Magnetic Resonance Studies of the Formation of the Ternary Phosphoenolpyruvate–Gadolinum–Muscle Pyruvate Kinase Complex†

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ABSTRACT: The water proton relaxation rates observed for the binary gadolinium–pyruvate kinase complex were examined as a function of temperature and frequency. The correlation times for the binary complex τ_c* over a variety of temperatures and frequencies ranged from 0.18 to 0.68 nsec as estimated from the Solomon–Bloembergen equations. These values are very similar to the values reported for the electron spin relaxation time (τ_s) for gadolinium. Thus it is concluded that the τ_c* for the binary enzyme complex is determined primarily by the τ_s of gadolinium. The vinyl protons of P-enolpyruvate are broadened in the presence of the gadolinium–pyruvate kinase complex indicating P-enolpyruvate binding, yet no change in the water proton relaxation enhancement is detected. On the basis of T_2^{-1} values of the vinyl protons of P-enolpyruvate measured by high-resolution proton magnetic resonance and calculation of the proton–paramagnetic ion distance, it is concluded that a ternary P-enolpyruvate complex does form, but the structure of the ternary complex cannot be uniquely determined at present.

The rare earth metal ions have recently been suggested as probes of electrostatic binding sites in proteins (Birnbaum *et al.*, 1970) and have been shown to activate α-amylase (Smolka *et al.*, 1971) and isoleucyl-tRNA synthetase (Kayne and Cohn, 1972). The usefulness of the rare earths as probes of enzyme–metal interactions lies in their spectroscopic and magnetic properties allowing use of visible absorption (Darnall and Birnbaum, 1970), difference absorption (Birnbaum *et al.*, 1970), fluorescence (Luk, 1971; Sherry and Cottam, 1973), water proton relaxation rate measurements (Valentine and Cottam, 1973; Sherry and Cottam, 1973; Reuben, 1971; Dwek *et al.*, 1971), and high-resolution proton magnetic resonance (Sherry *et al.*, 1972; Barry *et al.*, 1971) techniques. Since alkaline earths bind primarily through electrostatic bonds the rare earths should also prove useful in examining alkaline earth binding sites as recently shown with muscle pyruvate kinase (Valentine and Cottam, 1973).

Upon addition of the rare earth gadolinium to muscle pyruvate kinase a binary, metal-pyruvate kinase, complex is formed and the water proton relaxation rate (PRR) is enhanced, $\epsilon_b = 12 \pm 2$ (Valentine and Cottam, 1973). There are 3.7 ± 0.5 metal binding sites with a dissociation constant, K_D, of 26 ± 10 μM per enzyme of 237,000 daltons. A decrease in the water proton relaxation rate was observed when ATP was added to a solution containing gadolinium and muscle pyruvate kinase which suggests formation of the ternary, enzyme–gadolinium–ATP, complex. Yet when P-enolpyruvate was added to the binary gadolinium–enzyme complex only a slight decrease in PRR enhancement was observed in either the presence or absence of potassium ion which suggested that the ternary enzyme–gadolinium–P-enolpyruvate complex might not form.

The results of temperature and frequency dependent water proton relaxation rates in the binary complex suggest that the value of τ_c*, the correlation time for the enzyme–gadolinium complex, is determined by the value of the electronic spin-lattice relaxation of gadolinium, τ_s. Also, the high-resolution proton magnetic resonance spectra of the vinyl protons of P-enolpyruvate are broader in the presence of both pyruvate kinase and gadolinium than in the presence of either alone, suggesting the formation of a ternary P-enolpyruvate–gadolinium–pyruvate kinase complex.

Materials and Methods

The pyruvate kinase used in this study was isolated from fresh rabbit skeletal muscle (Tietz and Ochoa, 1958; Cottam *et al.*, 1969) and recrystallized to specific activities between 280 and 310 μmol min^{-1} mg^{-1}. The enzyme was stored as an ammonium sulfate suspension at 10–20 mg of protein/ml. Before use the protein was desalted on Sephadex G-50 columns equilibrated with the appropriate buffer. The protein concentration was determined using an extinction coefficient of 0.54 ml mg^{-1} cm^{-1} at 280 nm (Bücher and Pfleiderer, 1955). The catalytic activity of pyruvate kinase was determined by measuring the rate of appearance of pyruvate by following the oxidation of NADH at 340 nm using excess lactic dehydrogenase (Bücher and Pfleiderer, 1955) with reagents described previously (Cottam *et al.*, 1969).

The longitudinal and transverse water proton relaxation rates were measured by the null point method and the spin echo envelope (Carr and Purcell, 1954) with instruments operating at frequencies of 10.7, 24.3, and 33.8 MHz. The temperature of the samples (50 μl volume) was maintained constant (±0.5°) during the relaxation rate measurements. The observed enhancement (ϵ*) of the water proton relaxation rate is defined as $\epsilon^* = (T_{1P}^*)^{-1}/(T_{1P})^{-1}$, the ratio of the paramagnetic contribution to the relaxation rate in the presence of protein (*) to that in the absence of protein (Eisinger *et al.*, (1961). The paramagnetic contribution to the longitudinal relaxation rate is defined as $(T_{1P})^{-1} = (T_1)^{-1} - (T_{1(0)})^{-1}$, the difference in the observed relaxation rate in the presence and absence (0) of paramagnetic ion.

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High-resolution proton magnetic resonance spectra were obtained using a JEOLCo c60-HL high-resolution spectrometer operating in the external lock, field sweep mode. The rare earth solutions, P-enolpyruvate (Sigma grade), and cacodylate buffer were all dissolved in D_2O and the pyruvate kinase was extensively dialyzed against potassium cacodylate buffer prepared in D_2O, pD 6.0 (Glasoe and Long, 1960). All solutions were adjusted to pD 6.0 with DCl or NaOD prior to preparation of the reaction mixtures and obtaining the spectra. The probe temperature was 29 ± 1° and standard 5-mm sample tubes were used. All chemical shifts are reported relative to 2,2-dimethyl-1-silapentane-5-sulfonic acid (DSS).[1]

Results

Frequency and Temperature Dependent PRR Studies. In Figure 1 the temperature dependence of the water proton relaxation rates resulting from bound paramagnetic ion was examined at three frequencies (33.8, 24.3, and 10.7 MHz). The results presented as $(T_{1P}^*)_b^{-1}$ and $(T_{2P}^*)_b^{-1}$ have been normalized to 100 μM gadolinium bound to muscle pyruvate kinase for ease in comparison of the results. The reaction mixtures contained 270–770 μM enzyme sites (4 sites per 237,000 daltons), 91–100 μM gadolinium, and 50 mM sodium cacodylate buffer (pH 6.0). The value for $(T_{1P}^*)_b^{-1}$ was calculated by correcting for the contribution of free paramagnetic ion using a molar relaxivity of 12,200 M^{-1} sec^{-1} at 24.3 MHz for free gadolinium and a dissociation constant of 26 μM for the muscle pyruvate kinase-gadolinium binary complex (Valentine and Cottam, 1973).

In Figure 1, the relaxation rates observed at instrument frequencies of 33.8 and 24.3 MHz decrease as the temperature is increased. At 10.7 MHz, however, the relaxation rate increases slightly, then decreases as the temperature is increased. The results at 10.7 MHz could be explained by either a correlation time consisting of at least two components with different temperature coefficients or a region where $\omega\tau_s = 1$. For instance at the lower temperatures, both $1/\tau_s$(Gd) and the exchange rate of water into the primary coordination sphere of the bound gadolinium ion ($1/\tau_m$) may contribute to the value of τ_c^* since the rate constant for substitution of water into the inner coordination sphere of gadolinium, $1/\tau_m$, is 8×10^7 sec^{-1} (Cotton and Wilkinson, 1972). At the higher temperatures a rapid exchange of water molecules between the first coordination sphere of the bound paramagnetic ion and the bulk solvent is suggested by the large enhancements, the frequency dependence of the relaxation rates, and by $(T_{2P}^*)_b^{-1} \simeq 7/6\ (T_{1P}^*)_b^{-1}$. Thus at the higher temperatures, τ_m may be neglected in eq 1 and the Solomon-Bloembergen equation (eq 2) may

$$(T_{1P}^*)_b^{-1} = p^*q^*/(T_{1M}^* + \tau_m^*) \quad (1)$$

$$\frac{1}{T_{1M}} = \frac{2S(S+1)}{15r^6}[g\beta\hbar\gamma_I]^2\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right] + \frac{2S(S+1)A^2}{3\hbar^2}\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right) \quad (2)$$

be utilized to calculate τ_c^*. In these equations p is the ratio of the concentration of the paramagnetic ion to the concentration of ligand, q is the number of ligands in the first coordination sphere, S is the electron spin quantum number for gadolinium (7/2), γ_I is the nuclear magnetogyric ratio (2.675×10^4 rad/(sec G)), g is the electronic "g" factor (2), β is the Bohr magneton (8.795×10^6 rad/(sec G)), $\hbar$ is Planck's constant divided by 2π (1.054×10^{-27} erg sec), r is the internuclear proton–paramagnetic ion distance, ω_I is the Larmor angular precession frequency for nuclear spin (1.526×10^8 rad/sec), and ω_s is the Larmor angular precession frequency for electron spins (1.003×10^{11} rad/sec). The second part of eq 2 which arises from modulation of the scalar interaction between the electron spins on gadolinium and the nuclear spins of the water protons may be ignored in the calculation of τ_c^* in gadolinium–enzyme systems (Dwek, 1972). Unlike manganese systems, this assumption is reasonable for gadolinium where the unpaired 4f electrons are shielded from coordinated water molecules thus giving negligible hyperfine contact interactions.

The calculated values of τ_c^*, assuming $q^* = 5$ in the gadolinium–enzyme complex, are tabulated in Table I. All of the calculated values of τ_c^* over a range of frequencies and temperatures are within 2–7 $\times 10^{-10}$ sec, which is similar to those values of 4–7 $\times 10^{-10}$ sec at 30 MHz reported for the electron spin relaxation time (τ_s) for gadolinium (Bloembergen and Morgan, 1961). Since τ_c may have contributions from the cor-

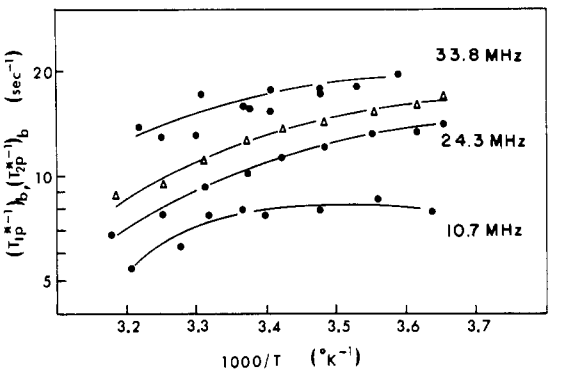

FIGURE 1: Water proton relaxation rates of gadolinium bound to pyruvate kinase as a function of temperature and frequency. The experiments contained: 50 mM sodium cacodylate buffer (pH 6.0), 270 μM enzyme binding sites (4 sites per 237,000 daltons), 91–100 μM gadolinium at the temperatures indicated. The data refer to the paramagnetic contribution of bound gadolinium to the longitudinal relaxation rate (●) of water protons at operating frequencies at 33.8, 24.3, and 10.7 MHz (top to bottom) and the transverse relaxation rate at 24.3 MHz (△). The values have all been normalized to 100 μM gadolinium bound to muscle pyruvate kinase as described in the text.

TABLE I: Calculated Values of the Correlation Time, τ_c^*, for the Gadolinium–Pyruvate Kinase Complex.[a]

	τ_c^* (nsec)		
1000/T (°K^{-1})	10.7 MHz	24.3 MHz	33.8 MHz
3.2	0.18	0.24	0.42
3.3	0.23	0.30	0.50
3.4	0.26	0.35	0.58
3.5	0.28	0.42	0.64
3.6	0.29	0.46	0.68

[a] The values of τ_c^* were calculated from eq 1 and 2, the data in Figure 1, and using $q = 5.0$, $r = 2.8 \times 10^{-8}$ cm, $S = 7/2$, $\gamma_I = 2.675 \times 10^4$ rad/(sec G), $g = 2.0$, $\beta = 8.795 \times 10^6$ rad/(sec G), $\hbar = 1.0544 \times 10^{-27}$ erg sec, ω_I (10.7 MHz) = 0.674×10^8 rad/sec, ω_I (24.3 MHz) = 1.526×10^8 rad/sec, ω_I (33.8 MHz) = 2.124×10^8 rad/sec, ω_s (10.7 MHz) = 0.443×10^{11} rad/sec, ω_s (24.3 MHz) = 1.003×10^{11} rad/sec, ω_s (33.8 MHz) = 1.397×10^{11} rad/sec.

[1] Abbreviation used is: DSS, 2,2-dimethyl-1-silapentane-5-sulfonic acid.

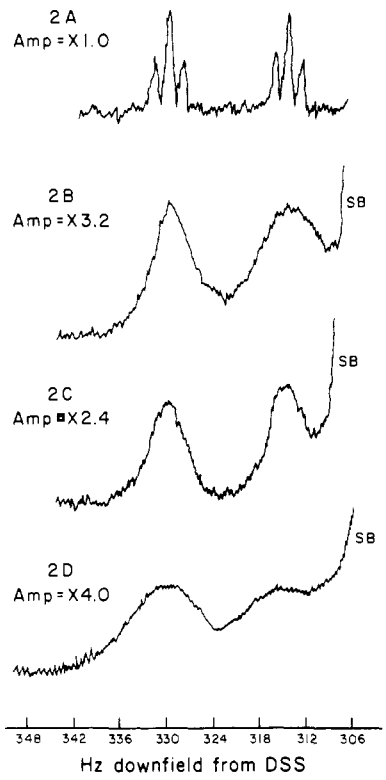

FIGURE 2: The effect of the addition of gadolinium and pyruvate kinase on the high-resolution proton magnetic resonance spectra of P-enolpyruvate. All solutions were prepared in D_2O and adjusted to pD 6.0. Sample A contained 73 mM potassium P-enolpyruvate and 50 mM potassium cacodylate as buffer. In addition the other samples contained: (B) 107 μM gadolinium; (C) 270 μM muscle pyruvate kinase binding sites (4 sites per 237,000 daltons); (D) 107 μM gadolinium plus 270 μM enzyme binding sites.

relation time for molecular rotation, τ_r, the residence lifetime of bound water, τ_M, and the electron spin relaxation time, τ_s (see eq 3), contributions to τ_c from τ_r and τ_M cannot be completely excluded. However, τ_r and τ_M are frequency independent whereas τ_s may exhibit a frequency dependence (when $\omega_s\tau_v > 1$) as shown in eq 4. τ_v is a correlation time related to the rate at which the zero field splitting is modulated by impact of the solvent molecules on the complex ($\tau_v < \tau_s$) and B is a constant relating the values of the electronic spin and the zero field splitting parameters (Bloembergen and Morgan, 1961). Since $\omega_s\tau_v$ increases with increasing frequency, we would expect τ_s to also increase with increasing frequency. As can be seen in Figure 1 and Table I, the value of τ_c* increases as the frequency is increased suggesting that τ_s dominates τ_c*. From the data in Table I a value of 3.5 ± 0.5 kcal/mol is calculated for the activation energy, E_A, of the relaxation process.

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_M} + \frac{1}{\tau_s} \quad (3)$$

$$\frac{1}{\tau_s} = B\left[\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right] \quad (4)$$

TABLE II: Calculated Values of τ_c* for the Gadolinium–Pyruvate Kinase Complex as q is Varied.[a]

q	τ_c* (nsec)	q	τ_c* (nsec)
1	1.91	6	0.29
2	0.90	7	0.25
3	0.59	8	0.22
4	0.44	9	0.19
5	0.35		

[a] Calculated from the data in Figure 1 at 24.3 MHz at 296°K, using eq 1 and 2 and constants listed in Table I.

The effect of changing q on the calculated values of τ_c* is illustrated in Table II. The values of τ_c* are calculated from the 24.3 MHz data in Table I at a temperature of 296°K ($1000/T = 3.1$). If the value of τ_c* is determined by τ_s of gadolinium ($4\text{–}7 \times 10^{-10}$ sec, Bloembergen and Morgan, 1961) our data are consistent with values of q from 3 to 5.

High Resolution Nuclear Magnetic Resonance (nmr). The proton magnetic resonance spectrum of P-enolpyruvate (Figure 2A) consists of two sets of overlapping doublets resulting from the splitting of each vinyl proton by one nonequivalent proton and one phosphorus nucleus ($J_{HH} \simeq J_{PH} \simeq 4$ Hz). The downfield proton resonance (330 Hz downfield from DSS) was previously assigned to the proton trans to the phosphate moiety while the resonance at 314 Hz was assigned to the cis vinyl proton (Cohn *et al.*, 1970). When gadolinium is added to the solution of P-enolpyruvate, each triplet collapses into a broadened single peak (Figure 2B). The relationship between nuclear magnetic resonance proton line widths and the corresponding distances between each proton and the paramagnetic metal center is apparent in this spectrum. The obvious larger paramagnetic broadening in the upfield triplet (proton cis to the phosphate) reflects the binding of gadolinium to the phosphate group of P-enolpyruvate. When muscle pyruvate kinase is added to the P-enolpyruvate (Figure 2C), the triplets also collapse into broadened single peaks. This broadening must result from an increase in the rotational correlation time of the substrate as it binds to the enzyme. The change in solution viscosity when the enzyme is added is of little significance in this experiment because no collapse of the overlapping doublets and only slight broadening of the individual resolved peaks are observed when the viscosity of a P-enolpyruvate solution is increased up to 2.33 cP with glycerol. The effect of addition of both muscle pyruvate kinase and gadolinium on the P-enolpyruvate protons is shown in Figure 2D. Both peaks broaden considerably more than in the previous spectra suggesting the enzyme-bound gadolinium lies near the P-enolpyruvate binding position. Under the conditions of this experiment more than 85% of the gadolinium is bound to the enzyme as calculated from the dissociation constant of 26 μM (Valentine and Cottam, 1973).

In order to estimate distances between the paramagnetic metal binding position and the P-enolpyruvate protons using nuclear magnetic resonance line-broadening data, one must be able to measure the paramagnetic broadening contribution to one isolated line of each overlapping doublet. This was accomplished using a Du Pont Model 301 curve resolver. Four Lorentzian curves of identical height and line width were fitted to the observed spectra of the low field P-enolpyruvate overlapping doublet. A mathematical addition of the two center peaks results in the simulation of the low-field triplet. Changing the line widths of each generated peak by an equal amount allows simulation of the broadened low-field peaks in Figure 2B–D. Each individual peak of the four under the simulated spectrum was then isolated and its full peak width at half-height measured. These results, along with the water (HOD), cacodylate, and DSS line widths, are tabulated in Table III. The line widths of the upfield P-enolpyruvate proton resonances are not

TABLE III: High-Resolution Proton Magnetic Resonance Line Widths.[a]

Sample Contains	P-enol-pyruvate	HOD	Cacodyl-ate	DSS
73 mM PEP	1.1	1.5	1.5	1.2
73 mM PEP + 0.107 mM Gd^{3+}	2.5	1.9	2.1	1.7
73 mM PEP + 0.06 mM MPK	2.4	1.6	1.8	1.9
73 mM PEP + 0.06 mM MPK + 0.107 mM Gd^{3+}	5.3	3.9	2.3	2.7

[a] The numbers represent the average full peak width in Hz at half-peak height of each tabulated proton resonance taken from a number of spectra. The reproducibility of the line widths is ±0.2 Hz. The line widths under P-enolpyruvate are those corresponding to a single peak from the downfield set of overlapping doublets as obtain using a Du Pont curve resolver (see text). Every sample contained 0.05 M sodium cacodylate buffer (pD 6.0), 0.1% internal standard, DSS, 73 mM P-enolpyruvate (PEP), and concentrations of muscle pyruvate kinase (MPK) and gadolinium as noted.

included because of difficulties arising from overlap of the HOD spinning bands.

The addition of 0.107 mM gadolinium to P-enolpyruvate in the absence of enzyme results not only in a paramagnetic broadening of the substrate protons but also a 30–40% increase in the peak widths of HOD, cacodylate, and DSS. This broadening results from a combination of outer sphere bulk susceptibility effects and inner sphere relaxation of these molecules when they are bound in the first coordination sphere of the gadolinium ion. When enzyme is added to P-enolpyruvate in the absence of gadolinium, only small changes are observed in the line widths of the HOD and cacodylate peaks, while the DSS line width increases approximately 40% (which may reflect some binding of DSS to the protein) and the P-enolpyruvate line width increases by 120%. The large increase in this latter line width reflects the binding of substrate molecules to the enzyme. These spectra may be recorded in any sample order with only a deviation of ±0.2 Hz in the line widths suggesting that these changes in peak width are not caused by field-inhomogeneity effects. When gadolinium is added to the enzyme–P-enolpyruvate sample, the cadodylate and DSS peaks are broadened by an additional 30–40% (similar to the addition of gadolinium to P-enolpyruvate alone) while the HOD and substrate peaks broaden by 140 and 120%. The broadening in the HOD peak reflects the known enhancement in the water proton relaxation rate when gadolinium binds to the enzyme (Valentine and Cottam, 1973) while the broadening in the substrate protons reflects the binding of gadolinium near the P-enolpyruvate site in the ternary enzyme complex.

The paramagnetic contribution to the spin–spin relaxation of the P-enolpyruvate protons can be determined knowing

$$(T_{2P})^{-1} = (\pi\Delta\nu)_{\substack{\text{presence of}\\ \text{gadolinium}}} - (\pi\Delta\nu)_{\substack{\text{absence of}\\ \text{gadolinium}}} \quad (5)$$

where $\Delta\nu$ = full peak width at half-height. The distances between the observed broadened protons and the gadolinium ion bound to the enzyme may be estimated assuming $\tau_M < T_{2M}$ in eq 6 and neglecting the scalar term in eq 7 (Dwek, 1972). In

TABLE IV: Effect of Terbium on the Chemical Shift of P-enolpyruvate Protons.[a]

Sample	Terbium (μM)	Pyruvate Kinase Sites (μM)	Chemical Shift (Hz) Cis Proton	Chemical Shift (Hz) Trans Proton
A			314	330
B	28		317	332
C	309		325	336
D		384	316	330
E	309	384	317	331

[a] The reaction mixtures all contained 75 mM P-enolpyruvate and 38 mM potassium cacodylate. All solutions were prepared in D_2O and adjusted to pD 6.0. The enzyme site concentration is calculated assuming 4 sites per 237,000 daltons. The chemical shifts of the protons cis and trans to the phosphate group of P-enolpyruvate are reported as Hz downfield from DSS. Temperature of the samples was 29 ± 1°. The spectra were recorded at −38 db radiofrequency power.

the ternary enzyme–gadolinium–P-enolpyruvate complex, a proton–metal distance of 7.9 ± 0.3 Å (proton trans to the phosphate group) was calculated from the 60-MHz data using ω_I = 3.77 × 10^8 rad/sec, ω_s = 2.476 × 10^{11} rad/sec, and assuming

$$(T_{2P})^{-1} = \frac{f}{T_{2M} + \tau_M} \quad (6)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}\frac{S(S+1)}{r^6}[\gamma_I g\beta\hbar]^2\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2}\right) + \frac{1}{3}\frac{S(S+1)A^2}{\hbar^2}\left(\tau_e + \frac{\tau_e}{1+\omega_S^2\tau_e^2}\right) \quad (7)$$

$\tau_c^* = \tau_s = 1 \times 10^{-9}$ sec as extrapolated from the frequency dependence of the values of τ_c^* obtained at 10.7 and 24.3 MHz and f is the mole fraction of ligands in the first coordination sphere. The error limits on the distance reflect a possible 50% uncertainty in the values of τ_c^* or τ_s used in the calculations.

The effect of the binding of terbium to P-enolpyruvate is shown in Table IV. The addition of 28 μM terbium causes a downfield lanthanide induced shift (LIS) of 2 and 3 Hz to the low-field and high-field triplets, respectively. Increasing the terbium concentrations to 309 μM results in a collapse of the splitting and a total downfield shift of 6 and 11 Hz. Again, these data suggest the high-field proton (cis to the phosphate) is nearest the terbium in the P-enolpyruvate–metal complex. Also shown in Table IV is the effect of the addition of 384 μM pyruvate kinase binding sites on the protons of P-enolpyruvate. This results in the collapse of the triplets and line broadening, as seen previously in Figure 2C, and little chemical shift. With the addition of both muscle pyruvate kinase (384 μM binding sites) and terbium (309 μM) no paramagnetic shift is observed in the P-enolpyruvate proton peaks.

Discussion

The addition of P-enolpyruvate to a solution containing manganese and pyruvate kinase is known to result in formation of a ternary complex which has been detected by a decrease in the water PRR enhancement (Mildvan and Cohn, 1966; Nowak and Mildvan, 1972; Cottam *et al.*, 1972; James *et al.*, 1973), protein ultraviolet difference spectrum (Kayne and

Suelter, 1965), and electron paramagnetic resonance spectra of manganese (Reed and Cohn, 1973). Two conformational forms of the ternary P-enolpyruvate complex were recently detected by PRR enhancements (James *et al.*, 1973). One conformation has a ternary enhancement of 2.3 and the other has an enhancement of 17 which is very similar to the binary enhancement of 22.

In contrast to the results with manganese activated pyruvate kinase, no change in the PRR enhancement was detected upon addition of P-enolpyruvate to solutions containing gadolinium and muscle pyruvate kinase (Valentine and Cottam, 1973). This study was initiated to answer the question of whether the ternary P-enolpyruvate–lanthanide ion–enzyme complex forms and whether its structure could be examined by utilization of the paramagnetic properties of lanthanide ions and their effect upon the high-resolution proton magnetic resonance (pmr) spectrum of P-enolpyruvate. An accurate value of the correlation time, τ_c*, was also determined to allow estimation of the substrate proton–gadolinium ion distance in the enzyme complex.

The values of τ_c* for the binary pyruvate kinase–gadolinium complex measured over a variety of temperatures and frequencies range from 0.2 to 0.7 nsec. These τ_c* values agree with τ_c* = 0.22 nsec for a gadolinium–lysozyme complex (Dwek *et al.*, 1971) and the electron spin relaxation time of gadolinium, τ_s, of 0.4–0.7 nsec (Bloembergen and Morgan, 1961). Since the correlation time for the enzyme–manganese complex is much larger, 3.2 ± 2.4 nsec (Nowak, 1973; Reuben and Cohn, 1970), and is primarily dominated by the electron spin relaxation of manganese, any contribution of τ_r* to the gadolinium–enzyme complex should be negligible. Thus the correlation time of the gadolinium–enzyme complex is determined by τ_s of gadolinium. Therefore, to change the observed enhancement one must alter the number of water molecules in the first coordination sphere or change the value of τ_s.

When one compares the high-resolution pmr spectra of the vinyl protons of P-enolpyruvate, the peaks are broader in the presence of gadolinium and enzyme than in the presence of either alone which suggests ternary complex formation. The vinyl proton–gadolinium internuclear distance estimated from the line-width data is 7.9 Å. This value is similar to the maximum internuclear vinyl proton–gadolinium ion distance as measured from Dreiding models.

The data provide evidence of the formation of a ternary complex but are not conclusive about the direct binding of the phosphate in the primary coordination sphere of the gadolinium ion. If the phosphate is binding in the primary coordination sphere of the gadolinium ion, the exchange rate of P-enolpyruvate on and off the bound gadolinium must be at least as rapid as the water exchange rate between the primary coordination sphere and the bulk solvent. This condition would result in little change in the binary enhancement upon the addition of P-enolpyruvate to the gadolinium–enzyme complex. However, it is more reasonable to assume the phosphate does not enter the coordination sphere of the bound gadolinium but rather lies at an approximate second coordination sphere position.

The LIS data with terbium are consistent with the gadolinium broadening data. Of those lanthanides ions tested all bind to muscle pyruvate kinase with a $K_D \leq 64$ μM (Valentine and Cottam, 1973). Thus under the conditions of this experiment, essentially all of the 309 μM Tb^{3+} is expected to be bound to the enzyme. The fact that no LIS is observed in the presence of enzyme (Table IV) also suggests that terbium binds to pyruvate kinase, otherwise a LIS comparable to that seen in the absence of enzyme would be expected. The lack of a measurable LIS in the ternary enzyme–terbium–P-enolpyruvate complex could result from (a) a smaller binding constant of P-enolpyruvate to the terbium–enzyme complex as compared to free terbium, (b) the P-enolpyruvate binds at a position far enough away from the terbium ion in the ternary complex to give immeasurably small LIS's, or (c) the principle symmetry axis in the ternary complex is considerably different from that in the binary terbium–P-enolpyruvate complex. The magnitude of the observed LIS depends upon the distance between the metal ion and each proton and the angle between a principle symmetry axis of the metal complex and each proton as shown in eq 8.

$$\Delta H = K[(3\cos^2\theta - 1)/r^3] \quad (8)$$

ΔH is the observed LIS in Hz and K is a constant which includes the magnetic spin quantum number and a contribution from the anisotropy in the g value. In the binary terbium–P-enolpyruvate complex the principle symmetry axis is determined by the bound ligand whereas in the ternary enzyme–terbium–P-enolpyruvate complex symmetry axis may be determined by the enzyme. Such changes in the principle symmetry axis have recently been observed when comparing substrate–europium and substrate–europium(EDTA) complexes (J. T. Lewis, A. D. Sherry, and H. Hung, manuscript in preparation). Thus relatively small changes in r, θ, or both could drastically lower the LIS. Presumably, if the LIS were larger in the free P-enolpyruvate–metal complex, a shift would also be observed in the ternary complex.

All of the data are consistent with a model where the P-enolpyruvate binds in the enzyme pocket at a position in which the substrate phosphate group is close to the primary coordination sphere of the lanthanide ion. However, the large size of the lanthanides may prevent the P-enolpyruvate-induced conformational change observed in the active manganese ion system (Kayne and Suelter, 1965).

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Kinetic and Magnetic Resonance Studies of the Interaction of Oxalate with Pyruvate Kinase†

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ABSTRACT: The interaction of the oxalate dianion with rabbit muscle pyruvate kinase has been invesigated by kinetic inhibition and by magnetic resonance techniques. Oxalate inhibits the pyruvate kinase reaction competitively with respect to the substrate, phosphoenolpyruvate, with an inhibitor constant of ~6 μM. The inhibition constant does not change upon substitution of Mn(II) for Mg(II) as the divalent cation activator. Formation of the ternary complex, Mn–enzyme–oxalate, lowers the enhancement of the proton relaxation rate of water from a value of 24 (measured at 24.3 MHz and 25°) for the binary Mn–enzyme complex to a value of 9 for the ternary complex with oxalate. Titration experiments give dissociation constants for oxalate from the ternary complex with Mn(II) and pyruvate kinase of the order of 1.2 μM. Neither the dissociation constant for oxalate nor the enhancement of the ternary complex, ϵ_t, is influenced by substitution of nonactivating $(CH_3)_4N^+$ for the activator, K^+, in the solution. The electron paramagnetic resonance (epr) spectrum for Mn(II) in the ternary complex with oxalate and pyruvate kinase differs from that of the binary Mn–enzyme complex and this indicates that oxalate binding changes the symmetry of the Mn(II) coordination sphere. However, direct Mn(II)–oxalate binding is not established. The spectrum for the oxalate ternary complex is much less anistropic than spectra for the corresponding complexes with pyruvate or the phosphoenolpyruvate. Furthermore, the spectrum of the oxalate complex is not influenced by subsequent addition of the nucleotide substrates, ADP or ATP, or by addition of the enzymatic enolization cofactors, K^+ and P_i. The potent interaction of oxalate with pyruvate kinase may arise because of structural similarities between oxalate and the enolate form of the substrate, pyruvate.

Pyruvate kinase (EC 2.7.1.40) catalyzes the reversible transfer of a phosphoryl group from P-enolpyruvate[1] to ADP to form pyruvate and ATP (Boyer, 1962; Kayne, 1973). Pyruvate kinase also catalyzes the phosphorylation of fluoride ion (Tietz and Ochoa, 1958) and of hydroxylamine (Kupiecki and Coon, 1960) by ATP. The enzyme requires both a divalent and a monovalent cation as obligatory cofactors in each of its catalytic reactions, while the latter two reactions, in addition, require bicarbonate as a cofactor.

Rose (1960) has shown that the enzyme catalyzes an exchange of protons in the methyl group of pyruvate with protons of the solvent. This exchange reaction requires both the divalent and monovalent cation activators of the normal enzymatic reaction and has an additional requirement for a cofactor containing a "phosphate-like" moiety (Rose, 1960). More recently Robinson and Rose (1972) have examined the exchange of tritium, labeled in C-3 of P-enolpyruvate, with solvent protons during the enzymatic reaction in the forward direction. These studies showed that the rate of tritium exchange exceeds the turnover rate in the forward direction. The exchange reaction of pyruvate kinase has implicated the enolate of pyruvate as an

† From the Johnson Research Foundation, Department of Biophysics and Physical Biochemistry, The School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received March 27, 1974.* This work was supported by grants from the U. S. Public Health Service (GM 12446) and the National Science Foundation (GB 32168X) and by a Research Career Development award to G. H. R. (1KO4-AM 70134). Presented in part at the 166th National Meeting of the American Chemical Society, Chicago, Ill., Aug 1973.

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[1] Abbreviations used are: PRR, proton relaxation rate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ϵ_a, ϵ_b, and ϵ_t are, respectively, the characteristic PRR enhancements for the Mn(II)–substrate, Mn(II)–enzyme, and Mn(II)–enzyme–substrate complexes; P-enolpyruvate, phosphoenolpyruvate.