Nuclear Magnetic Resonance Studies of the Function of Potassium in the Mechanism of Pyruvate Kinase†

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ABSTRACT: Phosphoenolpyruvate and its analogs, D-phospholactate, L-phospholactate, and phosphoglycolate, bind to the Mn^{2+} complex of muscle pyruvate kinase as previously detected by kinetic studies and by titrations measuring the longitudinal relaxation rate ($1/T_{1p}$) of water protons (Nowak, T., and Mildvan, A. S. (1970), *J. Biol. Chem. 245*, 6057). The present work shows that K^+ raises the affinity of pyruvate kinase–Mn^{2+} for P-enolpyruvate, pyruvate, D-phospholactate, L-phospholactate, and phosphoglycolate by factors of 14, 4, 5, 3, and 22, respectively, and decreases the enhancement of their ternary complexes (ϵ_T). Conversely, P-enolpyruvate, D-phospholactate, L-phospholactate, and phosphoglycolate raise the affinity of pyruvate kinase for K^+ by one or two orders of magnitude, indicating an interdependence of the binding of K^+ and P-enolpyruvate and its analogs to pyruvate kinase–Mn^{2+}. Potassium decreases the affinity of pyruvate kinase–Mn^{2+} for substrates or for analogs in which the carboxyl group is lacking (monofluorophosphate and methylphosphonate) or is blocked (methyl ester of D-phospholactate). From measurements of $1/T_{1p}$ of the methyl or methylene protons of the analogs, using the correlation time estimated for water protons in the same complexes, Mn to proton distances are calculated which are consistent with pyruvate kinase–Mn^{2+}–analog bridge complexes involving phosphoryl coordination to the enzyme-bound Mn^{2+}. Phosphoryl coordination is confirmed by ^{31}P nuclear magnetic resonance of phosphoglycolate. In the absence of K^+, the distances from enzyme-bound Mn to the methyl protons of L-phospholactate (5.4 Å), D-phospholactate (6.6 Å), the methylene protons of phosphoglycolate (6.3 Å), and the phosphorus of phosphoglycolate (3.5 Å), can be fit by a self-consistent composite superposition of models of the three complexes. The addition of K^+ to the enzyme increases the distance between the enzyme-bound Mn^{2+} and the methyl group of L-phospholactate to 6.7 Å, but has little effect on the distance to the methyl group of the D isomer (6.9 Å), consistent with a 1.0-Å movement of the carboxyl group of the composite analog and a 60° intramolecular rotation about the P–OMn bond. These results suggest coordination of the carboxyl group of P-enolpyruvate and its analogs by enzyme-bound K^+ which changes the conformation of the enzyme–Mn–P-enolpyruvate complex to its catalytically active form.

Pyruvate kinase was the first enzyme which was shown to require a monovalent cation for activity (Boyer *et al.*, 1942). As pointed out by Suelter (1970), a monovalent cation requirement has since been found for more than 60 enzymes. To date, however, the mode of action of the monovalent cation has not been elucidated in any of the systems studied. The general role proposed for monovalent cations has been to induce a conformational change of the enzyme yielding the active form (Kachmar and Boyer, 1953; Happold and Beechey 1958; Evans and Sorger, 1966). The primary bases for this suggestion were the correlation of enzymatic activity with the ionic radii of the ions, and the high concentrations of monovalent cations required for maximal effect (5–100 mM). The observations that activating cations could change the spectroscopic (Suelter *et al.*, 1966) and immunoelectrophoretic (Sorger *et al.*, 1965) properties of the enzyme or the nuclear magnetic resonance properties of an enzyme–Mn–substrate complex (Mildvan and Cohn, 1964) lent support to this theory. These observations, however, are also compatible with a more direct role for monovalent cations in catalysis, in addition to their effects on enzyme conformation. Lowenstein (1960) first proposed a direct role for K^+ in transphosphorylation reactions on the basis of model studies which showed that K^+ stimulated the phosphorylation of orthophosphate by MnATP. Lowenstein suggested that K^+ formed a ternary complex with MnATP which, by partial charge neutralization and, by inducing conformational changes of the polyphosphate chain, facilitated nucleophilic attack by orthophosphate. From an extensive survey of those enzyme reactions requiring monovalent cations, Suelter (1970) has proposed a direct role for K^+ in stabilizing an enolate intermediate formed during catalysis. Support for a direct role of the monovalent cation in catalysis by pyruvate kinase was obtained by Tl^+ nuclear magnetic resonance (nmr) studies which indicated that the monovalent activator binds within 8 Å of the Mn^{2+} binding site on pyruvate kinase (Kayne and Reuben, 1970). The divalent cation is believed to be at the reaction center coordinating the phosphoryl group undergoing transfer (Mildvan *et al.*, 1967).

We have previously shown by kinetic and binding studies (Nowak and Mildvan, 1970) that the P-enolpyruvate[1] analogs, D-phospholactate, and phosphoglycolate, are potent, stereoselective inhibitors of pyruvate kinase. The inhibitor, L-phospholactate, binds to the enzyme with only $1/20$th the affinity, presumably due to steric interference between the methyl group of L-phospholactate and the base on the enzyme which stereoselectively protonates P-enolpyruvate (Rose, 1970; Nowak and Mildvan, 1970, 1972). The present study

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[1] Abbreviations used are: PRR, the water proton longitudinal relaxation rate; P-enolpyruvate, phosphoenolpyruvate; TMA, tetramethylammonium cation.

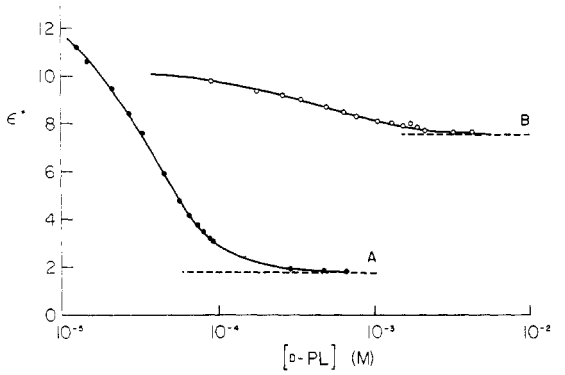

FIGURE 1: Titrations of pyruvate kinase–Mn^{2+} with D-phospholactate in the presence (A) and absence (B) of KCl. A plot of the enhancement (ϵ^*) *vs.* the concentration of D-phospholactate is shown. In curve A, pyruvate kinase (47.5 μM) in the presence of 50 μM $MnCl_2$–0.05 M Tris-Cl (pH 7.5) and 0.1 M KCl in 50 μl was titrated with a solution containing 0.20 mM D-phospholactate in addition to the same concentration of enzyme, $MnCl_2$, and KCl. In curve B, pyruvate kinase (65.4 μM), in the presence of 50 μM $MnCl_2$ and 0.15 M Tris-Cl (pH 7.5) in 50 μl, was titrated with a solution containing 4.67 mM D-phospholactate in addition to the same concentration of $MnCl_2$ and Tris-Cl. Temperature = 24 ± 0.5°. The points represent the observed enhancement values and the curves are computed using the K_3 and ϵ_T values given in Table I. The dashed lines represent operational end points obtained by graphical methods.

examines the effect of K^+ on the interaction of these analogs of P-enolpyruvate with the pyruvate kinase–Mn^{2+} complex and provides evidence for a direct role of the monovalent cation in binding the carboxyl group and adjusting the conformation of P-enolpyruvate analogs on the enzyme. A preliminary report of this work has been published (Nowak, 1971).

Experimental Section

Materials. Rabbit muscle pyruvate kinase and lactate dehydrogenase were purchased from Boehringer and Sohne (Mannheim, West Germany). The NADH and P-enolpyruvate were purchased from Sigma and 2-phosphoglycolate was purchased as the tricyclohexylammonium salt from General Biochemicals, Chagrin Falls, Ohio. Spectroscopically pure MgO was obtained from Johnson Matthey Chemicals, Ltd. (London, England). Sodium monofluorophosphate was purchased from K & K Laboratories, Plainview, N. Y., and was purified at 2° as follows. A 10-g sample of Na_2FPO_3 was dissolved in 25 ml of water, the pH was adjusted to 7.3 with 1 N KOH, and the solution was filtered. When the solution was kept at 2° overnight, fine crystals formed. One volume of cold ethanol was then added and mixed and, after standing 30 min, the upper layer was discarded. The ethanol procedure was repeated and the crystals formed in the bottom layer were then collected by filtration, washed with cold ether, and air-dried. The recrystallized Na_2FPO_3 was further purified by passage through a Dowex 1-Cl column and eluted with 50 mM NH_4HCO_3. The principal peak which contained no inorganic phosphate was collected and lyophilized. The solid residue was redissolved in 1 ml of H_2O and passed through a Dowex 50-H column. The acid fraction was neutralized with either KOH or TMA-OH to form the desired salt. The samples were found to be free of inorganic phosphate. D- and L-phospholactate were synthesized as previously described (Nowak and Mildvan, 1970), and the methyl ester of D-phospholactate was a side product of that synthesis. Methylphosphonic acid was a generous gift of Dr. Alexander Hampton. These compounds were converted to their K^+-free form by passage through a Dowex 50-H column and the material collected as the free acid. The acid form was neutralized either with TMA-OH or with Tris base, as desired.

Methods. Pyruvate kinase, assayed as previously described (Tietz and Ochoa, 1958), had a specific activity of 110–150 units/mg and was judged by acrylamide gel electrophoresis to be at least 98% pure. Binding studies were done by the proton relaxation rate method as previously described (Nowak and Mildvan, 1970) and the titrations were performed with little or no dilution of the enzyme–Mn^{2+} solution. When necessary, the observed paramagnetic contributions to the relaxation rates were corrected for dilution. The titrations were evaluated by computer fit using the program written by Reed *et al.* (1970) and adapted for the Wang 700 computer by Dr. Ilana Tamir.

In the analyses of the data, the previously reported dissociation constants (K_1) and enhancement factors (ϵ_a) of the binary Mn^{2+}–analog complexes were used (Mildvan and Cohn, 1966; Miller *et al.*, 1968), as were the corresponding parameters of the Mn^{2+}–enzyme complex, K_D, and ϵ_b (Mildvan and Cohn, 1965; Reuben and Cohn, 1970; Cottam and Mildvan, 1971). The dissociation constants which were computed are defined as K_3 = [EM][S]/[EMS] and K_s = [E][S]/[ES]. Values of K_3 and K_s were varied systematically and the values chosen were those which minimized the per cent standard deviation (% sd) about the mean of ϵ_T, the enhancement of the ternary enzyme–Mn^{2+}–analog complex (Reed *et al.*, 1970). Most of the titration curves were fit with a % sd of less than 5% although some curves were fit with a % sd of up to 10%. The values of K_3, K_s, and ϵ_T reported are averages obtained from several titrations.

In the cases where the system was kept K^+-free, KCl was replaced by either TMA-Cl or Tris-Cl, with indistinguishable results. All PRR experiments were performed on a Nuclear Magnetic Resonance Specialties PS60W pulsed nmr spectrometer operating at 24.3 MHz at 24 ± 1°. In the nmr experiments in which the relaxation rates of the carbon-bound protons or the phosphorus of the P-enolpyruvate analogs were measured, the enzyme was desalted as previously described (Nowak and Mildvan, 1970) and either lyophilized and redissolved in distilled 99.8% D_2O (General Dynamics) or concentrated by vacuum dialysis in collodion bags several times with the addition of 50 mM Tris-Cl (pH 7.5) in D_2O. For proton nmr studies the lyophilization and solution in D_2O were repeated twice. The nmr spectra were taken either on a Varian HA-100-15 nmr spectrometer or on a Varian XL-100-15 spectrometer which was operated at 30 ± 1°. For the ^{31}P nmr of phosphoglycolate, the Varian XL-100-15 nmr spectrometer was used at 40.5 MHz, with 12-mm sample tubes to increase the signal level. An internal deuteron lock was used since the solutions contained ≥90% D_2O. The $1/T_2$ data were obtained from measurements of the half-width of the resonance signal at half-height at 5 dB or more below saturation and $1/T_1$ was obtained by measuring the power at which the signal saturates. The paramagnetic contributions to the relaxation rates ($1/T_{1p}$, $1/T_{2p}$) were calculated by subtracting the relaxation rates observed in absence of Mn^{2+} from those observed in its presence. The values of $1/T_{1p}$ and $1/T_{2p}$ were normalized by the factor, p = [Mn]/[ligand], as previously described (Mildvan *et al.*, 1967; Mildvan and Cohn, 1970).

Results

Binding of P-enolpyruvate and Its Analogs to Pyruvate Kinase–Mn in the Presence and Absence of Potassium Ion. To determine the influence of the activating potassium ion on the binding of P-enolpyruvate and its analogs to pyruvate kinase–Mn, the binary enzyme–Mn complex was titrated with the ligand in the presence or absence of potassium measuring the $1/T_1$ of water. When the titrations were performed in the absence of potassium, the nonactivating cations, TMA or Tris, were substituted. A set of experiments obtained with D-phospholactate in the presence and absence of potassium is shown in Figure 1. In the presence of potassium, a significantly lower concentration of D-phospholactate is required to obtain a half-maximal effect in the titration and a significantly lower enhancement factor of the ternary complex (ϵ_T) is observed. The titration parameters (K_3, K_s, and ϵ_T) used to generate the solid curves of Figure 1 are summarized in Table I, which also contains the corresponding parameters for P-enolpyruvate and its other analogs, as determined by computer analysis. A marked effect of K^+ upon the dissociation constant, K_3, and the ϵ_T of each of the ternary complexes is observed. In each case, the dissociation constant of the ligand from the enzyme–Mn–ligand ternary complex is decreased by potassium, as is the value of ϵ_T for each complex. Similarly, K^+ appears to lower K_s, the dissociation constant of the binary enzyme–analog complexes (Table I). However, this point is less certain since K_s is less directly and less accurately determined than is K_3 from PRR titrations.

Effect of P-enolpyruvate and Its Analogs on the Binding of Potassium to the Enzyme. Since the ϵ_T measured in the absence of K^+ was different from that measured in its presence (Table I), each enzyme–Mn–ligand ternary complex could be titrated with K^+ to determine the dissociation constant of the monovalent cation from the ternary complexes. From equations previously derived (Mildvan and Cohn, 1966), the concentrations of K^+ required to produce a half-maximal decrease in ϵ_T approximate the dissociation constants (K_D) of K^+ from each quaternary enzyme–Mn–K–analog complex. These values, summarized in Table II, indicate that K^+ binds more tightly to the ternary complexes of P-enolpyruvate and its tight binding analogs, D-phospholactate and phosphoglycolate, than to the ternary complex of the weaker ligand, L-phospholactate. All of the dissociation constants in Table II are much lower than the dissociation constant of the binary pyruvate kinase–K^+ complex (0.12 M), determined by uv difference spectroscopy (Suelter *et al.*, 1966). This difference may be due, in part, to the presence of Mn^{2+} which lowers the dissociation constant for K^+ by an order of magnitude (0.014M) as determined kinetically (Suelter *et al.*, 1966). Tables I and II suggest a mutual qualitative and quantitative relationship: K^+ raises the affinity of the enzyme for P-enolpyruvate and its analogs and, conversely, P-enolpyruvate and its analogs raise the affinity of the enzyme for K^+. This interrelationship suggests the formation of a ligand–K^+–enzyme bridge complex. Since the enzyme-bound divalent cation may act by coordinating the phosphate group (Mildvan *et al.*, 1967), the possibility that K^+ might act by coordinating the carboxyl group of P-enolpyruvate and its analogs was investigated. If this were the case, K^+ would not be expected to increase the affinity of the enzyme for P-enolpyruvate analogs lacking a free carboxyl group.

Effect of Potassium on the Binding of P-enolpyruvate Analogs Lacking a Free Carboxyl Group. As shown in Figure 2, the methyl ester of D-phospholactate and methylphosphonate

TABLE I: Effect of Potassium on the Binding of P-enolpyruvate and Analogs to the Pyruvate Kinase–Mn Complex.[a]

Ligand	With K^+				Without K^+				$K_3 - K^+/K_3 + K^+$
	K_3 (μM)	K_s (mM)	ϵ_T	% Sd Range	K_3 (μM)	K_s (mM)	ϵ_T	% Sd Range	
P-enolpyruvate[b]	0.58 ± 0.03	0.13 ± 0.03	1.85 ± 0.05	5.4–9.3	3.9 ± 0.4	0.15 ± 0.05	5.63 ± 0.14	2.1–3.1	6.7 ± 0.8
D-Phospholactate	2.5 ± 1.0	0.40 ± 0.10	2.00 ± 0.05	0.5–3.0	12.0 ± 5.0	5.0 ± 1.0	9.01 ± 1.04	4.5–6.0	4.8 ± 2.8
L-Phospholactate	42.0 ± 2.0	1.5 ± 0.9	4.62 ± 0.22	9.2–10.6	110 ± 40	3.5 ± 1.5	10.24 ± 0.74	1.6–2.5	2.6 ± 1.0
Phosphoglycolate	15.0 ± 1.0	0.10 ± 0.01	3.30 ± 0.33	6.6–8.5	330 ± 130	1.2 ± 0.6	6.00 ± 0.83	2.7–5.9	22.0 ± 8.8

[a] These parameters were obtained by computer analysis of PRR titrations (Reed *et al.*, 1970). The PRR enhancement of the binary enzyme–Mn complex (ϵ_b = 25.0 ± 2.6) was not affected by the replacement of K^+ with either TMA^+ or $Tris^+$. The dissociation constants (K_I) and enhancements (ϵ_a) of the binary Mn^{2+}–ligand complexes used in the analyses were those previously reported for P-enolpyruvate (K_I = 1.9 mM; ϵ_a = 1.15, Mildvan and Cohn, 1966) and phospholactate (K_I = 2.1 mM; ϵ_a = 1.1, Miller *et al.*, 1968). For phosphoglycolate, as determined by epr and PRR at 25° and μ = 0.1, K_I = 1.7 ± 0.2 mM and ϵ_a = 1.06 ± 0.03. [b] Similar observations with P-enolpyruvate have recently been made by J. Reuben, G. Reed, and M. Cohn (private communication).

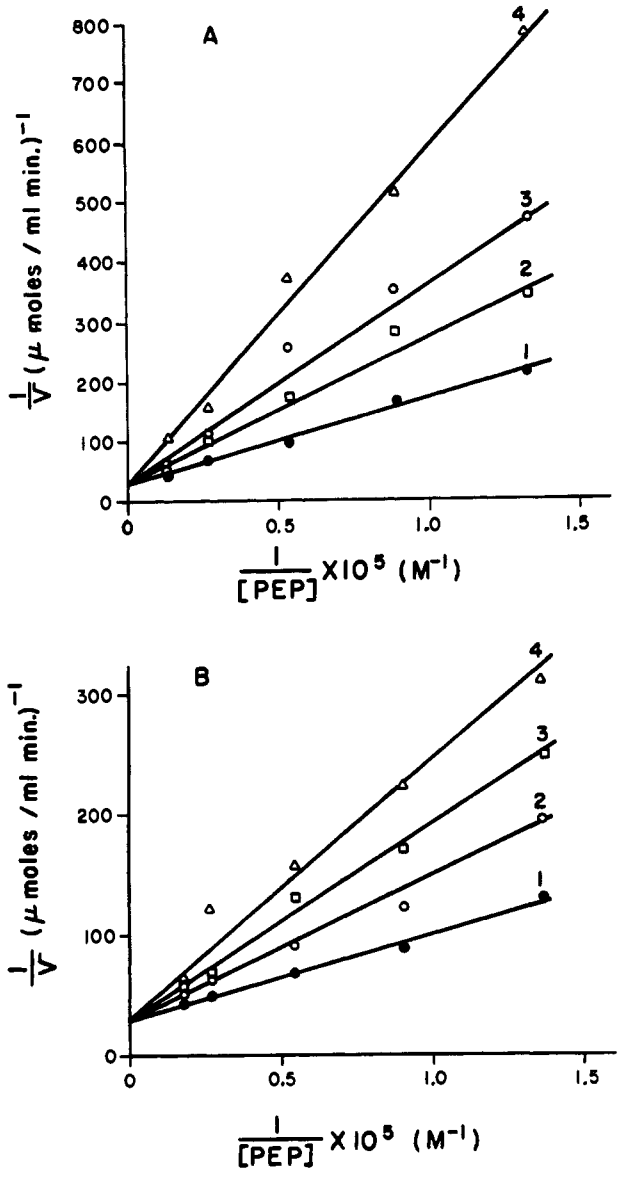

FIGURE 2: Double-reciprocal plots of the initial velocity of the pyruvate kinase reaction as a function of P-enolpyruvate concentration in the presence of varying concentrations of the methyl ester of D-phospholactate (Me-D-P-lactate) and CH_3PO_3. (A) The reactions contained the following concentrations of Me-D-P-lactate: line 1, 0 mM; 2, 0.42 mM; 3, 0.84 mM; 4, 1.26 mM. (B) The reactions contained the following concentrations of CH_3PO_3: line 1, 0 mM; 2, 2.65 mM; 3, 5.30 mM; 4, 10.6 mM. In addition, the reaction vessels all contained 100 mM Tris-Cl buffer (pH 7.5), 100 mM KCl, 4 mM $MnCl_2$, 1 mM ADP, 0.16 mM NADH, 25 μg of lactate dehydrogenase, and P-enolpyruvate as indicated. The reactions in A were initiated by the addition of 0.103 μg of pyruvate kinase and in B by the addition of 0.247 μg of pyruvate kinase to give a final volume of 1 ml. The temperature was maintained at 25°.

($CH_3PO_3^{2-}$) were found to be linear competitive inhibitors of the Mn-activated pyruvate kinase reaction with respect to P-enolpyruvate. Similar behavior was previously observed with fluorophosphate (Mildvan *et al.*, 1967). The inhibitor constants (Table III) indicate that these analogs are poorer inhibitors than the analogs possessing free carboxyl groups. Typical PRR titrations of pyruvate kinase–Mn with the methyl ester of D-phospholactate in the presence and absence of K^+ are shown in Figure 3, where little difference is observed between the two titration curves. The dissociation constants

TABLE II: Potassium Ion Binding to Pyruvate Kinase Ternary Complexes.[a]

Ligand	K_d (mM)
P-enolpyruvate	0.80 ± 0.05
D-Phospholactate	1.6 ± 0.3
L-Phospholactate	14.0 ± 3.0
Phosphoglycolate	1.0 ± 0.2

[a] K_d without Me^{2+}, 120 mM determined by difference spectroscopy (Suelter *et al.*, 1966). K_d with Mn^{2+}, 14 mM determined kinetically (Suelter *et al.*, 1966).

TABLE III: Inhibition of Pyruvate Kinase by Various P-enolpyruvate Analogs.

Inhibitor	K_I (mM)
Me-D-PL[a]	0.58 ± 0.02
FPO_3^{2-}	3.4 ± 1.0[b]
$CH_3PO_3^{2-}$	3.8 ± 0.1

[a] Methyl ester of D-phospholactate. [b] From Mildvan *et al.* (1967).

(K_3) of the three analogs from pyruvate kinase–Mn^{2+} obtained by computer analysis (Table IV) reveal that in the presence of the monovalent cation, the enzyme–Mn^{2+} complex has a lower affinity for these ligands. The monovalent cation, however, does not produce a change in the ϵ_T values. The presence of K^+ has essentially no effect on the K_s values of these analogs (Table IV).

With such weak binding ligands, the uncertainty in ϵ_T is high since an end point in the titration is seldom observed and the relative contribution of the ligand removing the metal from the protein is increased. Also, with these complexes the titration of enzyme–Mn is taking place over a narrower range of the measured parameter, ϵ^*. The dissociation constants of the enzyme–Mn–analog complexes obtained by PRR titrations (Table IV) in the presence of K^+ are in order of magnitude agreement with the K_I values obtained kinetically (Table III).

The ϵ_T value for FPO_3^{2-} obtained by computer analysis (32.2 ± 2.4, Table IV) differs by an order of magnitude from the previously reported value estimated by graphical analysis (1.5 ± 0.5, Mildvan *et al.*, 1967) while the K_3 values obtained by both methods are in agreement. The computer fit which utilizes all of the points in the titration curve and all of the operative equilibria is deemed more reliable. Because of the high dissociation constant of the ternary complex, similar to that of the binary complex, the concentration of the latter exceeds that of the former as the titration proceeds, and the ternary complex becomes a minor component. Computer analysis of the previously published titration curves for FPO_3^{2-} yields ϵ_T values indistinguishable from 32.2 ± 2.4.

Effect of Potassium on the Binding of Pyruvate to the Enzyme. The binding of pyruvate to the pyruvate kinase–Mn^{2+} complex was measured by PRR titration in the presence of either TMA^+ or K^+. With TMA^+ present, pyruvate binds to the

TABLE IV: Effect of Potassium on the Binding of P-enolpyruvate Analogs Lacking a Free Carboxyl Group to the Pyruvate Kinase–Mn^{2+} Complex.[a]

Analog	With K^+				Without K^+				$\frac{K_3 - K^+}{K_3 + K^+}$
	K_3 (mM)	K_s (mM)	ϵ_T	% Sd Range	K_3 (mM)	K_s (mM)	ϵ_T	% Sd Range	
Me-D-PL	0.73 ± 0.46	1.3 ± 0.4	23.1 ± 5.5	1.4-2.7	0.40 ± 0.10	0.5 ± 0.2	28.6 ± 2.9	0.25-1.4	0.55 ± 0.37
FPO_3^{2-}	1.60 ± 0.50	1.0 ± 0.2	32.2 ± 2.4	3.4-6.5	0.33 ± 0.13	1.0 ± 0.2	27.0 ± 1.2	1.1-2.0	0.21 ± 0.10
$CH_3PO_3^{2-}$	0.47 ± 0.25	3.5 ± 0.5	17.7 ± 4.6	1.2-4.0	0.11 ± 0.02	3.2 ± 0.5	18.9 ± 3.0	2.6-3.4	0.23 ± 0.13

[a] These parameters were obtained by computer analysis of PRR titrations (Reed *et al.*, 1970). The dissociation constants (K_I) and enhancements (ϵ_a) of the binary Mn–ligand complexes used in the analyses were $K_I = 2.0 \pm 0.2$ mM and $\epsilon_a = 1.0 \pm 0.1$ based on the values previously reported for FPO_3^{2-} (Mildvan *et al.*, 1967) and the similarity of these constants for various monophosphate ligands (Table I).

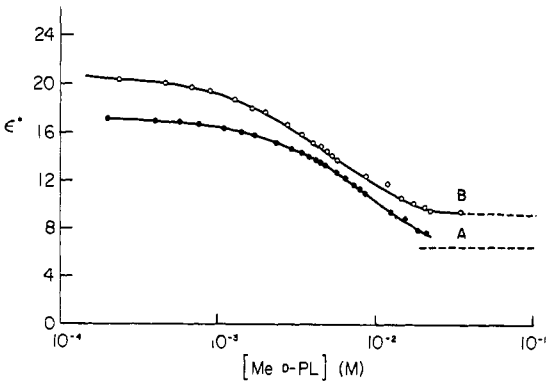

FIGURE 3: Titrations of pyruvate kinase–Mn^{2+} with Me-D-P-lactate in the presence (A) and absence (B) of KCl. A plot of the enhancement (ϵ^*) *vs.* the concentration of the inhibitor added is shown. In curve A, pyruvate kinase (67.5 μM) in the presence of 50 μM $MnCl_2$, 0.1 M KCl, and 0.05 M Tris-Cl (pH 7.5) in 50 μl was titrated with a solution containing 10 mM Me-D-P-lactate in addition to the same concentration of enzyme, $MnCl_2$, and KCl. In curve B, pyruvate kinase (67.5 μM) in the presence of 50 μM $MnCl_2$, 0.1 M TMA-Cl, and 0.05 M Tris-Cl (pH 7.5) in 50 μl was titrated with a solution containing 12 mM Me-D-P-lactate in addition to the same concentration of enzyme, $MnCl_2$, and TMA-Cl. Temperature 24 ± 0.5°. The points represent the observed enhancement values and the curves are computed using the K_3 and ϵ_T values given in Table IV. The dashed lines represent operational end points obtained by graphical methods.

enzyme with a K_3 of 0.66 ● 0.17 mM and an enhancement factor of 6.33 ± 1.08 for the ternary complex. The presence of K^+ increases the affinity of pyruvate kinase–Mn^{2+} by a factor of 3.5 ($K_3 = 0.19 \pm 0.04$ mM) but has no significant effect on the ϵ_T value (5.25 ± 0.56).

Paramagnetic Effects of Mn^{2+} and Pyruvate Kinase–Mn^{2+} on the Protons of D- *and* L-*Phospholactate and Phosphogly-*

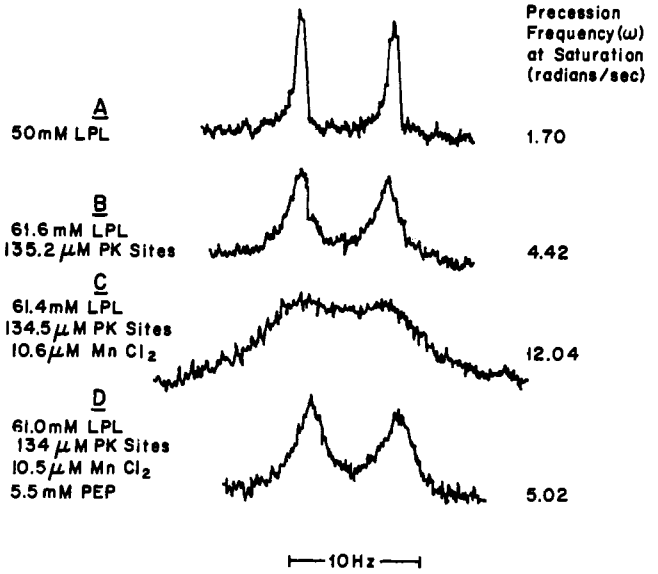

FIGURE 4: The effect of Mn–pyruvate kinase on the proton nuclear magnetic resonance spectra of L-phospholactate (L-PL) in the absence of KCl at 100 MHz. The L-PL was present as the Tris salt in 99.6% D_2O (A). Enzyme was added as a solution in 0.05 M Tris-Cl, pH 7.5 buffer in D_2O (B). $MnCl_2$ was added in D_2O (C). P-enolpyruvate was added as the Tris salt (pH 7.5) in D_2O (D). The initial volume was 0.375 ml (B) and the final volume was 0.379 ml (D). The temperature was 30 ± 1°.

TABLE V: Effect of Pyruvate Kinase–Mn on the Relaxation Rates of the Protons of the P-enolpyruvate Analogs.

Inhibitor	Additions	$sec^{-1} \times 10^{-3}$ $1/pT_{1p}$	$1/pT_{2p}$	ϵ_1	ϵ_2
L-Phospholactate	Mn^{2+}	14.9 ± 1.7	15.4 ± 0.9		
	PK[c] + Mn^{2+}	25.0 ± 2.0	77.0 ± 7.0	1.7	5.1
	PK + Mn^{2+} + K^+	3.63 ± 0.40	10.8 ± 1.0	0.24	0.70
D-Phospholactate	PK + Mn^{2+}	6.77 ± 0.70	13.8 ± 1.4	0.45[a]	0.90[a]
	PK + Mn^{2+} + K^+	1.36 ± 0.15	6.61 ± 0.65	0.09[a]	0.43[a]
Phosphoglycolate	Mn^{2+}	13.0 ± 0.5	13.0 ± 1.0		
	PK + Mn^{2+}	6.9 ± 0.8	15.0 ± 4.0	0.53	1.15
	PK + Mn^{2+} + K^+	≤ 0.9[b]	≤ 2.0[b]		

[a] Relaxation rates for Mn-L-PL used as denominator to calculate enhancement factors. [b] Probably exchange limited as discussed in the text. [c] PK = pyruvate kinase.

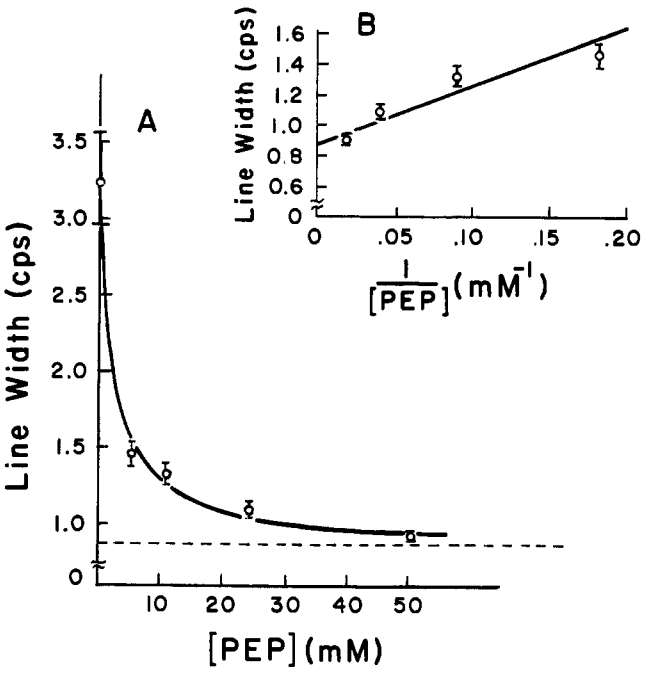

FIGURE 5: P-enolpyruvate competition with L-phospholactate (L-PL). (A) The effect of P-enolpyruvate on the line width of the methyl resonance of Tris L-PL (61.4 mM) in the presence of pyruvate kinase (134.6 μM sites), $MnCl_2$ (10.6 μM), and 50 mM Tris-Cl buffer (pH 7.5). Temperature = 30°. The points represent the experimental line widths measured after each addition of P-enolpyruvate. The curve is computed by assuming simple competition between P-enolpyruvate (K_3 = 4.0 μM) and L-PL (K_3 = 110 μM) for pyruvate kinase–Mn. (B) Reciprocal of P-enolpyruvate concentration *vs.* line width. Extrapolation to infinite concentration of P-enolpyruvate gives a line width of 0.87 cps, which is indistinguishable from that observed in the absence of enzyme and Mn^{2+}.

colate. The proton nmr spectrum of L- (and D-) phospholactate at 100 MHz has previously been described (Nowak and Mildvan, 1970). Only the methyl doublet (δ = 1.82 ppm downfield from tetramethylsilane, J = 7 cps) was suitable for relaxation rate measurements (Figure 4A). Metal-free pyruvate kinase was found to broaden the methyl resonance of L-phospholactate and to increase the R_F precession frequency at saturation (Figure 4B). These effects, which are decreased by 0.1 M K^+, but are unaffected by the diamagnetic divalent cation, Mg^{2+} (7 mM), are shown elsewhere to be due to a stereoselective diamagnetic effect of the protein on the relaxation rates of the methyl resonance of L-phospholactate (Nowak and Mildvan, 1972). The present paper considers only the additional paramagnetic effect of enzyme-bound Mn^{2+} on these resonances (Figure 4C). The paramagnetic effects of pyruvate kinase–Mn^{2+} on the longitudinal and transverse relaxation rates of the analogs, calculated by subtracting the respective diamagnetic effects from the total effects, are summarized in Table V.

In the absence of K^+, large paramagnetic effects of the enzyme-bound Mn^{2+} on the protons of all of the analogs are observed. With L-phospholactate, the presence of the enzyme enhances the effect of Mn^{2+} on the longitudinal and transverse relaxation rates of the methyl protons ($\epsilon_1 > 1$; $\epsilon_2 > 1$), while with D-phospholactate and phosphoglycolate, deenhancements of these rates are observed ($\epsilon_1 < 1$; $\epsilon_2 < 1$).

The presence of a saturating concentration of K^+ decreases all the paramagnetic effects, due in part to slower ligand exchange, as reflected in tighter ligand binding (Table I). With phosphoglycolate no paramagnetic effects are detected in the presence of enzyme, Mn^{2+}, and K^+ at 30 or 40° consistent with very slow exchange of the analog.

In all cases where paramagnetic effects of enzyme-bound Mn^{2+} are observed, $1/pT_{1p}$ is significantly less than $1/pT_{2p}$. It will be shown in the Discussion that $1/pT_{1p}$ can therefore be used to calculate Mn–proton distances on the ternary enzyme–Mn–ligand complexes. In the binary Mn–ligand systems, the longitudinal and transverse relaxation rates are equal, leaving open the possibility of exchange limited relaxation rates. Hence, in these cases only an upper limit to the distance can be calculated.

Competition between the Analogs in Ternary Enzyme–Mn^{2+}–Ligand Complexes with Phosphoenolpyruvate and ATP. As indicated in Figure 4D, the substrate P-enolpyruvate decreases the paramagnetic effects on the $1/T_1$ and $1/T_2$ relaxation rates of the analog L-phospholactate presumably by competing with the analog for the enzyme-bound Mn^{2+}. A titration study of this effect with the analog L-phospholactate (Figure 5) in absence of K^+ indicates that the results can be fit by simple competition between P-enolpyruvate and L-phospholactate for the same site on the enzyme. The solid theoretical curve which fits the data was obtained by assuming competition using a K_3 for P-enolpyruvate of 4.0 μM

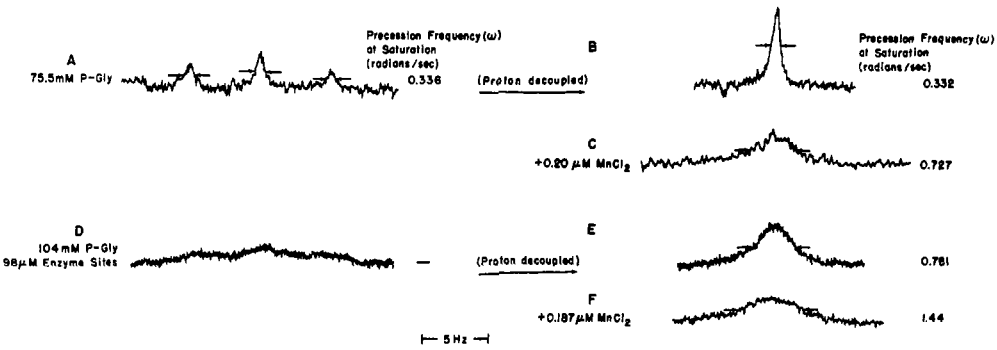

FIGURE 6: The effect of Mn^{2+} and pyruvate kinase–Mn^{2+} on the ^{31}P nmr spectrum of phosphoglycolate at 40.5 MHz. The phosphoglycolate was present as the TMA salt (K^+ free) in 99.6% D_2O, pH 7.5 (A). Proton decoupling simplified the spectrum without affecting the relaxation rates (B); $MnCl_2$ was added (C). Pyruvate kinase was added to phosphoglycolate in a solution containing 50 mM Tris-Cl (pH 7.5) (D) and the methylene protons were again decoupled from ^{31}P (E). $MnCl_2$ was added to this solution (F). The volume used in these experiments was 2.0 ml in a 12-mm nmr tube and the temperature was 30°.

TABLE VI: Effects of Mn^{2+} and Pyruvate Kinase–Mn^{2+} on the Relaxation Rates of the Phosphorus of Phosphoglycolate.[a]

	$sec^{-1} \times 10^{-3}$			
	$1/pT_{1p}$	$1/pT_{2p}$	ϵ_1	ϵ_2
Phosphoglycolate (57.5 mM) + Mn^{2+}	11.3 ± 0.7	3360 ± 240		
Phosphoglycolate (89.7 mM) + pyruvate kinase (138 μM sites) + Mn^{2+}	26.2 ± 4.2	4690 ± 470	2.32	1.40

[a] Mn^{2+} titrations were carried out over a concentration range of 0.06–0.3 μM $MnCl_2$.

and a K_3 for L-phospholactate of 110 μM, in agreement with the values of the dissociation constants determined independently by PRR titrations (Table I). This further substantiates the hypothesis that L-phospholactate binds at the same site on pyruvate kinase as does P-enolpyruvate and indicates that the continuous wave nmr, PRR, and previous kinetic studies (Nowak and Mildvan, 1970) are observing the same complex.

In other experiments, $1/pT_{2p}$ of D-phospholactate (60.6 mM) in the presence of pyruvate kinase (168 μM), $MnCl_2$ (25 μM), and KCl (31 mM) was decreased 20% by 13 mM ATP and an additional 17% by 31 mM P-enolpyruvate, consistent with competition between D-phospholactate, ATP, and P-enolpyruvate for a common site on the enzyme with affinities of the same order of magnitude. Competition between P-enolpyruvate and ATP on pyruvate kinase was first detected kinetically with the Mg^{2+}-activated enzyme (Reynard *et al.*, 1961) and confirmed with the Mn^{2+}-activated enzyme (Mildvan and Cohn, 1966). Similarly $1/pT_{2p}$ of phosphoglycolate (74 mM) in the presence of 340 μM pyruvate kinase sites and 25 μM $MnCl_2$ was decreased by 76% by 32.5 mM P-enolpyruvate consistent with competition for a common site on the enzyme between ligands of comparable affinity.

Effect of Pyruvate Kinase and Mn^{2+} on the ^{31}P Nuclear Magnetic Resonance Spectrum of Phosphoglycolate. To determine whether the phosphoryl groups of the tighter binding analogs were directly coordinated to the enzyme-bound Mn^{2+}, phosphoglycolate was studied. The ^{31}P nmr spectrum of this analog at 40.5 MHz consists of a triplet (J = 5.02 Hz) (Figure 6) which is 3.02 ppm downfield from the resonance of a separate sample of H_3PO_4 (64%) in D_2O (20% by volume) similarly locked on deuterons. The addition of pyruvate kinase to phosphoglycolate causes a broadening of the resonance and an increase in the precession frequency at saturation due to a diamagnetic effect of the protein on the relaxation rates of phosphorus (Nowak and Mildvan, 1972). The addition of Mn^{2+} greatly increases both parameters (Figure 6A) and the effects are diminished by the substrate, P-enolpyruvate (Figure 6B), consistent with competition at a common site.

Table VII, which summarizes the paramagnetic effects of pyruvate kinase bound Mn^{2+} on the ^{31}P relaxation rates of phosphoglycolate, reveals an enhancement due to the protein on $1/pT_{1p}$ and $1/pT_{2p}$ of the analog, consistent with phosphoryl coordination by the enzyme-bound Mn^{2+}. The latter will be confirmed by Mn^{2+} to phosphorus distance calculations.

Discussion

There is much evidence in the literature, based on uv difference spectroscopy (Suelter *et al.*, 1966), immunoelectrophoresis (Sorger *et al.*, 1965), and thallium nmr (Kayne and Reuben, 1970; Reuben and Kayne, 1971) for the direct binding of the activating monovalent cation to pyruvate kinase, with an affinity that agrees with its activator constant (Suelter *et al.*, 1966).

The observation that K^+ raises the affinity of pyruvate kinase for P-enolpyruvate and its analogs (Table I) and, conversely, that P-enolpyruvate and its analogs tighten the binding of K^+ to the enzyme (Table II), suggests an enzyme–K^+–P-enolpyruvate bridge complex. The observation that K^+ in fact actually decreases the affinity of the enzyme–Mn^{2+} for P-enolpyruvate analogs lacking a free carboxyl group (Table IV) and the evidence that the Mn^{2+} binds the phos-

phoryl group being transferred (Mildvan *et al.*, 1966) suggests that the carboxyl group of P-enolpyruvate and its analogs are coordinated by the enzyme-bound K^+.

Consistent with this hypothesis is the observation that K^+ increases the affinity of pyruvate kinase–Mn^{2+} for pyruvate by a factor of 3.5 but has little effect on the structure as reflected in the enhancement of the ternary complex. Proper binding of pyruvate in a ternary complex, however, probably does not occur unless ATP or an analog of ATP is present. Thus the enzyme does not catalyze the enolization of pyruvate in the absence of a group which will bind at the phosphoryl transfer site, *i.e.*, ATP, P_i (Rose, 1960), and pyruvate is not competitive with P-enolpyruvate in the absence of ADP (Mildvan and Cohn, 1966).

In the presence of pyruvate kinase the $1/pT_{1p}$ values of the protons (Table V) and phosphorus (Table VI) of the ligands are significantly less than the $1/pT_{2p}$ values. Hence,

$$1/pT_{1p} \leq 0.5(1/pT_{2p}) \tag{1}$$

$1/pT_{1p}$ contains a significant contribution from $1/T_{1M}$, the relaxation rate of the bound ligand, and may be used to calculate Mn^{2+} to proton and Mn^{2+} to phosphorus distances in the ternary complexes. This may be shown as follows. The equations describing Mn–ligand interactions (Luz and Meiboom, 1964; Swift and Connick, 1962), neglecting outer sphere contributions which are usually small, and assuming one analog bound per active site are

$$1/pT_{1p} = 1/(T_{1M} + \tau_M) \leq 1/\tau_M \tag{2}$$

$$1/pT_{2p} = 1/(T_{2M} + \tau_M) \leq 1/\tau_M \tag{3}$$

where T_{1M} and T_{2M} are the respective longitudinal and transverse relaxation times of the nuclei in the coordination sphere of the metal and τ_M is the residence time of the ligand in the coordination sphere. From the relationships 1, 2, and 3, we may write

$$1/(T_{1M} + \tau_M) \leq 0.5(1/\tau_M) \tag{4}$$

which, upon inversion, becomes

$$\tau_M \leq T_{1M} \tag{4a}$$

Hence, $1/pT_{1p}$ approximates $1/T_{1M}$. Even if relation 4a were an equality, the distances calculated by assuming $1/pT_{1p} = 1/T_{1M}$ would be too large by only 12% because of the sixth root relationship between the calculated distance (r) and $1/T_{1M}$ in the Solomon–Bloembergen equation for T_1 (Solomon, 1955; Solomon and Bloembergen, 1956). The dipolar term of the Solomon–Bloembergen equation for Mn to proton distances simplifies to

$$r\ (\text{in Å}) = 812[T_{1M} \cdot f(\tau_c)]^{1/6} \tag{5}$$

and for Mn to phosphorus distances is

$$r\ (\text{in Å}) = 601[T_{1M} \cdot f(\tau_c)]^{1/6} \tag{6}$$

where the correlation function is

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2} \tag{7}$$

In eq 7, τ_c is the correlation time for dipolar interaction and ω_I and ω_S are the nuclear and electron resonance frequencies, respectively. The correlation time is assumed to be identical with that for the $Mn \cdot H_2O$ interaction in the same complex, an assumption which is supported by the observation that the correlation time, τ_c, is dominated by τ_s, the electron spin relaxation time of Mn in the binary pyruvate kinase–Mn complex (Reuben and Cohn, 1970). The correlation function of each ternary complex, $f(\tau_c)^*$, is obtained from the respective ϵ_T values for H_2O in the same complex (Table I), using the relationship (Eisinger *et al.*, 1962)

$$\epsilon_T = \frac{f(\tau_c)^*}{f(\tau_c)} \frac{q^*}{q} \tag{8}$$

where ϵ_T is the enhancement of the complex and q is the coordination number for water on Mn^{2+}. The starred symbols refer to the complex under observation and the unstarred symbols refer to $Mn(H_2O)_6^{2+}$ for which the correlation function is $3\tau_c$ or 8.7×10^{-11} sec (Bloembergen and Morgan, 1961). In the binary complex of Mn with pyruvate kinase, q^* has been shown to be 3 (Reuben and Cohn, 1970). In the ternary complexes of all of the analogs, q^* has been assumed to be 2. Because of the sixth-root relationship in eq 5 and 6, a 50% error in q^* would lead to only a 7% error in the distance.

The longitudinal and transverse relaxation rates of the protons in the binary Mn–phospholactate complexes are equal (Table V), suggesting that $1/pT_{1p}$ is exchange limited and sets a lower limit to the value of $1/T_{1M}$. Hence, only upper limits to the Mn–proton distances in the binary Mn^{2+}–phospholactate complexes could be obtained (Table VII). For the protons and phosphorus of phosphoglycolate, the values of $1/pT_{1p}$ cannot be exchange limited since they are two orders of magnitude lower than $1/pT_{2p}$ of phosphorus (Tables V and VI). Hence $1/pT_{1p} = 1/T_{1M}$ and may be used to calculate distances directly (Table VII). The distances in the binary Mn–ligand complexes are consistent with monodentate or bidentate phosphoryl coordination. Phosphoryl coordination in the binary Mn–phosphoglycolate complex is established by the Mn to phosphorus distance of 2.8–2.9 Å (Table VII). From crystallographic data, typical Mn to phosphorus distances in undistorted Mn–phosphate complexes with metal–O–P bond angles of 109.5° are in the range 2.9–3.2 Å (International Tables for X-ray Crystallography, 1962). Simultaneous carboxyl coordination in the binary complexes which cannot be excluded by the distance calculations seems unlikely since the dissociation constant of Mn–phospholactate (2.1 mM) (Miller *et al.*, 1968) and Mn–P-enolpyruvate (1.9 mM) as determined by electron paramagnetic resonance (epr) agree with those of $Mn–FPO_3$ (1.8 mM) and $Mn–PO_4$ (4.7 mM) (Mildvan *et al.*, 1967).

The Mn^{2+} to proton and Mn^{2+} to phosphorus distances in the ternary complexes and their maximal errors are given in Table VIII. These distances are consistent only with phosphoryl coordination by the enzyme-bound Mn^{2+}. Carboxyl coordination which would yield Mn^{2+} to proton distances $\leq$5.4 Å is excluded by the calculated distances. The Mn^{2+} to phosphorus distance in the ternary enzyme–Mn–phosphoglycolate complex (3.5 ± 0.3 Å, Table VIII) appears to be slightly beyond the range of values expected for undistorted Mn–phosphate complexes but is significantly less than the value of 5.7–6.0 Å expected for a second sphere complex in which a water molecule intervenes between the Mn^{2+} and the phosphate.

In distorted complexes, including pentavalent phosphorus

TABLE VII: Distances of the Nuclei of P-enolpyruvate Analogs from Mn^{2+} in the Binary Mn–Ligand Complexes.

Analog	ϵ_a	q Assumed	τ_c[a] (sec × 10^{11})	r (Å)
L-Phospholactate	1.10 ± 0.01	5	3.83	≤3.61 ± 0.07[b]
		4	4.79	≤3.75 ± 0.08[b]
Phosphoglycolate	1.06 ± 0.03	5	3.69	3.67 ± 0.04[c]
		4	4.61	3.81 ± 0.04[c]
		5	3.69	2.78 ± 0.03[d]
		4	4.61	2.89 ± 0.03[d]

[a] Correlation time for water protons in the same complex determined from the enhancement (ϵ_a) of the binary complex. [b] Mn^{2+} to methyl protons. [c] Mn^{2+} to methylene protons. [d] Mn^{2+} to phosphorus.

TABLE VIII: Distances of Nuclei of P-enolpyruvate Analogs from the Pyruvate Kinase Bound Mn^{2+}.[e]

Analog	KCl	τ_c[a] (sec × 10^{10})	r (Å)
D-Phospholactate	−	7.95	6.6 ± 0.8[b]
	+	1.74	6.9 ± 0.5[b]
L-Phospholactate	−	9.07	5.4 ± 0.4[b]
	+	4.05	6.7 ± 0.5[b]
Phosphoglycolate	−	5.48	6.3 ± 0.4[c]
	−	5.48	3.5 ± 0.3[d]

[a] Correlation time of water protons in the same complex. [b] Mn^{2+} to methyl protons. [c] Mn^{2+} to methylene protons. [d] Mn^{2+} to phosphorus. [e] The error values given are maximal as discussed in the text.

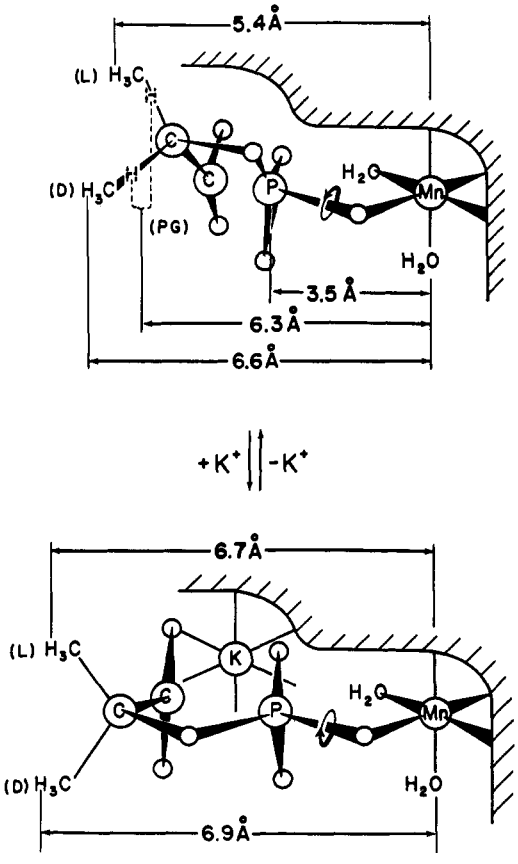

FIGURE 7: (A) Composite structure of the ternary complexes of pyruvate kinase–Mn–P-enolpyruvate analogs. The structure of the composite complex is consistent with each of the calculated distances shown and was computed with the aid of an ORTEP Fortran thermal-ellipsoid plot program for crystal structure illustrations which takes into account interatomic van der Waals interactions within the molecule to give the best molecular structure consistent with the distances. The addition of KCl to (A) gives (B) which was also drawn with the aid of the same program. To obtain B from A in the simplest manner, keeping only the position of Mn^{2+} constant, a 60° rotation about the (P–O) bond, as seen in the figure, is required. Upon binding to the enzyme, the activating monovalent cation, K^+, could coordinate the carboxyl group of the analogue and reorient the ligand to an active conformation.

compounds where the axial O–P bond can be as long as 1.79 Å, and metal–O–P bond angles of >120° are possible (Hamilton *et al.*, 1967; Ramirez and Ugi, 1971), a Mn-to-phosphorus distance of 3.4–3.8 Å is predicted. Thus the Mn-to-P distance in the pyruvate kinase–Mn–phosphoglycolate complex (3.5 ± 0.3 Å) suggests that the phosphate may be distorted in this complex, toward a pentacoordinate conformation in which the Mn coordinates an axial oxygen[2].

In absence of K^+, the distance from the enzyme-bound Mn^{2+} increases in the order, phosphoglycolate phosphorus < L-phospholactate methyl protons < phosphoglycolate methylene protons < D-phospholactate methyl protons (Table VIII). From the four distances calculated in the absence of K^+, a self-consistent composite structure may be obtained (Figure 7A) by superimposing the carboxyl, phosphate and C-2 carbon atoms of the analogs, D- and L-phospholactate and phosphoglycolate, with the aid of the ORTEP computer program. The self-consistency of the hypothetical model supports the validity of the assumptions made in the distance calculations.

Because of slow exchange of phosphoglycolate into its ternary complex, only two Mn to proton distances could be calculated in the presence of K^+. The monovalent cation has no significant effect on the distance between Mn^{2+} and the methyl protons of D-phospholactate but increases the Mn^{2+} to methyl distance in the L-phospholactate complex by 1.3 ± 0.6 Å. This change in distance may be obtained most simply in the composite model by a 60° rotation of the model about the P–OMn bond (Figure 7B). When this rotation occurs, the carboxyl group of the model moves upward by 1.0 ± 0.4 Å,

[2] We are grateful to Professor Oleg Jardetzky for suggesting this possibility to us.

presumably becoming coordinated by the bound K^+. The composite model (Figure 7B) predicts a Mn to K^+ distance of 4.5–6.0 Å in agreement with the Mn to Tl^+ distance obtained by Tl^+ nmr in the P-enolpyruvate complex (4.3–5.5 Å) (Reuben and Kayne, 1971). Since the substrate P-enolpyruvate occupies the same binding site as the analogs (Nowak and Mildvan, 1970, 1972) and its structure is similar to that of the analog (Watson and Kennard, 1966), the data suggest that the monovalent cation activates pyruvate kinase by coordinating the carboxyl group of P-enolpyruvate, and thereby properly aligns its phosphoryl group for nucleophilic attack. This single event may suffice to activate the enzyme. We have previously shown that pyruvate kinase immobilizes P-enolpyruvate analogs at the reaction center phosphorus, which would permit such orientational effects to operate in enzymatic rate accelerations (Nowak and Mildvan, 1972).

Finally, it should be pointed out that these considerations are based on studies of the structure of complexes containing only enzyme, Mn, K^+, and P-enolpyruvate analogs. Higher complexes containing ADP in addition to the above components have recently been detected and their structural properties are currently under investigation.

Acknowledgments

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