Conformational Changes Required for Pyruvate Kinase Activity as Modulated by Monovalent Cations*

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The interaction of a series of alkylamines with muscle pyruvate kinase was investigated by kinetic and physical studies in order to understand the mechanisms by which certain monovalent cations can activate the enzyme and to define several of the important conformational changes necessary for catalytic activity. Monomethylammonium ion interacts with pyruvate kinase to activate the enzyme. Dimethyl- and trimethylammonium ions do not activate, but are competitive inhibitors against activating cations. Tetramethylammonium ion neither activates nor inhibits pyruvate kinase activity.

When the enzyme is in the presence of monomethylammonium ion or dimethylammonium ion, a conformational change is observed by ultraviolet difference spectroscopy. This conformational change is similar to that observed with other activating cations and appears to be a necessary but not sufficient conformational change in the formation of an active complex. The interaction of the substrate phosphoenolpyruvate with the pyruvate kinase-Mn\(^{2+}\) complex in the presence of these cations was studied by water proton relaxation rate measurements. The affinity of the enzyme-Mn\(^{2+}\) complex for phosphoenolpyruvate is decreased by a factor of 5 in the presence of any of the alkylamines compared to the affinity measured in the presence of K\(^+\) or NH\(_4\)^+. No change in the K\(_m\) of phosphoenolpyruvate is observed however when it is measured in the presence of monomethylammonium ion, suggesting that the decrease in affinity for the substrate is not the reason for lack of enzymic activity. The conformation of the ternary enzyme-Mn\(^{2+}\)-phosphoenolpyruvate complex about the bound Mn\(^{2+}\), as reflected by the enhancement values (\(e_T\)) measured, differs depending upon the nature of the monovalent cation. The \(e_T\) values measured in the presence of the alkylamines are larger (\(e_T = 5.7 \pm 0.2\)) than those measured in the presence of K\(^+\) or NH\(_4\)^+ (\(e_T = 1.9 \pm 0.1\)). The environment about the bound Mn\(^{2+}\) reflected in the low enhancement values, however, is not in itself a prerequisite for an active complex. The effect of bound Mn\(^{2+}\) on the 1/\(T_1\) and 1/\(T_2\) of the methyl protons of the alkylamines was measured in the presence of the binary and ternary complexes of pyruvate kinase. The identical results which were measured in the presence of the binary complex for monomethylammonium ion and dimethylammonium ion demonstrate that they bind at the same site on the enzyme, 8.7 Å from Mn\(^{2+}\), forming an active conformation. Trimethylammonium ion binds 1.2 Å further from Mn\(^{2+}\) and is not able to elicit the required conformational change. Tetramethylammonium ion appears not to bind at all. In the formation of the ternary complex the 1/\(T_1\) value for monomethylammonium ion increases by a factor of 7 reflecting a 2.2-Å movement closer to the Mn\(^{2+}\) and provides a necessary change within the catalytic site to form the active complex. The 1/\(T_1\) value for the dimethylammonium ion increases only by a factor of 2 and reflects a 1-Å movement in the formation of the ternary complex. This change is insufficient to form an active complex.

Thus, in the interaction of pyruvate kinase with the ligands required for catalytic activity, it has been demonstrated that monovalent cations of only a certain size can induce a conformational change required of this enzyme. This complex must then be able to undergo a substrate-induced conformational change to obtain the activated complex which is required for catalytic activity.

Muscle pyruvate kinase was the first enzyme shown to have an absolute requirement for certain monovalent cations for enzymatic activity (1, 2). Initial investigations were consistent with the proposal that the activating monovalent cations induced a conformational change in the protein, giving the active form of the enzyme (3, 4). Recently however, data including binding studies (5) and NMR results (5-8) have indicated a more direct role for monovalent cations in pyruvate kinase activity. A direct role for monovalent cations implies a
specific site of interaction on the enzyme and it has been demonstrated by Tl⁺ binding studies (9) that there are four specific binding sites for monovalent cations on pyruvate kinase. This is in accord with the enzyme also having 4 divalent cations/mol (10, 11) and being a tetrameric enzyme (12). The monovalent cation site has been demonstrated to be in the vicinity of the divalent cation site (6-8). The divalent cation has been shown to be at the active site of pyruvate kinase, presumably acting as a metal bridge in binding the phosphoryl group of P-enolpyruvate* (5, 13, 14).

Two primary roles for the monovalent cation have been suggested: to act as a bridge to help bind P-enolpyruvate to the enzyme in its active conformation via its carboxyl group (5) or to act in stabilizing the enolate form of pyruvate (13) which has been shown to form after phosphor transfer occurs (16).

Several other less likely roles have also been proposed (17). By investigating the interaction of a series of alkylamines with pyruvate kinase by kinetics, ultraviolet difference spectroscopy, PRR studies, and 1H relaxation rate measurements, the nature of the required conformational changes taking place on the protein have been defined. A minimum of two conformational changes within the active site have thus been shown to occur in order to form the active enzyme complex. The roles which the monovalent cations must play in the formation of the active conformation of pyruvate kinase have been clarified.

MATERIALS AND METHODS

Rabbit muscle pyruvate kinase and lactate dehydrogenase were purchased from Boehringer and Sohne. The mono-, di-, tri- and tetramethylammonium chloride salts were purchased from Eastman and were recrystallized from water before use. The NADH, P-enolpyruvate, ADP, and Tris were purchased from Sigma. P-enolpyruvate and ADP were converted to their tetramethylammonium salts as previously described (5, 18). All other reagents were of the highest purity available.

Pyruvate kinase was assayed according to the method of Tietz and Ochoa (19) as previously modified (5). Both pyruvate kinase and lactate dehydrogenase were desalted by gel filtration using Sephadex G-25 fine resin. The enzyme used for high resolution NMR studies was from Eastman and was recrystallized from water before use. The NADH, P-enolpyruvate, ADP, and Tris were purchased from Sigma. P-enolpyruvate and ADP were converted to their tetramethylammonium salts as previously described (5, 18). All other reagents were of the highest purity available.

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The ultraviolet difference spectra were recorded on a Cary model 15 spectrophotometer using a 0 to 0.1 A scale expansion. The binding of P-enolpyruvate to the pyruvate kinase-Mn⁺ complex was performed by measuring the longitudinal PRR of water (1/T₁) by the Carr-Purcell sequence as described (5, 21) using a Seimco pulsed NMR spectrometer at 24.3 MHz at 24°. The paramagnetic contribution to the relaxation rate (1/T₁m) was calculated from the difference between the relaxation rate measured in the presence of the enzyme-Mn⁺-ligand complex (1/T₁,observed) and the relaxation rate measured for the same complex in the absence of Mn⁺ (1/T₁,monovalent). The enhancement values, ε, were calculated as the ratio of 1/T₁m measured in the presence of the enzyme-Mn⁺ complex, or enzyme-Mn⁺-ligand complex (1/T₁,observed) to the 1/T₁ value measured for the same concentration of Mn⁺ in free solution (1/T₁,free).

The titrations were performed as previously described (5) and the data was plotted as ε versus concentration of P-enolpyruvate added. The titration data was analyzed by a theoretical fit to the data (22). The values for the dissociation constant of the substrate from the enzyme-Mn⁺ complex, Kₛ, and the dissociation constant of the substrate from the free enzyme, Kₛ', were varied until the best fit to the data is obtained. The value for the enhancement factor of the ternary enzyme-Mn⁺-P-enolpyruvate complex εₜₗ was obtained as calculated at each point in the titration and corresponded to the enhancement value measured at saturating concentrations of P-enolpyruvate. The calculated values for εₜₗ and the values for Kₛ and Kₛ' were routinely obtained with a standard deviation of equal to or less than 5%. High resolution NMR spectra were taken on a Varian XL-100-15 spectrometer using a TTI pulse package and a Nicolet 1080 computer system.

The 1/T₁ values were measured by the line width at half-height. The paramagnetic contribution to the relaxation rates (1/T₁m and 1/T₁d) were calculated from the slopes of a plot of relaxation rate versus the concentration of Mn⁺ used. The values of 1/T₁m and 1/T₁d were normalized by the factor p where p = [Mn⁺]/[ligand] as described (25).

RESULTS

Kinetic Studies of Methylamine Interactions—Monomethylammonium chloride has been shown to be an activator of pyruvate kinase with a Michaelis constant, Kₐ = 20.5 ± 3.3 mM (8). However, neither dimethyl-, trimethyl-, nor tetramethylammonium chloride was found to activate pyruvate kinase. Dimethylammonium chloride and trimethylammonium chloride were found to be linear competitive inhibitors against K⁺ (Fig. 1), with Kᵢ values of 9.8 ± 0.5 mM and 31.1 ± 3.5 mM, respectively. Tetramethylammonium chloride inhibited pyruvate kinase activity in the presence of 20 mM KCl only at levels in excess of 200 mM. This inhibition can be attributed to an ionic strength effect since inhibition is also observed with activating cations at a similar ionic strength.

The effect of using CH₃NH₄⁺ in place of K⁺ on the Kₛ values of the substrates was measured using Mn⁺ as the divalent cation. The Kₛ of P-enolpyruvate was 30.5 ± 7.5 μM with CH₃NH₄⁺, the same as that measured in the presence of K⁺ (26 ± 5 μM) and as previously reported (26). The Kₛ for ADP, 570 ± 10 μM, increased 5-fold, however, as compared to the Kₛ of ADP measured in the presence of K⁺ (120 ± 10 μM).

Ultraviolet Difference Spectra Induced by Monovalent Cations—It has previously been shown that activating monovalent cations elicit a change in the ultraviolet spectrum of pyruvate kinase (4). The ultraviolet difference spectra of pyruvate kinase were measured in the presence of the various methylammonium cations against tetramethylammonium ion as shown in Fig. 2. In the experiments shown, the difference spectrum in the presence of K⁺ is virtually identical with that previously observed (4). In the presence of the activating cation CH₃NH₄⁺, a qualitatively similar spectrum is observed; however, the extinction coefficient is somewhat changed. The presence of dimethylammonium cation, although a nonactivating cation, gives an almost identical difference spectrum as observed with CH₃NH₂⁺. The presence of trimethylammonium ion gives a very small, nearly negligible difference spectrum, quite unlike that observed with the mono- and dimethylammonium ions. If these spectra are taken a day or so later, the shape and extinction coefficients of these spectra change, including that in the presence of K⁺. Qualitatively, the differences observed remain. These changes as a function of time have not been investigated further. However, no change in specific activity of the enzyme was measured while these changes in the difference spectra were observed.

Effect of Methylammonium Ions on Binding of P-Enolpyruvate to Enzyme-Mn⁺ Complex—The pyruvate kinase-Mn⁺ complex was plotted as ε versus concentration of ε-p-enolpyruvate added. The titration data was analyzed by a theoretical fit to the data (22). The values for the dissociation constant of the substrate from the enzyme-Mn⁺ complex, Kₛ, and the dissociation constant of the substrate from the free enzyme, Kₛ', were varied until the best fit to the data is obtained. The value for the enhancement factor of the ternary enzyme-Mn⁺-P-enolpyruvate complex εₜₗ was obtained as calculated at each point in the titration and corresponded to the enhancement value measured at saturating concentrations of P-enolpyruvate. The calculated values for εₜₗ and the values for Kₛ and Kₛ' were routinely obtained with a standard deviation of equal to or less than 5%. High resolution NMR spectra were taken on a Varian XL-100-15 spectrometer using a TTI pulse package and a Nicolet 1080 computer system. The 1/T₁ values were measured via the inversion recovery method (23, 24) and 1/T₁ values were measured by the line width at half-height. The paramagnetic contribution to the relaxation rates (1/T₁m and 1/T₁d) were calculated from the slopes of a plot of relaxation rate versus the concentration of Mn⁺ used. The values of 1/T₁m and 1/T₁d were normalized by the factor p where p = [Mn⁺]/[ligand] as described (25).
Active Site Changes during Pyruvate Kinase Activation

FIG. 1. Double reciprocal plots of the effects of (CH₃)₂NH·Cl and (CH₃)₄NH·Cl on the initial velocity of the pyruvate kinase reaction at varying concentrations of KCl. The reaction mixtures contained: 50 mM Tris-Cl buffer, pH 7.5; 1.5 mM tetramethylammonium-ADP; 1 mM tetramethylammonium-P-enolpyruvate; 5 mM MgCl₂; 0.16 mM NADH; 10 μg of lactate dehydrogenase; 1.12 μg of pyruvate kinase; and KCl as indicated. The concentration of total KCl and tetramethylammonium chloride was kept at 0.1 M. The total volume of the reaction mixtures was 1.0 ml. In Plot A, the reaction mixtures also contained (CH₃)₂NH·Cl in concentrations of: 0 mM, ○ -- ○; 10 mM, □ -- □; 20 mM, Δ -- Δ; and 40 mM, × -- ×. In Plot B the reaction mixtures also contained (CH₃)₄NH·Cl in concentrations of: 0 mM, ○ -- ○; 50 mM, □ -- □; 100 mM, ○ -- ○; and 100 mM, Δ -- Δ. The reactions were carried out at 25°C.

FIG. 2. Ultraviolet difference spectra of pyruvate kinase containing various alkylamines. Each spectrum is calculated to give a change in absorbance on a molal basis. These spectra were calculated from spectra measured on samples which contained 25 mM Tris-Cl buffer, pH 7.5, and 0.72 mg of pyruvate kinase in a final volume of 0.5 ml run against a blank containing the same amounts of enzyme and buffer containing 0.1 M (CH₃)₂NH·Cl. The cuvettes in the sample chamber contained: 0.1 M KCl; 0.1 M CH₃NH·Cl; 0.1 M (CH₃)₂NH·Cl; and 0.1 M (CH₃)₄NH·Cl, respectively. The instrument was balanced over this wavelength range prior to measuring each spectrum.

complex was titrated with P-enolpyruvate in the presence of 0.1 M KCl or 0.1 M of one of the alkylammonium chloride salts (Fig. 3). The data were analyzed by computer treatment as previously described (5, 22) to determine the values of $K_s$, $K_s$, and $cT$, which provided the best fit to the experimental data. The values obtained are summarized in Table I. Substitution of any of the alkylamines for K⁺ increases the dissociation constant, $K_s$, by a factor of about 5. The conformation about the bound Mn²⁺ in the ternary pyruvate kinase-Mn²⁺-P-enolpyruvate complex, as reflected by $cT$, changes upon substitution of any of the alkylamines for K⁺.

FIG. 3. Water relaxation titration studies measuring the effect of P-enolpyruvate on the $1/T_1$, of water protons in the presence of pyruvate kinase-Mn²⁺ containing various monovalent cations. Each reaction contained, initially: 50 mM Tris-Cl, pH 7.5; 50 μM MnCl₂; 0.1 M salt; and 0.33 mg of pyruvate kinase in a total volume of 0.05 ml. To this solution was titrated an identical solution which also contained either 90.9 μM (in the presence of KCl) or 303 μM tetramethylammonium-P-enolpyruvate. The end points were obtained by titrating with a more concentrated P-enolpyruvate solution and correcting for dilution. The curves represent titrations containing: KCl, ○ -- ○; CH₃NH·Cl, □ -- □; and (CH₃)₂NH·Cl, × -- ×. $T = 24$ °C.

TABLE I

<table>
<thead>
<tr>
<th>Monovalent cation</th>
<th>$K_s$ (μM)</th>
<th>$K_s$ (M)</th>
<th>$cT$</th>
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<tr>
<td>K⁺</td>
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<td>150</td>
<td>1.9</td>
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<td>CH₃NH⁺</td>
<td>4.0</td>
<td>400</td>
<td>5.3</td>
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<tr>
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<td>5.7</td>
</tr>
<tr>
<td>(CH₃)₄NH⁺</td>
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<td>150</td>
<td>5.6</td>
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</table>

* Values taken from Ref. 5.

Relaxation Rates of Carbon-bound Protons of Methylamines with Binary and Ternary Enzyme Complexes—It had been shown that the methyl protons of monomethylammonium ion provided an excellent NMR probe to monitor the monovalent cation interactions with the pyruvate kinase-Mn²⁺ complexes (8). These experiments were repeated using the pulsed, Fourier transform technique to measure $1/T_1$, of the ¹H nuclei. The interactions of each of the alkylammonium ions with the binary enzyme-Mn²⁺ and the ternary enzyme-Mn²⁺-P-enolpyruvate complexes were also studied. The methyl protons of each of the methylamines are well resolved from each other and easily amenable for high resolution studies (Fig. 4). The relaxation rate measurements with monomethylammonium chloride are shown in Fig. 5. The normalized values of the relaxation rates are summarized in Table II.

The binary enzyme-Mn²⁺ complex affects the $1/T_1$, values of monomethylammonium ion and dimethylammonium ion to the same degree, whereas the effect on trimethylammonium ion is halved. The effect on tetramethylammonium ion is barely above the noise level of the experiment. Upon the formation of the ternary enzyme-Mn²⁺-P-enolpyruvate complex, the $1/T_1$, values for the alkylammonium ions are differentially enhanced. For monomethylammonium ion, an
Active Site Changes during Pyruvate Kinase Activation

Fig. 4. 'H spectra of several alkylamines being investigated. High resolution 'H spectra were taken of 0.25 M solutions of various alkylamines in D₂O to be studied. The RF power was adjusted to give nearly identical peak heights. The spectra contain: A, CH₃NH₂Cl; B, (CH₃)₂NH₂Cl; C, (CH₃)₃NCI; and D, Tris-Cl. The most downfield line is the HDO line present at 5.2 ppm from tetramethylsilane.

Fig. 5. The effect of pyruvate kinase-Mn⁺⁺ on the 'H of CH₃NH₂Cl at 100 MHz. CH₃NH₂Cl was present at 0.1 M in D₂O containing 50 mM Tris-Cl, pH 7.5, and pyruvate kinase at 127 PM sites (A). MnCl₂ was added as a solution in D₂O to give a concentration of 14.9 PM (B) and tetramethylammonium-P-enolpyruvate was then added (C) to give a concentration of 1.5 mM. The initial volume was 0.40 ml. Temperature = 29 ± 1°C.

<table>
<thead>
<tr>
<th>Cation</th>
<th>Complex*</th>
<th>1/pT₁m s⁻¹</th>
<th>1/pT₁m s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃NH₂⁺</td>
<td>E-M</td>
<td>1,220 ± 125</td>
<td>41,700 ± 8,700</td>
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<tr>
<td>E-M·PEP</td>
<td>7,240 ± 140</td>
<td>144,000 ± 12,000</td>
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<tr>
<td>(CH₃)₂NH⁺⁺</td>
<td>E-M</td>
<td>1,130 ± 50</td>
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<td>E-M·PEP</td>
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<td>54,700 ± 8,800</td>
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<tr>
<td>(CH₃)₃N⁺⁺</td>
<td>E-M</td>
<td>620 ± 255</td>
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<tr>
<td>E-M·PEP</td>
<td>1,290 ± 300</td>
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<td></td>
</tr>
<tr>
<td>(CH₄)₄N⁺⁺</td>
<td>E-M</td>
<td>120 ± 40</td>
<td></td>
</tr>
<tr>
<td>E-M·PEP</td>
<td>320 ± 180</td>
<td></td>
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</tr>
</tbody>
</table>

* E·M denotes the effect of the pyruvate kinase-Mn⁺⁺ binary complex on the relaxation rates whereas E·M·PEP denotes the effect of the pyruvate kinase-Mn⁺⁺·P-enolpyruvate ternary complex.

It has been demonstrated that the longitudinal relaxation rate of a nucleus of a ligand complexed to or in the vicinity of a paramagnetic metal ion (1/T₁M) can be used to calculate the distance, r, between the nucleus and the metal ion (27, 28). When the nucleus measured is a proton and the paramagnetic metal ion used is Mn⁺⁺, the dipolar term of the Solomon-Bloembergen equation, for a 1:1 complex is simplified:

\[
r (\text{in } \text{Å}) = 812 \left[ \frac{T_{1M}}{f(r_c)} \right]^{1/4}
\]

where the correlation function is

\[
f(r_c) = \frac{3 r_c}{1 + \omega_1 \tau_c} - \frac{3 \tau_c}{1 + \omega_2 \tau_c}
\]

In this expression, \(r_c\) is the correlation time for the nuclear-electron dipolar interaction and \(\omega_1\) and \(\omega_2\) are the nuclear and electron resonance frequencies, respectively. Thus to use the Solomon-Bloembergen equation to calculate \(r_c\), the value of \(\tau_c\) must be obtained. As previously noted (29) the most rigorous method of determining \(\tau_c\) is from a frequency dependence of 1/T₁m. From such a frequency dependence of 1/T₁m a value for \(\tau_c\) of 3.3 ± 1.6 x 10⁻⁴ s is determined for both the binary and ternary complexes.² As noted previously (8) \(\tau_c\) does not appear to vary significantly in going from the binary to the ternary complex. This value for \(\tau_c\) agrees with the values estimated for pyruvate kinase-Mn⁺⁺ complexes measuring the frequency dependence of the Mn⁺⁺·H₂O interaction (10) and the Mn⁺⁺·pyruvate (30, 31) and Mn⁺⁺·α-(dihydroxyphosphinylmethyl)acrylate interactions (31).

As demonstrated previously (5) in the case where 1/pT₁p > 1/pT₁m, the value of 1/pT₁p is dominated by the relaxation rate of the measured nucleus in the vicinity of the paramagnetic species, 1/T₁M. Since 1/pT₁p > 1/pT₁m for these cations (Table II), the values of 1/pT₁p and \(\tau_c = 3.3 ± 1.6 \times 10^{-4}\) s are used to calculate \(r\) for the methyl protons of each of the monovalent cations in the absence or presence of the substrate P-enolpyruvate. The normalized relaxation rate values are summarized in Table III. The deviations given represent the maximum deviations calculated based on the extreme values for \(\tau_c\) and 1/T₁M. The data confirms the differences in interaction of these cations with the enzyme both in the binary and the ternary complexes.

**DISCUSSION**

A kinetic study of the activation of pyruvate kinase by a series of methylamines has shown that only monomethylammonium chloride activates pyruvate kinase (0.5 to 2% \(V_{max}\), measured with KCl) (8), whereas dimethyl-, trimethyl-, and tetramethylammonium chloride do not activate to within 0.001%. The dimethyl and trimethyl cations, however, are linear competitive inhibitors of activating cations (Fig. 1), indicating that they compete with activating cations for the same site on the enzyme. Tetramethylammonium ion does not act as a competitive inhibitor, however, indicating that this cation does not interact with pyruvate kinase or it interacts at a nongeneric site elsewhere on the protein.

Suellert et al. (4) have shown that activating monovalent...
rations elicit a conformational change on the protein as demonstrated by an ultraviolet difference change. Monomethylammonium chloride qualitatively elicits the same difference change as other activating cations (Fig. 2). Dimethylammonium chloride, however, elicits the identical spectral change indicating that, although this conformational change is necessary in the activation of the enzyme, it is not sufficient to give the catalytically active form. Trimethylammonium ion fails to elicit this ultraviolet change although it has been shown kinetically to compete for the monovalent cation site. Although the trimethyl cation can bind at the cation site, its size must prevent the conformational change reflected in the spectral change which occurs with activating cations

One important effect of K + is that it increases the binding of the substrate P-enolpyruvate to the enzyme-Mn2+ complex, as compared to the complex in the presence of tetramethylammonium chloride (5). To determine if this more tightly bound complex is required for enzymatic activity, the effect of each of the alkylamines on the K m of P-enolpyruvate was measured. The K m values measured in the presence of the alkylamines were greater, by a factor of 5, compared to the K m value measured in the presence of K +. The K m of P-enolpyruvate, however, measured in the presence of KCl or CH3NHCl is identical. Thus the increase in binding constant for the substrate is not reflected in any change in its K m, indicating that the increased K m is not a serious factor in preventing the formation of the active ternary complex. The K m of ADP is increased by a factor of 5 in the presence of CH3NH2+, although this cation does not affect the binding of ADP nor does ADP affect the position of CH3NH2+ within the active site.

The conformation about the bound Mn2+ in the ternary complex as reflected by T1 values is dependent upon the monovalent cation. The low value obtained in the presence of K+ (1.9) implies that the Mn2+ is greatly shielded from the solvent, while in the presence of the alkylamines, the greater T1 value (5.7 ± 0.2) implies a less shielded Mn2+ environment. Since the ternary complex in the presence of CH3NH2+ also yields a high T1 value, the more shielded Mn2+ complex is not an absolute necessity for catalysis. It is yet unclear if it is these two conformational forms which are the equilibrium forms measured by EPR (32).

The activating monovalent cations have been shown to bind within the active site of pyruvate kinase in the binary and ternary complexes of the enzyme (6-8). The effect of the bound Mn2+ on the relaxation rates of the carbon-bound protons of the methylamines was studied to obtain more detailed information concerning the interaction of these cations with pyruvate kinase. In the presence of pyruvate kinase there was no effect on the chemical shift, the longitudinal (1/T1), or transverse (1/T2) relaxation rates of the methyl protons. In the presence of the E-Mn2+ complex, the normalized relaxation rates measured for monomethyl- and dimethylammonium chloride are identical (Table II). This indicates that both cations bind at the same site in the enzyme-Mn2+ complex as indicated by kinetic and ultraviolet difference experiments. The trimethylammonium cation has one half the relaxation rate effect indicating this cation binds further from the Mn2+.

The marginal relaxation rates measured for tetramethylammonium ion support the evidence that this cation binds either very weakly or at a nonspecific site on the enzyme further from bound Mn2+. In the presence of the ternary enzyme-Mn2+-P-enolpyruvate complex the longitudinal relaxation rate of the monomethylammonium ion increases by 6.000 s -1 (Table II), indicating a dramatic movement within the active site as previously discussed (7,8). An increase in 1/T1 of only 1300 s -1 is observed for the dimethyl cation, indicating that the movement within the active site observed with the monomethylammonium cation does not take place with this cation.

The relaxation rates of the monomethylammonium cation have been measured in the presence of the binary and ternary enzyme complexes as a function of frequency and the correlation time, r c, for the electron-nuclear interaction was calculated. The value for the binary complex was 2.2 ± 0.6 × 10^-8 s and for the ternary complex was 4.3 ± 2.1 × 10^-8 s. Conservatively, there is no difference between these two values and a r c = 3.3 ± 1.6 × 10^-8 s was used to calculate the electron-nuclear distances in these complexes. This value is in agreement with other estimates of r c for pyruvate kinase (8, 10, 30, 31) with a smaller standard deviation. Since the values of 1/T1 are much larger than 1/T2, there is no significant effect of chemical exchange on 1/T1 (5) and therefore can be used to calculate the electron-nuclear distance r (Table III).

The data reported indicates that trimethylammonium ion which interacts at the monovalent cation site is 9.8 Å away from the divalent cation site and, presumably because of size, fails to elicit a conformational change on the enzyme. This conformational change, observed with monomethyl- and dimethylammonium cations by ultraviolet difference spectroscopy, involves a movement of the monovalent cation ≥1 Å closer to the divalent cation site. Failure to elicit this change results in the formation of an inactive complex. The binding of the substrate P-enolpyruvate induces a further change within the active site involving a 2.2 Å movement of the monovalent cation closer to Mn2+. Failure of the ternary complex to elicit this additional conformational change results in a non-activated complex as observed with dimethylammonium ion. The size of the dimethyl cation apparently precludes this change. This second conformational change may also involve a change about the catalytic site involving the positioning of the base which is responsible for the protonation of P-enolpyruvate during the catalytic sequence. Evidence implicating

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Table III

<table>
<thead>
<tr>
<th>Cation</th>
<th>Complex†</th>
<th>r</th>
</tr>
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<tbody>
<tr>
<td>CH3NH2</td>
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<td>CH3NH2·PEP</td>
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<tr>
<td>(CH3)3N+</td>
<td>E·M</td>
<td>9.8 ± 1.2</td>
</tr>
<tr>
<td>(CH3)3N·PEP</td>
<td>E·M·PEP</td>
<td>8.7 ± 0.7</td>
</tr>
</tbody>
</table>

†E·M denotes the distances calculated for the binary pyruvate kinase-Mn2+ complex and E·M·PEP denotes the distances calculated for the ternary pyruvate kinase-Mn2+·P-enolpyruvate complex.

*Unpublished observations.*
a monovalent cation role in the protonation step of the reaction stems from evidence demonstrating that the monovalent cation affects the partitioning of tritium from P-enolpyruvate to water and pyruvate (16) and the conformation of P-enolpyruvate in the active site changes in the presence of an activating cation (5). The ability of the monovalent cations to participate in these conformational changes within the active site of pyruvate kinase is crucial in forming the catalytically active form of the enzyme.

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Conformational changes required for pyruvate kinase activity as modulated by monovalent cations.

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