an effect similar in both magnitude and sign to that produced by protonation of $\mathrm{E_D\cdot Cd\cdot Glc\text{-}P_2}$. Binding of the second $\mathrm{Cd^{2+}}$ has no effect on enzymic activity (W. J. Ray, Jr., unpublished results).

substantially protonated, even at pH values significantly below 5, as would be expected if both interact strongly with positively charged groups.

As also might be expected, the ^{1}H NMR titration parameters for histidine residues in Tables I (free E_P), II (E_P·Cd complex), III (E_D·Cd·Glc-P_2 complex), and IV (E_D·Li·Glc-P_2 complex) show that some histidine residues are too far away to be affected by binding of metal ion or substrate at the active site of the enzyme, viz., histidine resonances c, d, and e. The correspondence of other histidines in the various complexes was ambiguous. Thus, although histidine resonances b and f have quite consistent pH titration parameters throughout Tables II–IV, they could not be correlated unambiguously with histidine resonances 1b and 1f in Table I on the basis of our available data, although they may well be the same residues. Similarly, residues a, g, and h could not be correlated in the different complexes of the enzyme. The low-pH perturbation in the titration curves of histidine resonances 1a, 1f, and 1h in E_P (Figure 1) suggests that these residues interact with a cluster of acidic groups that titrate cooperatively with a pH^*_{mid} around 5.8. Since the second transition was not observed in the ^{1}H NMR pH titration curves of E_P·Cd, this acidic cluster may comprise the metal ion binding site, or part of it. Binding of metal ion to such a cluster would decrease the pH_{mid} to well below the lowest pH at which observations were made, 5.0. Thus, no pH-dependent perturbation of histidines at low pH was expected for any enzyme complexes with bound metal ion, and none was observed.

The side chain of one histidine in E_D·Cd·Glc-P_2 (peak 5g, Figure 6) and probably the same residue in E_D·Li·Glc-P_2 (peak 7g, Figure 8) does not undergo a prototropic reaction (its chemical shift is not altered) between pH* 6.0 and 8.2 (Figures 6 and 8) although all observable histidine residues in E_P and E_P·Cd have begun to be protonated at pH* values below 7 (Figures 2 and 4). Because two histidines in E_P and three in E_P·Cd are not accounted for, histidine peak 5g (or 7g) could represent a residue that was not observed in E_P or E_P·Cd but, because of a substrate-induced change in solvent exposure, is observed in the ternary complexes (concomitant with the disappearance of another residue). An alternative explanation which we prefer is the that an oxygen ligand to the metal furnished by the enzymic phosphate group in E_P·M is replaced by a histidine imidazole nitrogen in E_D·M·Glc-P_2 as the consequence of a conformational rearrangement. As long as it remains coordinated with a metal ion, such a histidine would not be expected to protonate. This rationale is particularly attractive since earlier work (Rhyu et al., 1985) suggested that substrate binding leads to a metal ligand exchange whereby an oxygen ligand of bound Cd^{2+} is replaced by a nitrogen ligand. Other work (Rhyu et al., 1984) also indicated that the above process removes the phosphate group from the coordination sphere of the metal ion, and we presume that the departing oxygen ligand is that of the enzymic phosphate group. If the nitrogen ligand that replaces the departing phosphate were the abnormal imidazole residue observed in E_D·Cd·Glc-P_2 (peak 5g), the chemical shift of the histidine should be altered if the Cd^{2+} in this complex is replaced by Li^+. Such a change has been observed (Figures 6 and 8), and the chemical shifts are 7.98 for the Cd^{2+} complex and 8.39 ppm for the Li^+ complex.[5]

Either the substrate-binding step (E_P·M + Glc-P → E_P·M·Glc-P) or the subsequent change in chemical bonding (E_P·M·Glc-P → E_D·M·Glc-P_2) could produce the three active-site changes that have been observed: the elimination of the Cd^{2+}–phosphate interaction, the deshielding of the Cd^{2+} nucleus, and the appearance of an imidazole with an abnormally low pK_a. We will attempt to distinguish between these possibilities by studying substrate analogues that do not act as phosphate acceptors, i.e, ones that preclude the second of the above steps.

ACKNOWLEDGMENTS

We thank Dr. J. M. Puvathingal for preparing the enzyme.

Registry No. Cd, 7440-43-9; Li, 7439-93-2; phosphoglucomutase, 9001-81-4; histidine, 71-00-1; phosphoserine, 407-41-0.

REFERENCES

Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Wright, P. E. (1975) *FEBS Lett. 57*, 96–99.

Hoerl, M., Feldman, K., Schnackerz, K. D., & Helmreich, E. J. (1979) *Biochemistry 18*, 2457–2464.

Markley, J. L. (1973) *Biochemistry 12*, 2245–2250.

Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70–80.

Markley, J. L., & Porubcan, M. A. (1976) *J. Mol. Biol. 102*, 487–509.

Meiboom, S., & Gill, D. (1958) *Rev. Sci. Instrum. 29*, 688–691.

Porubcan, M. A., Westler, W. M., Ibañez, I. B., & Markley, J. L. (1979) *Biochemistry 18*, 4108–4116.

Ray, W. J., Jr. (1969) *J. Biol. Chem. 244*, 3740–3747.

Ray, W. J., Jr., & Long, J. W. (1976) *Biochemistry 15*, 4018–4025.

Ray, W. J., Jr., Szymanski, E. S., Ng, L. (1978) *Biochim. Biophys. Acta 522*, 434–442.

Ray, W. J., Jr., Hermodson, M. A., Puvathingal, J. M., & Mahoney, W. C. (1983) *J. Biol. Chem. 258*, 9166–9174.

Rhyu, G. I., Ray, W. J., Jr., & Markley, J. L. (1984) *Biochemistry 23*, 252–260.

Rhyu, G. I., Ray, W. J., Jr., & Markley, J. L. (1985) *Biochemistry 24*, 2536–2541.

Sudmeier, J. L., Evelhoch, J. L., & Jonsson, N. B. H. (1980) *J. Magn. Reson. 40*, 377–390.

Vogel, H. J., & Bridger, W. A. (1983) *Can. J. Biochem. 61*, 363–369.

Vogel, H. J., Bridger, W. A., & Sykes, B. D. (1982) *Biochemistry 21*, 1126–1132.

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[5] The downfield shift caused by lithium may result from its resemblance to the proton. We have found that the ring protons of imidazole shift downfield in the presence of 8 M LiCl, toward their position in the imidazolium ion (G. I. Rhyu, W. J. Ray, Jr., and J. L. Markley, unpublished results).