Structural Aspects of Manganese-Pyruvate Kinase Substrate and Inhibitor Complexes Deduced from Proton Magnetic Relaxation Rates of Pyruvate and a Phosphoenolpyruvate Analog*

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SUMMARY

The distance between enzyme-bound Mn(II) at the active site of rabbit muscle pyruvate kinase and the protons of bound substrate or inhibitor was determined in ternary and quaternary enzyme complexes. In particular, the NMR relaxation rates of pyruvate protons effected by bound Mn(II) were measured for (a) the ternary pyruvate kinase-Mn(II)-pyruvate complex; (b) the abortive quaternary complex containing ADP as the fourth component; and (c) the quaternary complex containing ATP as the fourth component, i.e., the equilibrium mixture. The relaxation rates of the three proton resonances of the inhibitor, \( \alpha \)-((dihydroxyphosphinylmethyl)acrylate, the methylene analog of P-enolpyruvate, were measured for the ternary enzyme-Mn(II)-inhibitor complex. Longitudinal \( T_1 \) and transverse \( T_2 \) NMR relaxation times were measured as a function of temperature and of Mn(II) concentration. Relaxation times were measured at two frequencies, 220 and 60 MHz, so that the correlation time and subsequently the distance of the substrate or inhibitor protons from the paramagnetic Mn(II) could be determined.

The correlation time for the ternary complex with the inhibitor, \( \alpha \)-((dihydroxyphosphinylmethyl)acrylate, was found to be \( 1.0 \times 10^{-9} \) s from the frequency dependence of \( T_1 \). The distance from Mn(II), determined to be 5.3 A to the methylene protons, 6.5 A to one vinyl proton, and 6.2 A to the other vinyl proton, provides evidence that the ternary P-enolpyruvate analog complex involves coordination of the phosphinyl group to Mn(II) bound to the enzyme. Although binding experiments indicate that P-enolpyruvate and \( \alpha \)-((dihydroxyphosphinylmethyl)acrylate compete for the same site on pyruvate kinase in the ternary complex, water proton relaxation rate data and EPR spectra show that the methylene analog of P-enolpyruvate forms a very different ternary complex than P-enolpyruvate. Consequently an extrapolation from the structure of the inhibitor complex to that of the substrate P-enolpyruvate is questionable.

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The pyruvate kinase-catalyzed phosphoryl transfer reaction in rabbit muscle

\[
\text{P-enolpyruvate} + \text{ADP} \rightarrow \text{Pyruvate} + \text{ATP}
\]  

(1)

has a monovalent and a divalent metal ion requirement. Mn(II) satisfies the divalent metal ion requirement and may function as a paramagnetic probe of the enzyme's active site (1). The equilibrium strongly favors formation of pyruvate and ATP (2).

Attempts to obtain quantitative structural information, i.e., interatomic distances in enzyme-substrate or product complexes from measurements of the paramagnetic relaxation effect of Mn(II) on substrate nuclei have been hampered by the relative magnitude of the various rate processes of this particular system. The complex formed with excess substrate, P-enolpyruvate, is not amenable to this approach because the rate-limiting step in the observed nuclear relaxation rate is the rate of chemical exchange between free and tightly bound substrate rather than the rate of the Mn(II)-induced nuclear relaxation process.
Therefore, more weakly bound substrates or inhibitors which do not suffer from this limitation have been substituted, including fluorophosphate (3), P-glycolate and P-lactate (4), and in the current investigation the methylene analog of P-enolpyruvate, α-(dihydroxyphosphinylmethyl)acrylate. The relevance of the structure of the substrate analog complexes to that of P-enolpyruvate will be considered.

The product, pyruvate, in its Mn(II)-enzyme complex enolizes if the phosphate site of the enzyme is occupied (5). In the active form of the complex, e.g. in the presence of ATP, enolization occurs and, at high enzyme concentrations, the methyl protons become rapidly deuterated when the solvent is the usual D2O, and proton NMR is no longer feasible. This report describes pyruvate NMR investigations of (a) the ternary pyruvate kinase-Mn(II)-pyruvate complex in D2O; (b) the abortive quaternary complex in D2O including ADP as the fourth component; and (c) the reactive quaternary complex in 10% H2O-90% D2O including ATP as the fourth component; this complex is the major species at equilibrium in the reaction.

For valid distance calculations between a paramagnetic ion and a nucleus to be obtained from the paramagnetic effect on the nuclear relaxation rate, several conditions must be met. (a) The binding of the paramagnetic ion must be specific for one site on the enzyme or enzyme-substrate complex. This prerequisite has been shown to be satisfied for pyruvate kinase (6). (b) The complex must exist in one structure. This requirement is also satisfied for the complexes described in this paper as evidenced by EPR experiments (7). (c) The nucleus under observation must undergo rapid chemical exchange between diamagnetic and paramagnetic environments. Experiments on frequency and temperature dependence of the relaxation rates will be described which bear on this point. (d) Data must be available to determine r, a parameter needed in the calculation of the distance r from the experimentally determined relaxation rates (cf. Equation 4). In some earlier investigations with rabbit muscle pyruvate kinase (3, 4), approximations were used to estimate the correlation time, rc. The approximation, which consists of estimating rc from the PRR enhancement of water in the same complex, may not hold. In particular it does not hold for the ternary pyruvate kinase-Mn(II)-P-enolpyruvate complex where it was found by a detailed study of the PRR dependence on temperature and frequency that, on an average, only 0.5 water molecule from the first coordination sphere of Mn(II) contributes to the proton relaxation rate of water (8). More confidence can be placed in distance determinations using correlation times estimated directly from the frequency dependence of the relaxation rates of nuclei on the substrate or inhibitor as, for example, in the NMR studies of [14C]pyruvate in complexes with Mn(II)-pyruvate kinase and pyruvate carboxylase (9). The variation of both temperature and frequency as detailed in the current investigation of proton relaxation rates are often needed to assign an unambiguous value for rc (1). The NMR longitudinal (T1) and transverse (T2) relaxation times of the proton resonances of the substrate pyruvate, or of the inhibitor, methylene analog of P-enolpyruvate, bound to pyruvate kinase were measured as a function of Mn(II) concentration and temperature. NMR measurements were made at two frequencies, 220 MHz and 60 MHz, so that the correlation time could be determined and the distance of paramagnetic Mn(II) ions from protons of pyruvate or of the P-enolpyruvate methylene analog could be calculated.

Materials and Methods

Sample Preparation—Pyruvate kinase was isolated from rabbit muscle according to the procedure of Tietz and Ochoa (10) with a final ammonium sulfate fractionation. The enzyme had a specific activity of 160 to 220 according to the assay of Tietz and Ochoa (10). The enzyme solution was dialyzed against three changes of 50 mM sodium cacodylate buffer (pD 7.0) followed by lyophilization. The residue was dissolved in deuterium oxide (99.97%), was allowed to stand at 4° for 3 hours to assure proton-deuterium exchange, and then was lyophilized again. This procedure was repeated once again and the lyophilized enzyme was dissolved in D2O and stored at 4°. The PDR refers to the reading on a Radiometer model 26 pH meter.

The sample of α-(dihydroxyphosphinylmethyl)acrylate acid was a generous gift from Dr. George L. Kenyon, University of California, San Francisco. Potassium pyruvate was prepared from distilled pyruvic acid (J. T. Baker Chemical Co.) and stored as a frozen solution in D2O. Solutions of ADP (P-L Biochemicals, Inc.) and sodium P-enolpyruvate (Sigma Chemical Co.) in D2O were used without further purification and stored frozen. D2O solutions of the cations studied were prepared from KCl, (CH3)4NCl, MnCl2·4H2O, and MgCl2·6H2O, all purchased from J. T. Baker Chemical Co. Solutions for NMR studies of the pyruvate kinase complex were prepared from stock solutions such that they were buffered with 50 mM sodium cacodylate (pD 7.0) in 99.97% D2O for the main complex and the abortive quaternary complex (with ADP) and in 90% D2O for the reactive quaternary complex (with ATP). The ATP for the reactive complex was generated in situ by addition of equivalent amounts of ADP and P-enolpyruvate. Solutions for studies of enzyme complexes with the P-enolpyruvate methylene analog were prepared in the same manner except that the solutions were all prepared in 99.97% D2O at pD 7.1. Measurements were made with identical solutions containing either magnesium chloride or manganese chloride. The results with solutions containing Mg(II) were used to correct for diamagnetic contributions to the NMR relaxation rates.

NMR Measurements—H NMR measurements were made at 220 MHz on a Varian HR-220 spectrometer equipped with pulse capabilities and a computer for time averaging and computing the Fourier transform to obtain the enhanced spectrum. Spectra at 60 MHz were obtained by using the same pulse unit and computer with a Varian DP-60 spectrometer. The temperature was regulated to ±1° with the Varian temperature controller. The pyruvate resonance was monitored at 2.47 ppm downfield from the methyl resonance of the external DSS reference. The peaks of α-(dihydroxyphosphinylmethyl)acrylate acid, were observed at the following chemical shifts from DSS: "a," 5.6 ppm; "b," 6.0 ppm; and "c," 2.8 ppm.

The transverse relaxation time (T2) for the pyruvate proton resonance was obtained from the linewidth at half-maximal amplitude, 1/T2 = eW0, and represents the average of at least nine spectra. Linewidths were not measured in the case of α-(dihydroxyphosphinylmethyl)acrylate acid due to the extensive spin-spin coupling.

NMR longitudinal relaxation times (T1) were measured by an improved 90°-90° Fourier transform method (11). This technique permits the rapid measurement of the longitudinal relaxation times of individual resonances which are time averaged for signal to noise enhancement. T1 was determined using the expression

$$\ln [\frac{1}{T_1} - 1/10^6] = -\frac{1}{T_1}$$

(2)

where M(τ) is the magnitude of the proton resonance for a certain pulse separation and M∞ was obtained with τ > 77τ. The values of T1 reported are averages of three individual measurements on the same sample.

Observation of Pyruvate Spectra in Equilibrium Mixture—Enolization of pyruvate occurs in the reactive pyruvate kinase-
Mn(II)-ATP-pyruvate complex (5). In the presence of 100% D₂O, the pyruvate methyl group becomes fully deuterated and a proton resonance is no longer observable. Ideally the spectra should be obtained with H₂O solutions. However, the pulsed Fourier transform method applied to dilute solutions with H₂O as solvent requires a receiver and computer with a very large dynamic range to obtain a reasonable signal to noise ratio for the dilute component in the presence of a strong water signal. Such a large dynamic range is impractical, so D₂O is generally used to attenuate the problem. As a compromise in this case, 90% D₂O is used. At least 20 hours at 4°C are allowed for the pyruvate to equilibrate in the 90% D₂O solution via the enolization reaction. The resultant pyruvate methyl proton signal intensity is 10% of its original intensity prior to enolization. Even the presence of 10% water (5.5 m) severely limits the signal to noise enhancement attainable for 30 mM residual pyruvate protons present in a solution of 100 mM pyruvate. The problem is somewhat alleviated by partial filtering of the HDO resonance, and the conditions yield useful spectra. Fig. 1 illustrates a sequence of pyruvate spectra used to calculate T₁. The example chosen is not typical, but the least favorable with the broadest line and lowest signal to noise that was used. Difficulties arise from the very broad line, use of 90% D₂O solvent, and the low proton concentration in the methyl group due to enolization of pyruvate catalyzed by pyruvate kinase.

The measurements with the ternary pyruvate complex and the abortive quaternary complex could be made with solutions in 99.97% D₂O since the enolization reaction is extremely slow with those complexes.

Paramagnetic Relaxation Theory—Theoretical considerations are briefly presented here; the references should be consulted for more detail. The paramagnetic contribution to the longitudinal relaxation rate 1/T₁ of a ligand nucleus is a weighted average for the coordinated and uncoordinated ligand and depends on the residence time Tₘ in the coordination sphere (1,12):

\[
\frac{1}{T_{1p}} = \frac{1}{T_{10}} + \frac{1}{T_{1p}} = \frac{1}{T_{1p}} + \frac{1}{T_{1M}} \tag{3}
\]

where T₀ is the ion-proton internuclear distance, ω₁ is the angular precession frequency for the nucleus, and B is a product of physical constants determined by the electron spin of Mn(II) and the magnetogyric ratio of the proton; the numerical value of B is 2.87 × 10⁻¹⁰.

The correlation time τₑ is given by:

\[
\tau_e^{-1} = \tau_r^{-1} + \tau_s^{-1} + \tau_M^{-1} \tag{6}
\]

where τᵣ is the correlation time for rotational motion and τₛ is the electron spin relaxation time. Plots of f₁(τₑ) and f₂(τₑ), the terms in parentheses in Equations 4 and 5 versus τₑ, are shown in Fig. 2A for 220 and 60 MHz.

It has been found (13) that the correlation times τₑ and τₘ may be described in terms of (Eₘ),; activation energies:

\[
\tau_e = \tau_{1p} = \tau_{1M} \tag{7}
\]

As the temperature decreases, τₑ and τₘ will increase, but τₛ may either increase or decrease (14). Usually, τₑ increases with increasing temperature for small complexes (15) and decreases with increasing temperature for large complexes (16). In general the activation energies for the processes will fall in the order (Eₛ)ₘ > (Eₘ)ₘ > (Eₛ)ₘ. 

RESULTS

Relative Magnitudes of Relaxation Rates (1/T₁ₘ, 1/T₃ₘ) and Chemical Exchange Rate (1/τₑ) of Pyruvate Protons in Reactive Complex—The longitudinal and transverse relaxation rates, 1/T₁ and 1/T₂, of the pyruvate methyl protons in the quaternary pyruvate kinase-Mn(II)-ATP-pyruvate complex were determined at 60 MHz and at 220 MHz. The values for 1/T₁ and 1/T₂ for the corresponding diamagnetic Mg(II) complexes were subtracted from the observed values to yield the paramagnetic contribution to the relaxation rates, 1/T₁ₘ and 1/T₃ₘ. The fraction of the diamagnetic contribution to the total relaxation rates ranged from 5 to 50% and 15 to 40% for 1/T₁ and 1/T₂, respectively, depending on Mn(II) concentration. Table I lists the normalized values of the relaxation rates, 1/pT₁ₘ and 1/pT₃ₘ, where p is the ratio of the concentration of Mn(II) to pyruvate.

If 1/Tₑₘ was determined by τₘ only (cf. Equation 3), i.e. if limited by the rate of chemical exchange 1/τₑ, then 1/T₁ₘ and 1/T₃ₘ would not change with frequency. It can be seen from Table I that the ratio of 1/pT₁ₘ (60 MHz) to 1/pT₃ₘ (220 MHz) is approximately 10, so the observed relaxation rate is not dominated by chemical exchange. To eliminate the possibility that τₘ partially contributes to the relaxation rate, we compare T₁ₘ and T₃ₘ. Since T₁ₘ (or T₃ₘ) is considerably longer than T₃ₘ (or T₁ₘ), one would expect T₃ₘ to be more affected by a τₘ contribution than T₁ₘ. Since τₘ has a large temperature coefficient, the ratio of T₁ₘ/T₃ₘ should increase with temperature. Examination of Table I reveals no such trend (Experiments 3 to 6). It is therefore concluded that τₘ ≪ T₁ₘ, T₃ₘ, and the rate of chemical exchange 1/τₑ > 10³ s⁻¹.
**FIG. 2.** A, theoretical dependence of the correlation functions, \( j_1(\tau_c) \) and \( j_2(\tau_c) \), on the correlation time, \( \tau_c \), based on Equations 4 and 5. Curves A1 and B1 show \( j_1(\tau_c) \) as a function of \( \tau_c \) at 220 and 60 MHz, respectively. Curves A2 and B2 show \( j_2(\tau_c) \) as a function of \( \tau_c \) at 220 and 60 MHz, respectively. B, dependence of the ratio \( pT_{1p}/pT_{2p} \) at 220 MHz and 60 MHz on the correlation time \( \tau_c \). C, dependence of the \( pT_{1p}(220)/pT_{1p}(60) \) ratio on the value of \( \tau_c \).

**TABLE 1**

| Experiment | Frequency (MHz) | Temperature | \( 1/pT_{1p} \) | \( 1/pT_{2p} \) | \( \tau_c \) | \( \tau_c \) |
|------------|----------------|-------------|----------------|----------------|-----------|-----------|
| 1          | 220            | 17          | 0.10           | 0.15           | 14        | 60        | 2.1 7.7   |
| 2          | 220            | 17          | 0.10           | 0.15           | 14        | 60        | 1.8 7.7   |
| 3          | 220            | 17          | 0.10           | 0.15           | 14        | 60        | 4.7 9.1   |
| 4          | 220            | 17          | 0.10           | 0.15           | 23        | 60        | 4.4 8.7   |
| 5          | 220            | 17          | 0.10           | 0.15           | 26        | 60        | 4.7 8.7   |
| 6          | 220            | 17          | 0.10           | 0.15           | 35        | 60        | 4.0 8.0   |
| 7          | 220            | 17          | 0.10           | 0.15           | 5         | 47        | 2.3 7.8   |
| 8          | 220            | 17          | 0.10           | 0.15           | 7.8       | 50        | 2.3 7.8   |
| 9          | 220            | 17          | 0.10           | 0.15           | 23        | 60        | 2.1 7.7   |
| 10         | 60             | 0.094       | 304.3          | 330.0          | 343.0     | 135.0     | 5.3 8.0   |
| 11         | 60             | 0.016       | 304.3          | 330.0          | 43.9      | 132.0     | 4.4 7.8   |

**Calculation of \( \tau_c \)** The ratio \( T_{1p}/T_{2p} \) may be used to calculate \( \tau_c \) from Equations 4 and 5 as shown in Fig. 2B. Provided (a) \( j_2(\tau_c) > j_1(\tau_c) \) (ref. Fig. 2A), i.e. \( \tau_c < 1/\omega_1 \); and (b) there is no hyperfine contribution to \( T_{2p} \). The values of \( \tau_c \) calculated from the \( T_{1p}/T_{2p} \) ratio for several solutions with varying Mn(II) concentration and temperature are given in Table I. It is apparent that there is no systematic temperature or concentration dependence of \( \tau_c \). It is also noteworthy that, within experimental error, the values of \( \tau_c \) for the pyruvate kinase-Mn(II) pyruvate ternary complex and pyruvate kinase-Mn(II)-ADP-pyruvate abortive quaternary complex are the same as \( \tau_c \) for the reactive quaternary complex. The likely assumption that \( \tau_M \) is also not limiting the relaxation rates for the ternary and abortive complexes was made in the calculations. The average value for \( \tau_c \) from all the \( T_{1p}/T_{2p} \) calculations is \( 3.5 \pm 1.2 \times 10^{-9} \) s.

The value of \( \tau_c \) may also be determined from the frequency dependence of \( pT_{1p} \) if \( \tau_c \gtrsim 1/(\omega_1) \), where \( \omega_1 \) is the lowest frequency used (cf. Fig. 2C). Using an average of the \( pT_{1p} \) results at 220 and 60 MHz, \( \tau_c \) is determined to be \( 2.7 \times 10^{-9} \) s in good agreement with the \( T_{1p}/T_{2p} \) calculations. This result validates the assumption that the hyperfine contact contribution to \( pT_{2p} \) is negligible. Those values of \( \tau_c \) are also consistent with values of \( \tau_c \) of the order of \( 10^{-9} \) s estimated from the Mn(II) EPR spectra of various pyruvate kinase-Mn(II)-complexes (7).

At the higher frequency, the temperature was varied over the range 5°-35°. In Fig. 3, the temperature dependence of \( 1/T_{1p} \) is shown for the experiments with Mn(II) concentration equal to 0.15 mM. Under these conditions, more than 95% of Mn(II) is bound in the quaternary complex, and consequently changes in the ratio of bound to unbound Mn(II) need not be considered in analysis of the temperature dependence. The small positive temperature dependence may be ascribed to the fact that \( \omega_1 \tau_c > 1 \). We may consider which rate process dominates the correlation time in Equation 6. A Debye-Stokes law calculation for the rotational reorientation time of the entire enzyme gives \( \tau_c = 10^{-7} \) s. If the pyruvate is rigidly bound to the enzyme, \( \tau_c \) is clearly too large to contribute to \( \tau_c \), which is \( \sim 3 \times 10^{-9} \) s. The very small temperature dependence lends further support to the choice of \( \tau_c \) as the pertinent correlation time since, as indicated earlier, \( \tau_c \) is much less dependent on

* In 99.97% D₂O with no nucleotide present.
* In 90% D₂O containing 1.1 mM ADP in place of ATP.
H-1 and 31P-1H coupling is not necessarily a valid measure of 7'H, and TBp values because the apparent linewidth including pyruvate, neither rM nor rc can be estimated from a comparison a, b, and c protons, respectively, as listed in Table II. Relaxation rates at 60 to 220 MHz are 2.8, 2.7, and 2.3 for the a and b vinyl protons and of the c methylene protons are not dominated by TIM. The ratios of the relaxation rates exhibit a frequency dependence indicating that the observed relaxation rate of its protons in the ternary enzyme-protein-Mn(II) distances. A search among less tightly bound substrate analogs revealed that (Z)-P-enolfluoropyruvate was unsuitable because its proton NMR relaxation rate in the ternary enzyme-protein-Mn(II)-inhibitor complex as expected for a rc value of the order of \(1 \times 10^{-9}\) s (cf. Fig. 1). Consequently, \(\tau_c\) must be estimated from the frequency dependence of \(T_1p\). The correlation time, \(\tau_c\), determined from the ratio of the paramagnetic contributions to the longitudinal relaxation rate at 60 MHz and 220 MHz according to Equation 8, is given in Table II for each of the proton resonances of the analog. In each case, \(\tau_c\) is calculated to be \(1 \times 10^{-3}\) s. Little temperature variation is observed at 220 MHz over the range of 63°-34° for the three proton resonances of the methylene analog in the ternary pyruvate kinase-Mn(II)-inhibitor complex has little influence on the calculated value of \(\tau_c\).

**TABLE II**

| Proton       | \(A/\tau_p\) \((260 \text{ MHz})\) | \(B/\tau_p\) \((60 \text{ MHz})\) | \(B/A\) | \(\tau_c\) | \(r\) |
|--------------|-------------------------------|-------------------------------|--------|----------|-----|
| Vinyl a      | 0.38                          | 1.08                          | 2.84   | 1.1      | 6.5 |
| Vinyl b      | 0.51                          | 1.35                          | 2.67   | 1.0      | 6.2 |
| Methylene c  | 1.26                          | 2.94                          | 2.32   | 0.92     | 5.3 |

\(T_2\). The estimated accuracy of the average distances is within \(\pm 0.5\) A.

**DISCUSSION**

The reaction pathway for the binary metal-enzyme complex in the pyruvate kinase reaction is shown schematically below (Scheme 1).
FIG. 4. Competition of P-enolpyruvate with α-(dihydroxyphosphinylmethyl)acrylate for the ternary complex formed with pyruvate kinase-Mn(II). The effect of added P-enolpyruvate on the longitudinal relaxation rate of the methylene resonance of α-(dihydroxyphosphinylmethyl)acrylate in a solution composed of 40 mg per ml of pyruvate kinase, 71.3 µM MnCl₂, and 60 mM α-(dihydroxyphosphinylmethyl)acrylate buffered with 10 mM sodium cacodylate, pH 7.2. The curve was computed assuming simple competition of P-enolpyruvate (Kₛ = 1.5 µM) with α-(dihydroxyphosphinylmethyl)acrylate (Kᵢ = 600 µM).

It is assumed in this scheme that the required monovalent cation potassium is present and that M(II) is the activating divalent metal ion, Mn(II) in the current investigation. Primary interest, of course, centers on the structure of the activated complex in the transition state between Complexes III and IV. Although the over-all equilibrium in this reaction favors the formation of ATP and pyruvate (2) (Kₑq ~ 2000), there is insufficient data available under conditions with stoichiometric amounts of enzyme present to determine the relative amounts of enzyme-bound substrates at equilibrium, i.e. Complexes III, IV, and V. Our calculations of distances are based on the assumption that at equilibrium the enzyme is entirely in the form of Complex V. However, even if Complex V concentration were only 50% of the total enzyme concentration, it would change the calculated distance a maximum of only 12%.

Possible modes of pyruvate bonding in Complexes V and VIA may be assessed by comparison of distances measured from molecular models and those calculated in Table I from the paramagnetic contribution to the proton relaxation rate. From the molecular model of the structure in which Mn(II) is complexed to the carboxyl group of pyruvate, the permissible values of Mn(II)-proton distance range from 2.5 to 4.8 Å. In contrast the value derived from Experiment 1, Table I is 7.7 Å unequivocally ruling out direct bonding between Mn(II) and pyruvate in Complex VIA. Similar conclusions have been reached from experiments on ³¹C relaxation rates in some [³¹C]pyruvate-pyruvate kinase complexes (9). On the basis of these results it is not possible to assign the position of the transferable phosphoryl group in the complex. It has been suggested that the phosphoryl group is coordinated to Mn(II) (9, 18). The experimentally derived values (Table I) of 8.2 ± 0.5 Å for the Mn(II) to pyruvate proton distance in the equilibrium complex (predominantly Complex V) is certainly large enough to accommodate a phosphate group intervening between pyruvate and Mn(II). On the other hand, the Mn(II) EPR spectra for the ternary pyruvate complex (Complex VIA) and the equilibrium complex are nearly identical, thus arguing against direct Mn(II) coordination to the phosphoryl group (7).

The structure of the Mn(II)-pyruvate kinase-P-enolpyruvate complex is not yet established. Evidence for a metal-phosphoryl bridge complex in pyruvate kinase-Mn(II) complexes was...
The two effects, however, found to be too large by more than an order of magnitude (A. S. Mildvan, private communication). The two effects, however, tend to cancel each other.

The proton relaxation rate of water in the complexes should be similar and the EPR spectrum should be similar. Although at the present time the differences in symmetry of the Mn(II) complexes with P-enolpyruvate or its analogs (7). In the presence of (CH₃)₂N⁺ rather than K⁺, two species are found to be in equilibrium for the P-enolpyruvate complex or for the P-glycolate complex, one species being characterized by an isotropic spectrum, the other by an anisotropic spectrum. In the presence of K⁺, the complex of P-enolpyruvate or P-glycolate exhibits only the anisotropic spectrum, but the methylene analog exhibits only the isotropic spectrum with either K⁺ or (CH₃)₂N⁺. At the present time, we have reliable Mn(II)-H distances only for the isotropic form of the ternary complex from the current study with the inhibitor α-(dihydroxyphosphinylmethyl)acrylate. This structure differs from Complex II A on the reaction path, and how the Mn(II)-H and Mn(II)-3¹P distances are affected cannot be assessed.

The Mn(II)-H distances in the K⁺ form of the ternary enzyme-Mn(II)-α-(dihydroxyphosphinylmethyl)acrylate complex (Table II) support a structure with Mn(II) bonding to the phosphinyl group. The three distances calculated for the three protons of the ligand strictly limit the possible structures. The structure of a feasible ternary complex constructed from molecular models is shown in Fig. 5 with the experimental PRR values cannot be quantitated in terms of distance changes between Mn(II) and the ligand nuclei under observation, it would be unsafe to assume that no distance changes are involved.

The necessity for an independent parameter for determining the identity of the structural species is well illustrated by experiments on the EPR spectra of ternary pyruvate kinase-Mn(II)-fluorophosphate complexes with P-enolpyruvate or its analogs (7). In the presence of (CH₃)₂N⁺ rather than K⁺, two species are found to be in equilibrium for the P-enolpyruvate complex or for the P-glycolate complex, one species being characterized by an isotropic spectrum, the other by an anisotropic spectrum. In the presence of K⁺, the complex of P-enolpyruvate or P-glycolate exhibits only the anisotropic spectrum, but the methylene analog exhibits only the isotropic spectrum with either K⁺ or (CH₃)₂N⁺. At the present time, we have reliable Mn(II)-H distances only for the isotropic form of the ternary complex from the current study with the inhibitor α-(dihydroxyphosphinylmethyl)acrylate. This structure differs from Complex II A on the reaction path, and how the Mn(II)-H and Mn(II)-3¹P distances are affected cannot be assessed.

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Fig. 6. A, geometry of the pyruvate kinase-Mn(II)-α-(dihydroxyphosphinylmethyl)acrylate complex. The distance indicated (6.3 ± 0.5 Å) is the experimentally determined value for the vinyl group. B, geometry of the pyruvate kinase-Mn(II)-ATP-pyruvate complex (Complex V). The distance indicated (8.2 ± 0.5 Å) is that which was experimentally determined.

On the basis of recently determined dissociation constants (4), it now appears that only a small percentage of the Mn(II) was in the form of the ternary enzyme-Mn(II)-fluorophosphate complex in the initial studies of the fluorophosphate complex. Furthermore, the correlation time used in the calculation was subsequently found to be too large by more than an order of magnitude (A. S. Mildvan, private communication). The two effects, however, tend to cancel each other.

The authors (20) point out. As for the methylene-vinyl proton coupling constants, the coupling to a proton could be greater than the coupling to b proton (21) contrary to the argument presented in Reference 20. The uncertainty in the assignment does not affect any of the conclusions in this paper since the distances to protons a and b are so similar (cf. Fig. 6).

The methylene analog of P-enolpyruvate, which has been shown here to compete with P-enolpyruvate for the same binding site on the enzyme (cf. Fig. 4), suffers from the same difficulty in equating the structure of its enzyme-metal complex with that of the true substrate. Not only does it, like fluorophosphate, have little effect on the water PRR enhancement (4) while the latter decreases the enhancement by an order of magnitude (18).

The assignment of the “a” and “b” vinyl protons to the upfield and downfield peaks, respectively (under “Materials and Methods”) is tentative since it is based on analogy to P-enolpyruvate (19). The opposite assignment has been made tentatively in a recent paper (20) based on the relative magnitude of coupling constants. The relative magnitude of the P-H coupling constants is not a definitive criterion, as the authors (20) point out. As for the methylene-vinyl proton coupling constants, the coupling to a proton could be greater than the coupling to b proton (21) contrary to the argument presented in Reference 20. The uncertainty in the assignment does not affect any of the conclusions in this paper since the distances to protons a and b are so similar (cf. Fig. 6).
values for $r$ given in parentheses. The values from the constructed model are still within experimental error of the NMR distance calculations. Nowak and Mildvan (4) recently reported distance calculations in the K$^+$ form of the ternary complexes of the $I'$-enolpyruvate analog, $P$-lactate, with pyruvate kinase-Mn(II) and also came to the conclusion that phosphate to manganese(II) ligand formation was present. However, their quantitative results are questionable since $r_2$ was not determined from any frequency or temperature dependence.

This study quantitates the difference between the spatial relationship of substrate and divalent metal ion activator in the quaternary pyruvate complex (V, Scheme 1) and of the inhibitor and divalent metal ion in the ternary inhibitor complex with the methylene analog of $P$-enolpyruvate. Structures for the two complexes are diagrammed in Fig. 6. Essential features are the coordination of Mn(II) to the phosphinyl group of the $I'$-enolpyruvate analog in the inhibitor complex and the displacement of the pyruvate substrate away from Mn(II) in the equilibrium complex.

The spatial relationship of Mn(II) to the phosphate group in Complex IIA on the reaction path and in the transition state remains an open question. Future experiments in which attempts will be made to observe the $^{31}P$ of bound $P$-enolpyruvate to avoid the limitation of slow exchange between free and bound $P$-enolpyruvate may yield data from which the spatial relationship between Mn(II) and $^{31}P$ in the $P$-enolpyruvate complex may be specified.

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