Nuclear Magnetic Resonance Studies of Selectively Hindered Internal Motion of Substrate Analogs at the Active Site of Pyruvate Kinase†

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ABSTRACT: The interactions of muscle pyruvate kinase with three competitive analogs of phosphoenolpyruvate, D- and L-phospholactate and phosphoglycolate, were studied by measuring the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the protons and phosphorus of the analogs. The enzyme alone or in the presence of Mg^{2+} elicits a stereoselective dipolar effect on the relaxation rates of the methyl protons of L-phospholactate to a much greater extent than on D-phospholactate. The relaxation rates yield a correlation time (τ_c) which is a typical time constant for hindered methyl rotation on the bound L isomer ($\tau_c = 1.7 \times 10^{-9}$ sec, $E_{act} = 1.8$ kcal/mole). The hindrance of methyl rotation is presumably due to steric interaction of the methyl group of the L isomer with the group on the enzyme which protonates phosphoenolpyruvate. Little or no hindrance of rotation of the methyl group of D-phospholactate is detected ($\tau_c \leq 5 \times 10^{-10}$ sec). With phosphoglycolate, hindered motion of the methylene protons ($\tau_c \geq 1.1 \times 10^{-8}$ sec) and the phosphate ($\tau_c = 6.6 \times 10^{-8}$ sec) are detected. The correlation time for phosphorus is indistinguishable from the rotation time calculated for the entire enzyme molecule ($\tau_r = 5.6 \times 10^{-8}$ sec). The τ_c values thus indicate progressively greater immobilization of the bound analogs as one approaches the reaction center phosphorus. Such immobilization or "freezing" of bound substrates at the reaction center would permit orientational or entropic effects to operate in enzyme catalysis.

The earliest nuclear magnetic resonance (nmr) studies of the binding of small molecules to macromolecules made use of the increased relaxation rates of the nuclei of the ligand when it binds to the macromolecule. Such dipolar effects on nmr line widths were used by Jardetzky and coworkers to study the interaction of various antibiotics and sulfonamides with serum albumin (Jardetzky, 1964; Jardetzky and Wade-Jardetzky, 1965) and have since been extended to studies of the binding of the coenzyme NAD to dehydrogenases (Hollis, 1967) and of substrate analogs to chymotrypsin (Sykes, 1969a,b), aspartate transcarbamylase (Sykes *et al.*, 1970), lysozyme (Sykes, 1969a,b; Studebaker *et al.*, 1971; Raftery *et al.*, 1968, 1969), and carbonic anhydrase (Lanir and Navon, 1971).

Although diamagnetic effects on relaxation rates are usually much smaller, and more difficult to interpret than paramagnetic effects (Jardetzky, 1964), they can yield structural information when supplemented by studies of the structure of the complex by independent methods such as X-ray diffraction or paramagnetic probe experiments. Thus, the diamagnetic effect of lysozyme on α- and β-*N*-acetylglucosamine in solution were interpreted in terms of the crystal structure of this enzyme (Blake *et al.*, 1965, 1967; Phillips, 1967; Phillips and Sarma, 1967) where the observed shifts were explained by proximity to the aromatic residue, tryptophan-108, and the larger dissociation constant of the α anomer can be explained by a steric interaction between the methyl group of the inhibitor and residues 52 and 109 at the active site of lysozyme (Sykes, 1969a,b).

The present work follows from our earlier observation (Nowak and Mildvan, 1970) that the compounds, L-phospholactate and D-phospholactate, analogs of the substrate, P-enolpyruvate,[1] bind to the pyruvate kinase–Mn complex with very different affinities. The 20-fold weaker binding of phospholactate, as compared with its stereoismer, D-phospholactate, was explained in terms of the stereochemistry of the pyruvate kinase reaction (Rose, 1970) by proposing van der Waals interaction of the methyl group of the L isomer with a proton donating group on the enzyme. Such a van der Waals interaction might be expected to produce a stereoselective dipolar effect on the relaxation rates of the methyl group of L-phospholactate, but not of D-phospholactate. The present paper reports such a stereoselective effect and examines its properties.

Experimental Section

Materials. Rabbit muscle pyruvate kinase and lactate dehydrogenase were purchased from Boehringer und Sohne (Mannheim, West Germany). The NADH and P-enolpyruvate were purchased from Sigma and 2-phosphoglycolate was purchased as the tricyclohexylammonium salt from General Biochemicals, Chagrin Falls, Ohio. Spectroscopically pure MgO was obtained from Johnson Matthey Chemicals, Ltd., London, England, and was neutralized with DCl to form contaminant-free $MgCl_2$. D- and L-phospholactate were synthesized as previously described (Nowak and Mildvan, 1970). The analogues of P-enolpyruvate were converted to their K^+-free form by passage through a Dowex 50-H^+ column and the material collected as the free acid. This was then

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[1] Abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; TMA, tetramethylammonium cation.

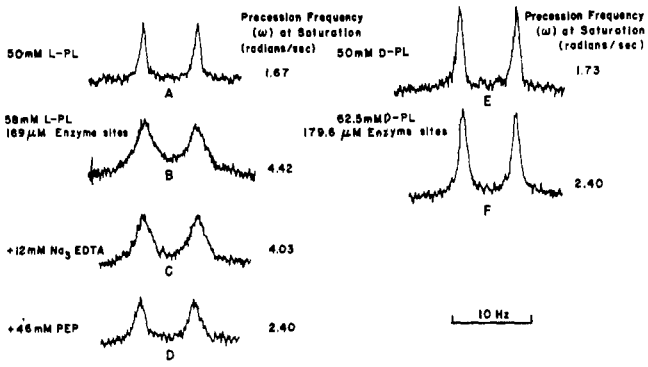

FIGURE 1: The effect of pyruvate kinase on the proton nuclear magnetic resonance spectra of the methyl groups L-phospholactate (L-PL) and D-phospholactate (D-PL) in the absence of a divalent cation and KCl at 100 MHz. The phospholactates were present as their Tris salts in 99.6% D_2O (A and E). Enzyme was added as a solution in 0.05 M Tris-Cl (pH 7.5) buffer in D_2O (B and F; EDTA was added as the trisodium salt in H_2O (C); and P-enolpyruvate added as the Tris salt in D_2O (D). The initial volumes were 0.4 ml and the temperature was 30 ± 1°.

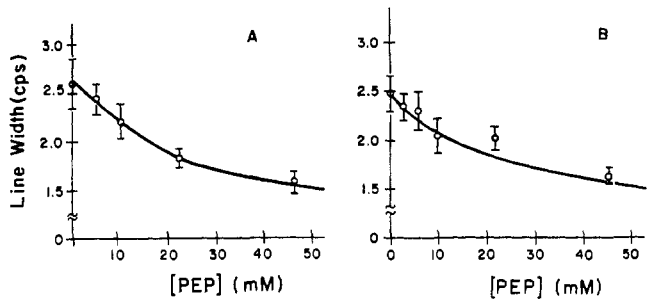

FIGURE 3: P-enolpyruvate competition with L-phospholactate for pyruvate kinase in the presence and absence of KCl. (A) The effect of 338 μM pyruvate kinase sites on the line width of the methyl resonance of the Tris salt of L-phospholactate (61.4 mM) in 99% D_2O (pH 7.5) is titrated with Tris-P-enolpyruvate added as a solution in D_2O (pH 7.5). The points represent the observed line widths and the solid curve is computed by assuming competition between P-enolpyruvate (K_s = 0.40 mM) and L-phospholactate (K_3 = 1.0 mM). (B) The effect of 135 μM pyruvate kinase sites on the line width of the methyl resonance of the K^+ salt of L-phospholacetate (50 mM) in 99% D_2O (pH 7.5) is titrated with P-enolpyruvate added as a solution in D_2O (pH 7.5). The points represent the observed line widths and the solid curve is computed by assuming competition between P-enolpyruvate (K_s = 0.15 mM) and L-phospholacetate (K_s = 0.20 mM). Temperature = 30 ± 1°.

neutralized either with tetramethylammonium hydroxide or Tris base.

Methods. Pyruvate kinase, assayed as previously described (Tietz and Ochoa, 1958), had a specific activity of 110–150 units/mg and was judged by acrylamide gel electrophoresis to be at least 98% pure. In the nmr experiments where the relaxation rates of the carbon-bound protons of the P-enolpyruvate analogs were measured, the enzyme was desalted as previously described (Nowak and Mildvan, 1970), lyophilized, and redissolved in distilled 99.8% D_2O. The lyophilization and solution in D_2O were repeated twice. In experiments where the relaxation rates of the ^{31}P nucleus were measured, the solutions were desalted, concentrated by vacuum dialysis, diluted with D_2O for field locking, and passed through a small Chelex column prior to use to remove any trace paramagnetic metal contaminants. The nmr spectra were taken either on a Varian HA-100-15 or a Varian XL-100-15 nmr spectrometer, and on the Varian HA-220 spectrometer. The $1/T_2$ data were obtained from measurements of the half-width of the resonance signal at half-height at 5 dB or more below saturation and $1/T_1$ was obtained by measuring the power at which the signal saturates, as described elsewhere (Mildvan and Cohn, 1970).

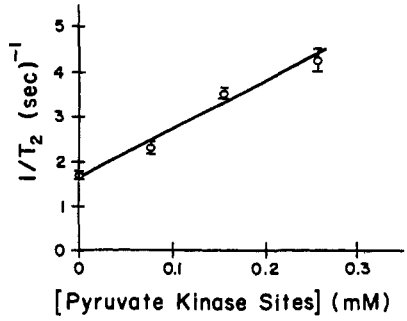

FIGURE 2: The effect of pyruvate kinase on the transverse relaxation rate of the methyl protons of L-phospholacetate. The transverse relaxation rate ($1/T_2$) of the methyl group of L-phospholacetate (50 mM) was measured at 100 MHz at varying concentrations of pyruvate kinase. Temperature = 30 ± 1°.

With the analogs, L-phospholactate and phosphoglycolate large diamagnetic effects of pyruvate kinase on the relaxation rates of the protons and phosphorus were observed, comparable in magnitude to paramagnetic effects (Nowak and Mildvan, 1972). These diamagnetic effects were expressed quantitatively in a manner analogous with paramagnetic effects (Mildvan and Cohn, 1970). Thus the diamagnetic effects on the relaxation rates ($1/T_{1d}$, $1/T_{2d}$) were calculated by subtracting the relaxation rates observed in absence of enzyme ($1/T_{1(0)}$, $1/T_{2(0)}$ from those observed in its presence ($1/T_{1(obsd)}$, $1/T_{2(obsd)}$), and the values of $1/T_{1d}$ and $1/T_{2d}$ so obtained were normalized by the factor f = [diamagnetic sites]/[ligand] (eq 1 and 2). The normalization assumes saturation by ligands

$$1/fT_{1d} = (1/T_{1(obsd)} - 1/T_{1(0)})[\text{ligand}]/[\text{diamagnetic sites}] \quad (1)$$

$$1/fT_{2d} = (1/T_{2(obsd)} - 1/T_{2(0)})[\text{ligand}]/[\text{diamagnetic sites}] \quad (2)$$

at equivalent, noninteracting sites, an assumption justified by the data. The site concentration was calculated assuming a protein molecular weight of 237,000 (Warner, 1958) and four active sites per molecule (Reuben and Cohn, 1970; Cottam and Mildvan, 1971).

Results and Discussion

Diamagnetic Effects of Pyruvate Kinase on the Relaxation Rates of the Methyl Protons of L- *and* D-*Phospholactate.* The proton nuclear magnetic resonance spectrum of the L- (and D-) phospholactate at 100 MHz has previously been described (Nowak and Mildvan, 1970). Only the methyl doublet (δ = 1.82 ppm downfield from tetramethylsilane; J = 7 Hz) was suitable for relaxation rate measurements (Figure 1). Metal-free pyruvate kinase broadens the methyl resonance of L-phospholactate due to an increase in the transverse relaxation rate ($1/T_2$) and increases the radiofrequency power required to saturate these resonances due to an increase in the longitudinal ($1/T_1$) and transverse relaxation rates of the

TABLE I: Effect of Pyruvate Kinase on the Relaxation Rates of the Magnetic Nuclei of Phosphoenolpyruvate Analogs.

Analog	Additions[a]	$sec^{-1} \times 10^{-3}$ $1/fT_{1d}$	$sec^{-1} \times 10^{-3}$ $1/fT_{2d}$	τ_c (sec $\times 10^9$)
L-Phospholactate[b]	PK[e]	0.65 ± 0.15	1.80 ± 0.20	1.7 ± 0.4
	PK + Na_3EDTA	0.36 ± 0.15	1.65 ± 0.20	2.6 ± 0.4
	PK + K^+	0.11 ± 0.03	0.87 ± 0.10	3.8 ± 0.1
	PK + K^+ + EDTA	0.11 ± 0.03	0.72 ± 0.10	3.4 ± 0.2
D-Phospholactate[b]	PK	0.23 ± 0.10	0.29 ± 0.07	≤0.5
Phosphoglycolate[c]	PK	0 ± 0.02	0.79 ± 0.10	≥11
		0.028 ± 0.003[d]	6.00 ± 1.0[d]	66 ± 6
	PK + K^+	0 ± 0.02	0.75 ± 0.10	≥10

[a] Conditions and concentrations are as described in Figure 1. K^+, when present, was added as KCl at a final concentration of 0.1 M. [b] The relaxation rates of the methyl protons in absence of enzyme are $1/T_1 = 1.05 \pm 0.08$ sec^{-1} and $1/T_2 = 2.93 \pm 0.10$ sec^{-1}. [c] The relaxation rates of the methylene protons in absence of enzyme are $1/T_1 = 1.05 \pm 0.10$ sec^{-1} and $1/T_2 = 3.52 \pm 0.38$ sec^{-1}. [d] Phosphorus nucleus studied at 40.5 MHz. The relaxation rates of this nucleus in absence of enzyme are $1/T_1 = 0.0205 \pm 0.0020$ sec^{-1} and $1/T_2 = 2.58 \pm 0.12$ sec^{-1}. [e] PK = pyruvate kinase.

methyl protons (Figure 1B). The observed transverse relaxation rate is directly proportional to enzyme concentration (Figure 2) indicative of an interaction of the ligand with the enzyme.

The diamagnetic effect of the enzyme on the relaxation rates of the methyl protons of L-phospholactate are decreased by the addition of P-enolpyruvate (Figure 1D). A titration of the effect of P-enolpyruvate on the diamagnetic line broadening in the absence of K^+ (Figure 3A) can be fit by assuming competition between P-enolpyruvate (K_s = 0.40 mM) and L-phospholactate (K_s = 1.0 mM). The K_s for P-enolpyruvate is similar to the value obtained independently, in the absence of activating monovalent and divalent cations, by ultraviolet (uv) difference spectroscopy (0.25 mM, Suelter *et al.*, 1966). The K_s value for L-phospholactate is in reasonable agreement with the dissociation constant estimated independently by analyses of titrations in which the proton relaxation rate of water was measured (3.5 ± 1.5 mM, Nowak and Mildvan, 1972).

In the presence of K^+ (Figure 3B) a titration of the L-phospholactate complex with P-enolpyruvate can also be fit by assuming competition between P-enolpyruvate (K_s = 0.15 mM) and L-phospholactate (K_s = 0.20 mM). The former value is in reasonable agreement with the dissociation constants determined by uv difference spectroscopy (0.08 mM) (Suelter *et al.*, 1966) and by kinetic analysis (0.05 mM) (Mildvan and Cohn, 1966).

The K_s of L-phospholactate is somewhat smaller than the value estimated by analysis of titrations in which the proton relaxation rate of water was measured (1.5 ± 0.9 mM, Nowak and Mildvan, 1972). However, the latter value is based on the concentration of minor components in an equilibrium which includes enzyme–Mn complexes. These competition studies thus suggest that the diamagnetic effects of the enzyme on the relaxation rates of the methyl protons of L-phospholactate take place at the P-enolpyruvate binding site of pyruvate kinase and represent an active-site phenomenon.

In sharp contrast with the marked diamagnetic effect on the methyl protons of L-phospholactate, a negligibly small diamagnetic effect is observed on the methyl protons of D-phospholactate (Figure 1F). Table I summarizes the diamagnetic effects of pyruvate kinase on the relaxation rates of the

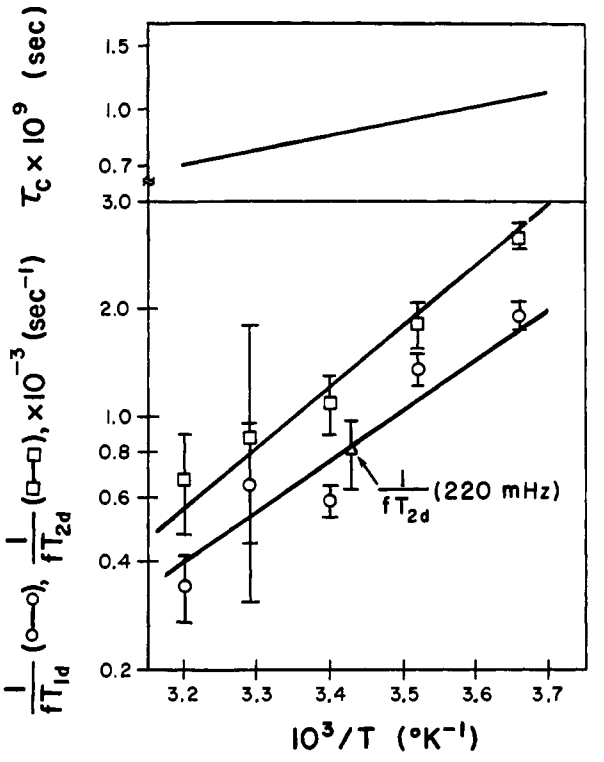

FIGURE 4: Arrhenius plot of the temperature dependence of the effect of pyruvate kinase on the $1/T_{1d}$ and $1/T_{2d}$ of the methyl group of L-phospholacetate. The reciprocal temperature dependences of the logarithm of the relaxation rates are fit by assuming that the correlation time (τ_c) of the methyl group decreases with increasing temperature with an activation energy (E_a) of 1.8 kcal/mole.

carbon-bound protons of the analogues. The enzyme produces significantly larger diamagnetic effects on the methyl protons of L-phospholactate than on those of D-phospholactate (Table I) (Figure 1). Thus the effect of pyruvate kinase on the methyl resonance of L-phospholactate is stereoselective. The weaker binding of the L isomer had previously been explained by steric interaction of its methyl group with a proton donat-

ing group on pyruvate kinase which protonated the substrate, P-enolpyruvate. Since the line broadening might be due to hindered rotation of the methyl group resulting from this steric interaction, a more detailed study of the diamagnetic effects of pyruvate kinase on the longitudinal ($1/fT_{1d}$) and transverse ($1/fT_{2d}$) relaxation rates of the methyl resonance of L-phospholactate was made.

Effect of EDTA and Activating Cations on the Diamagnetic Relaxation Rates of L-*Phospholactate.* As shown in Table I and Figure 1C, the relaxation rates are essentially unaffected by 10 mM EDTA which renders unlikely the possibility that they are due to trace paramagnetic metal contaminants. The monovalent activator significantly lowers the diamagnetic effects (Table I). In experiments not shown, 20 μM $MgCl_2$ prepared from spectroscopically pure MgO had no effect on $1/fT_{1d}$ and $1/fT_{2d}$ of L-phospholactate in the presence or absence of K^+, but 10 μM $MnCl_2$ increases both relaxation rates due to its paramagnetic effects as shown elsewhere (Nowak and Mildvan, 1972).

Effect of Temperature and Frequency on the Diamagnetic Relaxation Rates of L-*Phospholactate.* Figure 4 shows a negative temperature coefficient of $1/fT_{1d}$ and $1/fT_{2d}$ at 100 MHz and a decrease in $1/fT_{2d}$ upon changing the frequency of observation from 100 to 220 MHz, indicating that the observed line broadening is due to a change in the relaxation rate rather than to a change in the chemical shift of bound L-phospholactate. The negative temperature dependence is indicative of the rapid exchange of L-phospholactate into the diamagnetic environment of the enzyme, at a rate which is greater than $1/fT_{2d}$ ($>10^3$ sec^{-1}). From the relationship for the chemical shift mechanism with rapid exchange (Swift and Connick, 1962), we may write

$$\frac{1}{fT_{2d}} = \tau_B(\Delta\omega_B)^2 \tag{3}$$

where τ_B is the residence time and $\Delta\omega_B$ is the change in the chemical shift of the ligand in the diamagnetic environment of the enzyme. From eq 3 a 4.8-fold greater value of $1/fT_{2d}$ at 220 MHz than at 100 MHz would have been expected since $\Delta\omega_B$ is directly proportional to the frequency of observation. Because the opposite effect of frequency is observed (Figure 4), the shift mechanism is excluded. Hence, the dipolar relaxation mechanism is operative and $1/fT_{2d}$ and $1/fT_{1d}$ measure the relaxation rates of a bound molecule of L-phospholactate, for which the relevant equations for a three-spin system, derived in a manner similar to those previously derived for a two-spin system (Abragam, 1967; Solomon, 1955), are

$$\frac{1}{fT_{1d}} = \frac{2\gamma_I^4}{5}\frac{h^2}{\pi^2}\frac{I(I+1)}{r^6}\left(\frac{\tau_c}{1+\omega_I^2\tau_c^2} + \frac{8\tau_c}{1+4\omega_I^2\tau_c^2}\right) \tag{4}$$

$$\frac{1}{fT_{2d}} = \frac{3\gamma_I^4}{5}\frac{h^2}{\pi^2}\frac{I(I+1)}{r^6} \times \left(2\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{\tau_c}{1+4\omega_I^2\tau_c^2}\right) \tag{5}$$

In eq 4 and 5 γ_I is the gyromagnetic ratio of the proton, I is the nuclear spin quantum number (1/2 for protons), ω_I is the proton resonance frequency, r is the interproton distance, and τ_c is the correlation time for proton–proton dipolar interaction. From the ratio of $1/fT_{2d}$ at 100 and 220 MHz (1.5 ± 0.2) a correlation time, presumably the methyl rotation time[2] $\tau_c = 1.5 \pm 1.0 \times 10^{-9}$ sec, is calculated at 18° from eq 5. From the ratio of $1/fT_{1d}$ at 100 and 220 MHz (3.19 ± 0.24) a correlation time $\tau_c = 1.3 \pm 1.0 \times 10^{-9}$ sec is calculated from eq 4, in agreement with the value calculated from the T_2 data.

From the ratio of T_{1d}/T_{2d} at 100 MHz, a separate calculation of τ_c can be made from eq 6 which is derived from eq 4

$$\frac{T_{1d}}{T_{2d}} = \frac{8\omega_I^4\tau_c^4 + 23\omega_I^2\tau_c^2 + 6}{8\omega_I^2\tau_c^2 + 6} \tag{6}$$

and 5 above. Using eq 6 and the ratio $T_{1d}/T_{2d} = 1.62 \pm 0.18$ at 18° and at 100 MHz yields a value of $\tau_c = 0.9 \pm 0.2 \times 10^{-9}$ sec in agreement with the approximate values calculated from the frequency dependence. These values are reasonable time constants for a hindered methyl rotation. Hindered methyl rotation is the only motion in this system with a time constant of this order which could modulate dipolar proton–proton interactions and thereby serve as the correlation time.[2] Since the value of τ_c for unhindered rotation would be expected to be $<10^{-11}$ sec (Leffler and Grunwald, 1963), the binding of L-phospholactate to the enzyme has caused at least a two order of magnitude slowing of the rotation of its methyl group. Figure 4 gives the temperature dependences of $1/fT_{1d}$ and $1/fT_{2d}$ which are fit by assuming that the correlation time has an energy of activation of 1.8 kcal/mole, an enthalpy of activation $\Delta H^{\ddagger} = 1.2$ kcal/mole and an entropy barrier $-T\Delta S^{\ddagger} = 4.0$ kcal/mole. These kinetic parameters are typical for methyl rotation (Leffler and Grunwald, 1963). Since the correlation time is known, using eq 4, we may calculate r, the root-mean-sixth interproton distance,[3] to be 1.22 ± 0.12 Å. While this value is of the correct order of magnitude, it is significantly smaller than the root-mean sixth of the interproton distance in a methyl group (1.49 ± 0.08 Å) as calculated from known bond distances and angles (Pauling, 1960). This discrepancy could be due to the neglect of either hyperfine interaction, or intermolecular effects of the enzyme in the use of eq 4. The latter alternative is suggested by the results in the next section.

Effect of pH and Potassium on the Diamagnetic Relaxation Rates of L-*Phospholactate.* From the stereochemistry of the pyruvate kinase reaction, the methyl group of L-phospholactate interacts with the base which protonates P-enolpyruvate. Hence, protonation or deprotonation of this base might be expected to alter the rate of rotation of the methyl group of L-phospholactate and thereby to influence τ_c. No effects of pH on the relaxation rates of the methyl protons of L-phospholactate were observed from pH 6.2 to 10.2 as directly measured in D_2O with the glass electrode. Hence, no change in the T_{1d}/T_{2d} ratio beyond the overall experimental error of ±24% and of τ_c greater than ±40% occurred over this pH range. These results suggest that the pK of the base lies outside this range of pH values which would tend to exclude thiolate, phenolate, and α- and ϵ-amino groups. The role of α-amino groups is ruled out by the observation that the terminal

[2] Relaxation rates have previously been used to determine intramolecular rotation times (Brevard *et al.*, 1969; Brevard and Lehn, 1970; Anderson and Fryer, 1969). Hubbard (1970) has shown theoretically that methyl rotation would yield exponential relaxation behavior as has been assumed here.

[3] The term root-mean-sixth distance obtained from relaxation studies is actually the reciprocal of the sixth root of the average of $1/r^6$ values or $[\Sigma_i(1/nr_i^6)]^{-1/6}$.

amino groups of active pyruvate kinase are acylated (Cottam *et al.*, 1969). Although the carboxylate, imidazole, and guanidinium groups cannot be excluded by the pH study, the latter group seems unlikely because of the absence of an observed chemical shift of the methyl resonance of L-phospholactate. The maximum velocity of the overall reaction (Plowman and Krall, 1965) and the rate of the enolization of pyruvate (Rose, 1960) catalyzed by pyruvate kinase change markedly over this pH range. However, the rate controlling steps in these processes probably involve changes at the phosphoryl groups rather than at the proton-donating group (Rose, 1960; Robinson and Rose, 1972).

As indicated in Table I, the addition of the monovalent activator, K^+, decreases both $1/fT_{1d}$ and $1/fT_{2d}$ disproportionally; the T_{1d}/T_{2d} ratio yields a 2-fold greater value of τ_c, indicating that the methyl rotation may be more hindered in the active complex, presumably due to stronger interaction with the proton-donating group. The decreases in the relaxation rates despite the increase in τ_c observed in the presence of K^+ are probably due to the loss of intermolecular interactions with magnetic nuclei of the protein which do not hinder methyl rotation. In support of this view, a calculation of the root-mean-sixth interproton distance from the data obtained in the presence of K^+, using eq 4, yields a value of 1.50 Å in agreement with the correct value for a methyl group (1.49 ± 0.08 Å). Hence, in the presence of K^+ (but not in its absence) only an intramolecular dipolar process relaxes the methyl protons of L-phospholactate. We have suggested elsewhere that the monovalent activator forms a bridge between the enzyme and the carboxyl group of the analog reorienting the analog with respect to the enzyme (Nowak and Mildvan, 1972).

Thus it is concluded that pyruvate kinase elicits a stereoselective effect on the relaxation rates of the methyl resonance of L-phospholactate due to van der Waals interaction between the proton donating group on the enzyme and the methyl group of the analog (Figure 5), the rotation of which is slowed by more than an order of magnitude. This interaction is increased, and the methyl rotation further slowed by the monovalent cation activator. Other interactions between this methyl group and magnetic nuclei of the protein are eliminated by the monovalent activator.

Diamagnetic Effects of Pyruvate Kinase on the Relaxation Rates of Protons and Phosphorus of Other Analogs. Pyruvate kinase (179 μM sites) exerts a detectable diamagnetic effect on the transverse, but not the longitudinal, relaxation rate of the methylene protons of 76 mM phosphoglycolate (δ = 4.63 ppm downfield from tetramethylsilane; J = 5.0 Hz). From the limiting value of T_{1d}/T_{2d} (Table I) a limiting value of τ_c ($\geq 1.1 \times 10^{-8}$ sec) was calculated using the equations for a two-spin system (Solomon, 1955; Abragam, 1967). This value of τ_c suggests significantly hindered motion at the C_2 carbon atom of the analog such that the tumbling of the entire protein molecule ($\tau_r = 5.6 \times 10^{-8}$ sec as calculated from Stokes' law for rotational diffusion) may serve as the correlation time. The root-mean-sixth interproton distance for the methylene group of phosphoglycolate, calculated using the equations for a two-spin system (Solomon, 1955; Abragam, 1967) is ≥1.64 Å, which is in good agreement with the corresponding distance (1.59 ± 0.08 Å) calculated from known bond distances and angles (Pauling, 1960).

The ^{31}P nmr spectrum of phosphoglycolate at 40.5 MHz consists of a triplet (J = 5.0 Hz) which is 3.02 ppm downfield from the external standard 64% H_3PO_4 in 20% D_2O (Nowak and Mildvan, 1972). The diamagnetic effects of

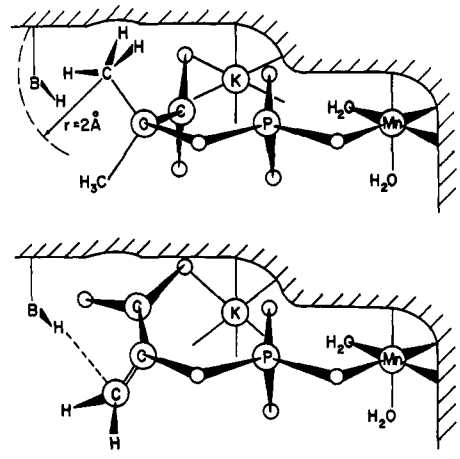

FIGURE 5: A composite model of the ternary analog complexes of pyruvate kinase (upper figure) is compared to the ternary complex with P-enolpyruvate (lower figure). The van der Waals radius of the methyl group (Pauling, 1960) of L-phospholactate is shown to interact with the group (BH) which protonates P-enolpyruvate. The composite model is based on magnetic resonance data (Nowak and Mildvan, 1972). The conformation of P-enolpyruvate is based on its crystal structure (Watson and Kennard, 1966, and personal communication), and on the stereochemistry of the pyruvate kinase reaction (Rose, 1970).

pyruvate kinase on the relaxation rates of phosphorus (Table I) yield a T_{1d}/T_{2d} ratio of 214 ± 43. From this ratio, using the equations for a two-spin system (Solomon, 1955; Abragam, 1967), a correlation time $\tau_c = 6.6 \pm 1.0 \times 10^{-8}$ sec is calculated. This value is in agreement with the calculated rotational time of the enzyme molecule which probably serves as the correlation time for this process. Thus the phosphate portion of enzyme-bound phosphoglycolate rotates with the tumbling time of the entire protein molecule, and may therefore be totally immobilized by the enzyme.

Very small diamagnetic effects of the enzyme on the methyl group of D-phospholactate are detected (Table I) yielding little evidence of hindered rotation of this group. An estimate the upper limit of $\tau_c \leq 5 \times 10^{-10}$ sec at 30° is obtained from the T_{1d}/T_{2d} ratio of 1.3 ± 0.5. Because of the experimental uncertainty in the T_{1d}/T_{2d} ratio, τ_c could be as low as 10^{-12} sec, a value consistent with unhindered rotation (Leffler and Grunwald, 1963).

A composite model of the three analogs bound at the active site of pyruvate kinase summarizing the correlation times estimated for the various groups is shown in Figure 6. The geometry of the composite model is based on kinetic and binding data, and on distances calculated from nuclear relaxation data on the Mn^{2+}-activated enzyme (Nowak and Mildvan, 1970, 1972). The composite model reveals more hindrance of rotation at the L-methyl position than at the D-methyl position and progressively greater loss of motion as one approaches the phosphate portion of the bound analog where all motion may be frozen. Thus motion is most hindered at the reaction centers where protonation and phosphoryl transfer are taking place. Such immobilization or freezing of bound substrates at the reaction center would permit enzymes to accelerate reactions by orientational or entropic effects. The present results thus provide experimental support for the feasibility of such hypotheses of enzyme catalysis (Bruice, 1970; Storm and Koshland, 1970; Reuben, 1971; Page and Jencks, 1971).

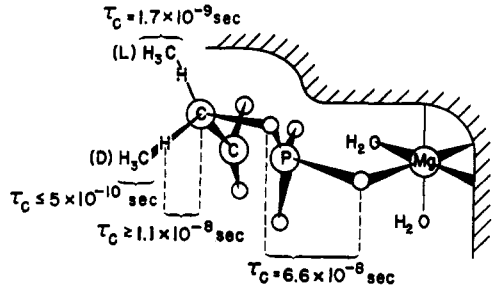

FIGURE 6: Composite model of the phosphoenolpyruvate analogs at the active site of pyruvate kinase, showing the correlation times. The correlation times (presumably the intramolecular rotation times at the indicated positions) were estimated from the T_{1d}/T_{2d} ratio of each nucleus measured. The rotation time of the entire pyruvate kinase molecule, calculated from Stokes' law, is 5.6×10^{-8} sec.

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