

Proton NMR Studies of the Histidine Residues of Rabbit Muscle Pyruvate Kinase and of its Phosphoenolpyruvate Complex*

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Proton NMR spectra at 250 MHz of rabbit muscle pyruvate kinase, a tetrameric protein of molecular weight 237,000, were obtained with 16 bit A/D conversion in order to study the roles of the histidine residues in catalysis. Six of the 14 histidine residues/subunit gave detectable C-2 proton signals, and three gave C-4 proton signals. Analysis of the upfield shifts of these resonances with increasing pH by least squares computer fitting indicated these signals to arise from single histidines with the following pK' values at 25 °C in the presence of the activating monovalent (K^+) and divalent (Mg^{2+}) cations: His-1, 6.7; His-2, 6.3; His-3, 6.2; His-4, 6.0; His-5, 5.9; and His-6, 5.6. The presence of a saturating level of the substrate P-enolpyruvate resulted in a 0.4 unit decrease in the pK' of His-3 to a value of 5.8, but no significant change in any of the other pK' values. Titration with P-enolpyruvate at a constant pH* (5.8) measuring the upfield shift of the His-3 resonance yielded a dissociation constant for P-enolpyruvate ($74 \pm 7 \mu M$) consistent with kinetic and other binding data, indicating active site binding. Both the monovalent (K^+) and divalent (Mg^{2+}) cation activators were found to be essential for the full P-enolpyruvate induced upfield shift. No effects of P-enolpyruvate were noted in the absence of both cations and only small effects of P-enolpyruvate were noted in the presence of either cation activator alone. The metal activators themselves induced smaller upfield shifts of the His-3 resonance. These findings indicate that the decrease in pK' of His-3 induced by the binding of P-enolpyruvate results directly from a stronger interaction of the metal ion activators with His-3, in the enzyme-substrate complex, or indirectly from a P-enolpyruvate induced conformational change in the environment of His-3.

Pyruvate kinase from rabbit muscle is a tetramer ($M_r = 237,000$) with four indistinguishable subunits (1). It has been shown that two divalent cations/subunit are necessary to activate pyruvate kinase, one of which is coordinated to the enzyme, forming second sphere substrate complexes (2) while the other is coordinated to the polyphosphate chain of the nucleotide and is not coordinated to the enzyme (3). In addition,

pyruvate kinase has a monovalent cation binding site (4). The binding of the substrate, P-enolpyruvate tightens the binding of the enzyme-bound divalent cation (5) and causes a 3.3-Å decrease (from 8.2 to 4.9 Å) in the distance between the enzyme-bound monovalent cation and the divalent cations, implying a conformational change at the active site (6).

While much is known about the location and roles of the metal ions, little direct information exists on the essential amino acid residues at the active site. Chemical modification studies have implicated a lysine (7) and a cysteine (8) at or near the nucleotide binding site (9). An affinity label based on ADP has detected a basic group with a $pK = 8.5$, either lysine or tyrosine, at or near the phosphoryl transfer site (10). In addition, the role of one or more histidines at the active site has been suggested by a pK of 6.8 in the binding of Mn^{2+} to the enzyme (11) and by chemical modification studies (12). Proton NMR is an effective and direct technique for studying the role of histidines in enzymes (13). Heretofore, such studies have been limited to proteins of $M_r \leq 68,000$ (13, 14). Although NMR spectra in the aromatic region of pyruvate kinase with and without P-enolpyruvate have been reported at a single pH (15), the spectral changes were not analyzed further. The availability of a midfield NMR spectrometer (250 MHz), together with high resolution (16 bit) analog to digital conversion, has enabled us to study the histidines of rabbit muscle pyruvate kinase.

In the present paper, six histidine C-2 proton resonance lines of pyruvate kinase without and with P-enolpyruvate were observed at 250 MHz and their behavior as a function of pH and substrate concentration were studied. From these observations, information relevant to the role of histidines in the active site was obtained and the effects of the binding of P-enolpyruvate were analyzed.

EXPERIMENTAL PROCEDURES

Materials—Rabbit muscle pyruvate kinase used in this study was obtained from Boehringer. Ammonium sulfate was removed by gel filtration using Sephadex G-25. The enzyme was concentrated and deuterated in a collodion bag by repeated vacuum dialysis at 4 °C. When assayed using the coupled lactic dehydrogenase reaction (16) the specific activity of pyruvate kinase (200 to 250 units/mg) was found to be consistent with that of the pure enzyme. Sodium dodecyl sulfate-gel electrophoresis of this enzyme gave a single band.

P-enolpyruvate and PIPES¹ were obtained from Sigma and used without further purification; 95% [3H]Tris- $^2H_{11}$, KO^2H , 2HCl and 99.85% 2H_2O were obtained from Stohler Isotope, Merck Sharp & Dohme and Norell Chemical Co., respectively.

Methods—The 250 MHz 1H NMR spectra of 0.6 to 1.0 mM pyruvate kinase subunits (i.e. sites) in 2H_2O solution containing 1.5 mM K-PIPES were obtained with a Bruker WM 250 pulse FT spectrometer at 25 °C. At least 1024 free induction decays were accumulated over 8192 data points using a 16-bit analog-digital converter and block

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¹ The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

averaging. DSS was used as an external reference for calculating chemical shifts.

^1H NMR spectra of pyruvate kinase (0.7 mM sites) with and without KCl (0.1 M) and MgCl_2 (5.0 mM) were obtained in the pH range 4.9 to 8.7. Similarly, pH titrations were also made of a solution of pyruvate kinase, KCl (0.1 mM), MgCl_2 (5.0 mM), and P-enolpyruvate (1.4 mM) in the pH range 4.6 to 8.1. Convolution difference spectra were obtained as described by Campbell *et al.* (17) using line broadenings of 5.0 and 0.5 Hz and an additive constant of 0.85.

Integration of the histidine C-2 resonances was done by computer simulation of the signals by Lorentzian lines, allowing for a linearly sloping base-line. Independent measurements of the area under the curves, by cutting out and weighing the peaks, gave indistinguishable results.

pH measurements of solutions in D_2O were made at 25 °C using a pH meter (Radiometer 2b) with an Ingold electrode. The meter was calibrated with two standard pH buffers (A. Thomas Co.). The pH values given are the actual meter readings and have not been corrected for the deuterium isotope effect at the glass electrode. The notation pH^* is used to designate uncorrected readings obtained in this manner and pK' designates the corresponding ionization parameter. It has been found that histidine pK' values determined directly from uncorrected glass electrode readings in D_2O agree well with pK values determined in H_2O (18, 19). Apparently the isotope effect on the pK value is cancelled by the opposite effect on the glass electrode (18, 19). Adjustment of pH was made by adding 0.1 M KO^2H or 0.1 M ^2HCl by means of a micropipette. When a small amount of protein precipitation in the sample occurred in the course of the pH titration, the precipitate was removed by centrifugation. The pH reading before and after the NMR measurements agreed within ± 0.02 pH unit. The back-titration to the original pH restored the initial spectra. The enzyme was found to have the same specific activity before and after the pH titrations.

Analysis of Titration Data—The histidine chemical shifts (δ_{obs}) are generally weighted averages of the chemical shifts of the protonated (δH^+) and unprotonated (δH^0) forms.

$$\delta_{\text{obs}} = \frac{\delta\text{H}^+ [\text{His}^+] + \delta\text{H}^0 [\text{His}^0]}{[\text{His}^+] + [\text{His}^0]} \quad (1)$$

The pK' value of histidine was obtained by fitting the NMR data as functions of pH to the Hill equation by nonlinear least squares calculations.

$$\frac{\delta\text{H}^+ - \delta_{\text{obs}}}{\delta\text{H}^+ - \delta\text{H}^0} = \frac{K_a^n}{K_a^n + [\text{H}^+]^n} \quad (2)$$

where K_a is the dissociation constant of histidine, $[\text{H}^+]$ is the hydronium (deuterium) ion concentration calculated from the pH^* measurement, and n is the Hill coefficient. Both four-parameter fits (K_a , δH^+ , δH^0 , n) and three parameter fits (with n fixed equal to 1) were calculated. When an ambiguity in assignment arose because of overlap of resonances during the titration, the least squares fit was attempted using several sets of assignments. The set giving rise to the best fit was used. This approach was necessary only in the pH titration in the presence of P-enolpyruvate.

P-enolpyruvate titration was carried out by a stepwise addition of the substrate to the NMR sample solution containing 0.55 mM pyruvate kinase, 0.1 M KCl and 5.0 mM MgCl_2 . The pH of the solution was adjusted when necessary prior to the NMR measurement.

The binding of P-enolpyruvate to pyruvate kinase was found to alter the chemical shift of a histidine C-2 proton. In fitting the titration data to a theoretical curve, the observed chemical shift (δ_{obs}) of the histidine proton is reasonably assumed to be the weighted average of the chemical shifts of the histidine in the free enzyme (δ_0) and in the enzyme P-enolpyruvate complex (δ_b).

$$\delta_{\text{obs}} = \frac{\delta_0[E_0] + \delta_b[E_b]}{[E_0] + [E_b]} \quad (3)$$

In equation 3, $[E_0]$ and $[E_b]$ are defined as the concentrations of free enzyme and the P-enolpyruvate-pyruvate kinase complex, respectively. The dissociation constant of P-enolpyruvate is defined as

$$K_D = \frac{[E_0] \cdot [S_{\text{free}}]}{[E_b]} \quad (4)$$

where $[S_{\text{free}}]$ is the concentration of free P-enolpyruvate. The relation of the substrate concentration and the substrate induced chemical shift is obtained as

$$\delta_{\text{obs}} = \delta_0 + (\delta_b - \delta_0) \frac{[E_b]}{[E]} \quad (5)$$

In equation 5, $[E_b]$ is defined as

$$[E_b] = 1/2 \{ K_D + [E] + [S] - \sqrt{K_D^2 + [E]^2 + [S]^2 + 2K_D[E] + 2K_D[S] - 2[E][S]} \} \quad (6)$$

where $[E]$ and $[S]$ are the total enzyme and total substrate (P-enolpyruvate) concentrations, respectively.

RESULTS

Histidine Resonances of Pyruvate Kinase—A typical 250-MHz ^1H NMR spectrum in the aromatic region of rabbit muscle pyruvate kinase at pH 5.8 is shown in Fig. 1A. Six resonance lines due to the C-2 imidazole protons of histidine in the low field region were observed. For future reference the signals will be referred to as histidines 1 through 6. The presence of the activating cations K^+ (0.1 M) and Mg^{2+} (5.0 mM) caused a small (0.03 ppm) upfield shift of the resonance labeled His-3, as shown in Fig. 2.

Integration of the spectrum reveals these six histidine C-2H lines to consist of essentially the same number of protons. Because His-1, His-2, and His-3 are quite sharp and well resolved, the integration shows these three to consist of the same number of protons within $\pm 10\%$. The line width of His-1 is 5.4 Hz which is very small compared with that found on other proteins (18), and His-1 is observed as a sharp line at each pH value in the course of the titration. Therefore, it was concluded that His-1 consists of one proton and His-2 and His-3 therefore also consist of one proton. Moreover the peaks of His-4, His-5, and His-6 appear also to be due to one proton

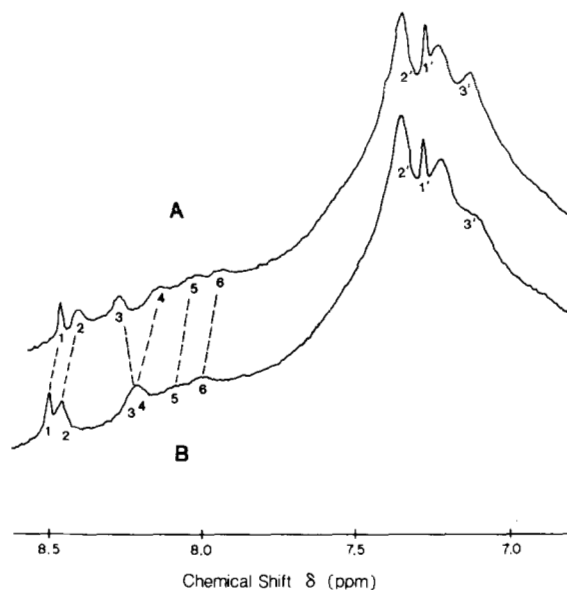


FIG. 1. ^1H NMR spectra in aromatic region of rabbit muscle pyruvate kinase at 250 MHz at 25 °C. NMR samples contain 0.65 mM pyruvate kinase with 0.1 M KCl and 5.0 mM MgCl_2 in 1.5 mM K-PIPES in $^2\text{H}_2\text{O}$ either without P-enolpyruvate at pH^* 5.8 (A) or with 2.0 mM P-enolpyruvate at pH^* 5.7 (B). The data was collected as 4096 transients in 16 blocks, using 16-bit analog-digital conversion, a sweep width of 5000 Hz, an acquisition time of 0.8192 s, and no other delays. Chemical shift is from external DSS. Six histidine C-2 ring proton resonances are resolved and numbered 1 through 6 as shown and as referred to in the text. Approximate positions of resolved C-4 ring protons are numbered 1' to 3' as shown and are referred to in the text.

although the errors in the integrated areas are greater ($\pm 25\%$). Pyruvate kinase has 14 histidine residues (20). Hence, the resonances of the other 8 histidines are either too broad for detection or occur elsewhere in the aromatic region.

Only three resonance lines due to the C-4 protons of histidine were observed at higher field. However, because of the intense peaks of other aromatic proton resonances in this region, these three resonances were not detected at all pH values (Fig. 3).

Effect of pH on Histidine Resonances of Pyruvate Kinase—Titration curves, measuring the changes in the chemical shifts (δ) of the histidine resonances with pH^* are shown in Fig. 3. The pK' values, obtained both with n fixed at unity and with n freely variable, and also the end points of the titrations are listed in Table I. As expected for histidine C-2 protons, the total change in chemical shift with pH^* ($\Delta\delta\text{H}^+$, H^0) of each of the six resonances is close to 1 ppm (Table I). The three resonance lines observed in the region 6.8 to 7.4 ppm assigned to the C-4 protons of histidines have much smaller values of δH^+ , H^0 , as expected (13). A comparison of the line shapes and intensities (Fig. 1A) and pK' values (Fig. 3; Table I) suggest that the C-4 histidine peaks detected correspond to

histidine 1, 2 and a superposition of several other histidines including histidine 3.

Histidine pH titrations of pyruvate kinase have also been carried out in the presence of 5.0 mM MgCl_2 in addition to 0.1 M KCl, which is a sufficient Mg^{2+} level to saturate the divalent cation binding site at pH 7.5 (11). Titration parameters are listed in Table II. Because of the limited pH^* range of the data available, the titration curves of the C-4 histidine resonances were analyzed only with n fixed to unity in accord with the findings that $n \sim 1$ for the corresponding C-2 protons. The presence of 5 mM MgCl_2 did not significantly alter the pK' values or the other titration parameters of the observed histidine resonances from those found in the presence of KCl alone (Table II).

Effect of P-enolpyruvate on Histidine Resonances of Pyruvate Kinase—A typical spectrum of pyruvate kinase in the presence of KCl, MgCl_2 , and a saturating level of the substrate P-enolpyruvate (2 mM) is shown in Fig. 1B. The presence of P-enolpyruvate has caused the C-2 resonance of His-3 to shift upfield by 0.11 ppm from that found with KCl and MgCl_2 so

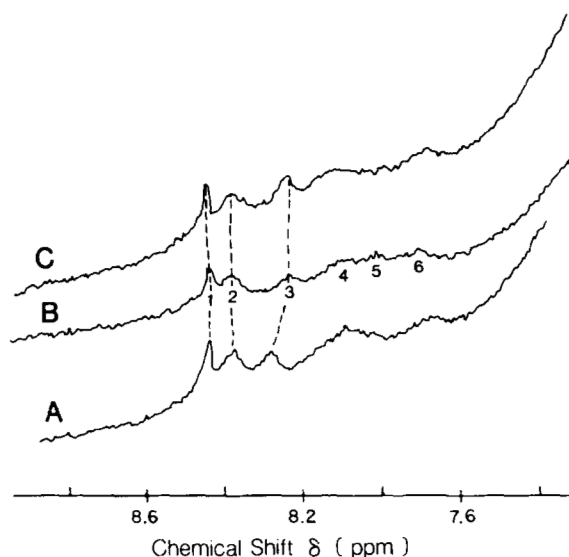


FIG. 2. Effect of K^+ or Mg^{2+} on the ^1H NMR spectra in histidine C-2 ring proton region of pyruvate kinase at pH 6.0. A, 0.60 mM pyruvate kinase alone in 1.5 mM K-PIPES; B, with 0.1 M KCl; C, with 5.0 mM MgCl_2 . The number of transient was 1024, collected in four blocks. Other conditions were as described in Fig. 1.

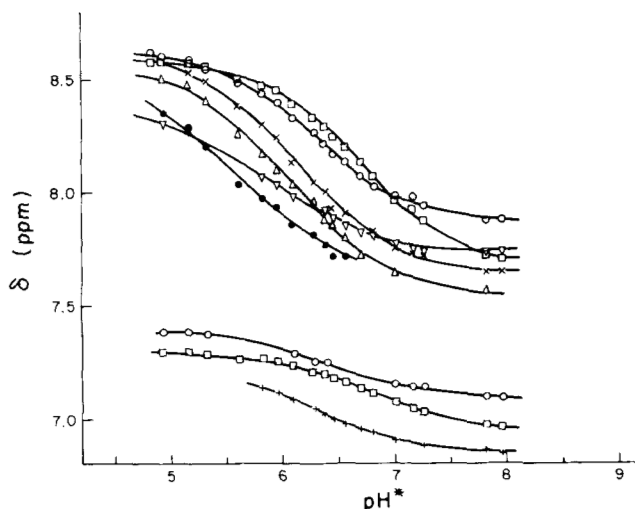


FIG. 3. ^1H NMR pH titration curves for the histidine C-2 and C-4 ring protons of pyruvate kinase in the presence of 0.1 M KCl. The data were obtained at 250 MHz using 0.6 mM pyruvate kinase in 1.0 mM K-PIPES at 25 °C. The symbols refer to the resonances of Fig. 1 as follows. C-2 protons: \square , His-1; \circ , His-2; \times , His-3; \triangle , His-4; ∇ , His-5; \bullet , His-6; lower 3 curves are C-4 protons: \square , His-1'; \circ , His-2'; $+$, His-3'. The C-2 protons 1 and 2, correspond to C-4 protons 1' and 2', respectively. The C-2 proton 3 corresponds to C-4 proton 3' which includes some other histidines, as discussed in the text.

TABLE I
Least squares analysis of pH titration data for histidine residues of pyruvate kinase in the presence of KCl (0.1 M)

Peaks	pK'		n^a	Chemical shift ^b		
	n fixed at 1	n fitted		δH^{+c}	δH^{0d}	$\Delta\delta\text{H}^+, \text{H}^0$
					ppm	
1	6.71 ± 0.01	6.72 ± 0.01	0.91 ± 0.03	8.60 ± 0.01	7.63 ± 0.01	0.97 ± 0.02
2	6.29 ± 0.04	6.29 ± 0.02	0.94 ± 0.01	8.64 ± 0.01	7.85 ± 0.01	0.79 ± 0.02
3	6.14 ± 0.02	6.14 ± 0.04	0.99 ± 0.05	8.62 ± 0.02	7.63 ± 0.02	0.98 ± 0.04
4	6.03 ± 0.02	6.01 ± 0.02	0.89 ± 0.07	8.62 ± 0.04	7.52 ± 0.01	1.09 ± 0.04
5	5.93 ± 0.04	5.89 ± 0.02	0.89 ± 0.05	8.39 ± 0.01	7.72 ± 0.01	0.67 ± 0.02
6	5.61 ± 0.05	5.55 ± 0.01	0.83 ± 0.05	8.59 ± 0.05	7.59 ± 0.02	1.00 ± 0.07
1'	6.77 ± 0.07			7.29 ± 0.02	6.94 ± 0.01	0.35 ± 0.03
2'	6.35 ± 0.05			7.39 ± 0.01	7.09 ± 0.01	0.30 ± 0.02
3'	6.29 ± 0.09			7.23 ± 0.04	6.84 ± 0.01	0.39 ± 0.005

^a Hill coefficient.

^b From external DSS.

^c Chemical shift of protonated histidine residue.

^d Chemical shift of deprotonated histidine residue.

TABLE II

Least squares analysis of pH titration data for histidine residues of pyruvate kinase in the presence of KCl (0.1 M) and MgCl₂ (5 mM)

Peaks	pK'		n	Chemical shift		
	n fixed at 1	n fitted		δH^+	δH^0	$\Delta\delta H^+, H^0$
					ppm	
1	6.73 \pm 0.01	6.73 \pm 0.01	0.94 \pm 0.02	8.60 \pm 0.01	7.62 \pm 0.01	0.98 \pm 0.01
2	6.30 \pm 0.02	6.30 \pm 0.02	0.91 \pm 0.04	8.65 \pm 0.01	7.82 \pm 0.01	0.82 \pm 0.02
3	6.21 \pm 0.02	6.21 \pm 0.02	0.99 \pm 0.05	8.60 \pm 0.02	7.61 \pm 0.01	0.99 \pm 0.02
4	5.98 \pm 0.03	5.98 \pm 0.06	0.88 \pm 0.24	8.61 \pm 0.11	7.56 \pm 0.05	1.05 \pm 0.16
5	5.85 \pm 0.10	5.88 \pm 0.05	1.19 \pm 0.11	8.36 \pm 0.03	7.71 \pm 0.01	0.65 \pm 0.04
6	5.74 \pm 0.10	5.55 \pm 0.06	0.73 \pm 0.09	8.64 \pm 0.05	7.55 \pm 0.03	1.09 \pm 0.08
1'	6.55 \pm 0.08			7.36 \pm 0.02	6.94 \pm 0.02	0.42 \pm 0.04
2'	6.50 \pm 0.07			7.43 \pm 0.02	7.07 \pm 0.01	0.35 \pm 0.03
3'	6.16 \pm 0.06			7.30 \pm 0.03	6.82 \pm 0.01	0.48 \pm 0.04

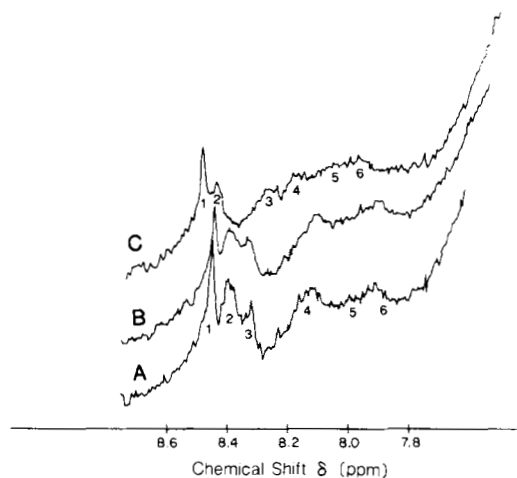


FIG. 4. Effect of P-enolpyruvate and K⁺ on the aromatic region of the 250-MHz proton NMR spectrum of pyruvate kinase. A, pyruvate kinase (0.6 mM) alone at pH* 5.8; B, pyruvate kinase with 1.2 mM P-enolpyruvate at pH* 5.8; C, pyruvate kinase with 1.2 mM P-enolpyruvate and 0.1 M KCl at pH* 5.7. K-PIPES (1.0 mM) was also present.

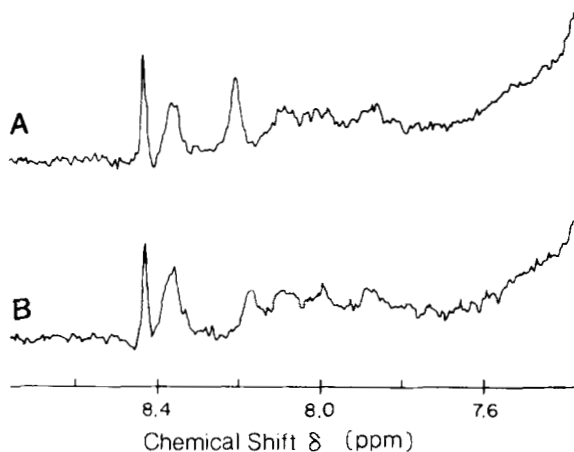


FIG. 5. Effect of P-enolpyruvate on the aromatic region of the 250-MHz proton NMR convolution difference spectrum of pyruvate kinase in the presence of Mg²⁺ and in the absence of K⁺. A, components present were pyruvate kinase (0.7 mM) MgCl₂ (5.0 mM), and deuterated Tris-Cl (0.1 M), pH* = 5.8; B, as in A with 2.0 mM P-enolpyruvate.

that it overlaps with that of His-4, while the other histidines shift downfield slightly due to a small decrease (0.1 unit) in pH*. Both K⁺ and Mg²⁺ must be present for a shift of this magnitude to occur as shown in Fig. 4. In the absence of activating metal ions little or no effect of P-enolpyruvate is

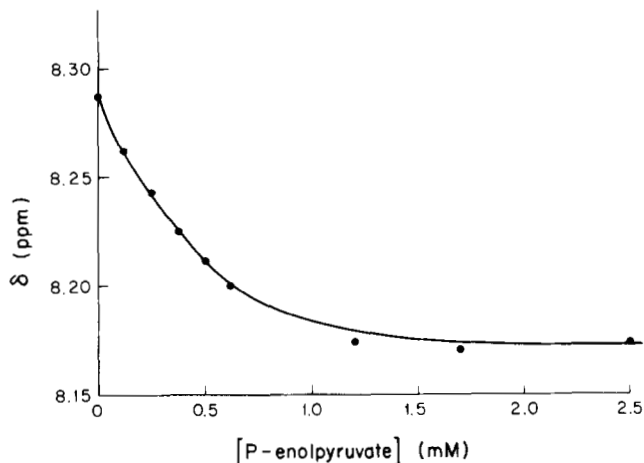


FIG. 6. Titration measuring the effect of P-enolpyruvate on the chemical shift of the C-2 ring proton of His-3 of pyruvate kinase at pH* 5.8. Components present were pyruvate kinase (0.55 mM), KCl (0.1 M) MgCl₂ (5.0 mM), and K-PIPES (1.5 mM). The curve is a computer fit to the data according to equation 5, obtained by a nonlinear least squares method, assuming a 1:1 stoichiometry between P-enolpyruvate and enzyme subunits.

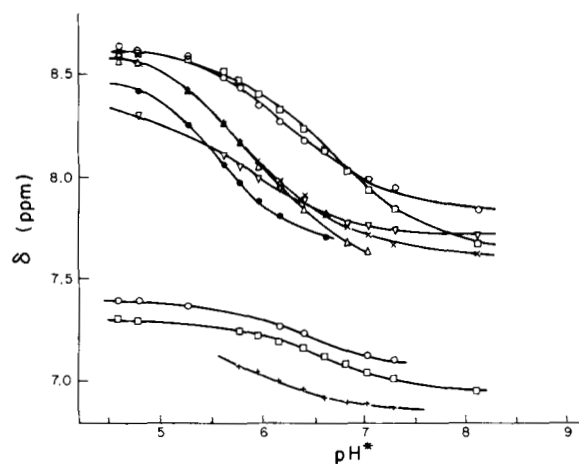


FIG. 7. ¹H NMR pH titration curves for the histidine C-2 and C-4 ring protons of pyruvate kinase in the presence of 0.1 M KCl, 5.0 mM MgCl₂, and 1.4 mM P-enolpyruvate. Components, conditions, and symbols are otherwise as described in Fig. 3.

seen (Fig. 4). In the presence of K⁺ alone only a small shift of 0.05 ppm is induced by P-enolpyruvate, and in the presence of Mg²⁺ alone a much smaller shift of 0.02 ppm is induced by P-enolpyruvate (Fig. 5). In the experiment with Mg²⁺ alone, the ionic strength was maintained at 0.1 M using deuterated Tris-Cl.

TABLE III

Least squares analysis of pH titration data for histidine residues of pyruvate kinase in the presence of KCl (0.1 M) MgCl₂ (5.0 mM), and P-enolpyruvate (1.4 mM)

Peaks	pK'		n	Chemical shift		
	n fixed at 1	n fitted		δH^+	δH^0	$\Delta\delta H^+, H^0$
					ppm	
1	6.59 ± 0.02	6.62 ± 0.01	0.86 ± 0.03	8.63 ± 0.01	7.64 ± 0.01	0.99 ± 0.02
2	6.21 ± 0.03	6.23 ± 0.03	0.95 ± 0.06	8.67 ± 0.02	7.83 ± 0.02	0.85 ± 0.04
3	5.83 ± 0.06	5.77 ± 0.04	0.76 ± 0.03	8.75 ± 0.03	7.61 ± 0.02	1.05 ± 0.04
4	5.91 ± 0.02	5.92 ± 0.03	0.89 ± 0.08	8.64 ± 0.03	7.54 ± 0.03	1.00 ± 0.06
5	5.88 ± 0.04	5.81 ± 0.02	0.78 ± 0.04	8.39 ± 0.02	7.70 ± 0.01	0.69 ± 0.02
6	5.54 ± 0.05	5.57 ± 0.02	1.10 ± 0.07	8.53 ± 0.03	7.65 ± 0.01	0.87 ± 0.03
1'	6.60 ± 0.07			7.30 ± 0.01	6.96 ± 0.02	0.34 ± 0.03
2'	6.37 ± 0.06			7.40 ± 0.01	7.07 ± 0.02	0.33 ± 0.03
3'	6.07 ± 0.09			7.20 ± 0.02	6.86 ± 0.01	0.34 ± 0.03

A titration with P-enolpyruvate at pH 5.8 in the presence of K⁺ and Mg²⁺, measuring the change in chemical shift of the C-2 proton resonance of His-3 is shown in Fig. 6. The dissociation constant of P-enolpyruvate from the enzyme·Mg²⁺·K⁺·P-enolpyruvate complex found by a nonlinear least squares fitted titration curve ($74 \pm 7 \mu\text{M}$) is in reasonable agreement with K_D values obtained by kinetic studies ($71 \pm 22 \mu\text{M}$ (21)) and by binding studies using the ultracentrifuge method ($75 \mu\text{M}$ (22)) or in competition with CrATP ($90 \pm 30 \mu\text{M}$ (23)), at somewhat higher pH values. These results confirm that the observed shift of His-3 results from P-enolpyruvate binding to the active site of pyruvate kinase.

Titration of the histidine resonances of pyruvate kinase with pH were carried out in the presence of a saturating level (1.4 mM) of P-enolpyruvate in addition to 0.1 M KCl and 5.0 mM MgCl₂. Titration curves obtained from 13 pH values (Fig. 7) and titration parameters obtained by computer fitting are listed in Table III. A large difference in the titration curve was observed only for His-3. The pK' value of His-3 decreased by 0.37 units to 5.77 on the addition of P-enolpyruvate (1.4 mM) in the presence of Mg²⁺ and K⁺, while those of the other histidines changed by ≤ 0.1 unit.

DISCUSSION

The proton NMR spectra of the aromatic region of pyruvate kinase showed resonances that were unexpectedly well resolved for a protein with a large molecular weight (237,000 (1)). The muscle pyruvate kinase molecule is composed of four indistinguishable subunits. Each subunit includes three domains and it has been found by x-ray analysis that the active site is located in the intersection of domains A and B (24). Some local freedom of motion of each domain could lead to sharper resonance lines near the active site (18). On the other hand, the motion of histidine residues located inside the protein may be hindered. As a result, most of the histidine resonance lines would be too broad to be observed. This may explain our detection of only six of the 14 histidines/subunit of pyruvate kinase.

The presence of histidine 3 at the active site is suggested by its change in chemical shift in response to the high affinity, active site binding of P-enolpyruvate. This change in shift actually results from a decrease in the pK' of His-3 by 0.4 units. Since the direct interaction of the P-enolpyruvate trianion with His-3 would increase the pK' value, this decrease results either from the binding of Mg²⁺ at or near His-3 or indirectly from a substrate-induced conformational change. Both the monovalent and divalent cation activators must be present for this effect of P-enolpyruvate, suggesting that the shift in pK' might result from the more direct interaction of the metal ions with His-3.

The overall effect of P-enolpyruvate, Mg²⁺, and K⁺ on the chemical shift of the C-2 proton of His-3 is 0.14 ppm at pH 5.8. The activating cations K⁺ or Mg²⁺ alone induce smaller upfield shifts of His-3, providing further evidence that His-3 is located at or near the metal binding site. Indeed the distance between the monovalent and divalent cation activators in the enzyme-P-enolpyruvate complex (4.9 Å) (6) is comparable to that expected for the two metals sharing a common imidazole ligand (5.8 Å), although this distance could result from other structures as well. The large additional effect of P-enolpyruvate on the chemical shift of His-3, which is due entirely to a shift in the pK' of His-3, is consistent with the finding that the substrate tightens the active site binding of both the monovalent and divalent cation activators (5, 25).

In the absence of P-enolpyruvate, the effects of the monovalent and divalent cation activators on the shift of His-3 are not additive. This finding is consistent with kinetic data which suggest that the monovalent cation can bind at the divalent cation site in absence of the divalent cation, and conversely (25). Further evidence for the direct interaction of a paramagnetic divalent cation activator with His-3 will be presented in the next paper in this series.

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