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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

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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.

intermediate in the normal phosphoryl transfer reaction of the enzyme.

Recent electron paramagnetic resonance (epr) studies of Mn(II)–pyruvate kinase complexes showed that in the ternary complex, enzyme–Mn–pyruvate, addition of the enolization cofactors, P_i and K^+, produced very minor changes in the electronic environment of the bound Mn(II) (Reed and Cohn, 1973). This observation indicated that either the environment of the Mn(II) was insensitive to enolization of the enzyme bound pyruvate or that the enolpyruvate complex represented only a minor species even in the presence of all the necessary cofactors. We have therefore sought a suitable analog of enolpyruvate in order to differentiate between these two possibilities and to better assess the possible changes in the active-site conformation upon enolization of the substrate, pyruvate.

Inhibition by the dianion of oxalic acid (pK_1 = 1.25, pK_2 = 4.21) of other enzymes which utilize pyruvate as a substrate (Schmitt *et al.*, 1966; Novoa *et al.*, 1959; Mildvan *et al.*, 1966), and the structural similarities of oxalate (I) and the enolate of pyruvate (II) have prompted an examination of the in-

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teraction of the oxalate anion with pyruvate kinase. The present paper reports the results of kinetic and magnetic resonance studies of the oxalate inhibition of the pyruvate kinase reaction.

Materials and Methods

Enzymes. Pyruvate kinase was isolated from rabbit muscle by the method of Tietz and Ochoa (1958) with a final ammonium sulfate fractionation at 55% saturation. The precipitate from the final fractionation was dissolved and stored in a 20 mM imidazole-HCl buffer (pH 7.0), containing 1 mM EDTA. The purified enzyme had a specific activity of ~250 μmol of product formed per minute per milligram of protein at pH 7.5 and 25°. Aliquots of the stock enzyme solution were dialyzed exhaustively against 50 mM Hepes[1] brought to pH 7.5 with either $(CH_3)_4NOH$ or KOH and made 75 mM in either $(CH_3)_4NCl$ or KCl. Lactate dehydrogenase was obtained from Boehringer-Mannheim.

Reagents. ADP and ATP were obtained from Sigma and Hepes from Calbiochem. Phosphoenolpyruvate (tricyclohexylammonium salt) was purchased from Sigma. Pyruvic acid (Eastman) was distilled under reduced pressure and neutralized with $(CH_3)_4NOH$ prior to use. Acrylic acid (sodium salt) was obtained from K&K Laboratories, and oxamic acid (sodium salt) from Aldrich Chemical Co.

Kinetic Assays. The pyruvate kinase reaction was followed spectrophotometrically using either the decrease in absorbance of P-enolpyruvate at 230 nm (Pon and Bondar, 1967) or the coupled assay involving lactate dehydrogenase (Bucher and Pfleiderer, 1955). Although oxalate has been reported as an inhibitor of lactate dehydrogenase (Novoa *et al.*, 1959), the inhibition constant is of the order of 100 μM at pH 7.5 (Novoa *et al.*, 1959). The highest level of oxalate used in these experiments was 30 μM, and no dependence of the initial velocity on the concentration of lactate dehydrogenase was observed at this level of oxalate. Higher precision was obtained with the coupled assay, and the results reported here were obtained by this method. The assays were carried out at 26 ± 0.4°, and the following conditions were used: total volume of 1 ml; 50 mM Hepes–KOH (pH 7.5)–75 mM KCl; 25 μg of lactate dehydrogenase; 200 μM NADH; 5.0 mM $MgCl_2$ or $MnCl_2$; with P-enolpyruvate as variable substrate 2.0 mM ADP was included; with ADP as variable substrate, 100 μM P-enolpyruvate was included.

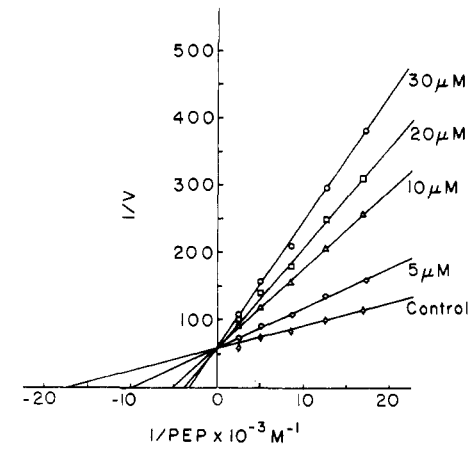

FIGURE 1: Double reciprocal plot of the influence of oxalate on the initial velocity of the Mn(II) activated pyruvate kinase reaction. Velocities are in units of micromoles of product formed per minute with 0.15 μg of protein. Other conditions are given under Materials and Methods; PEP, P-enolpyruvate.

Magnetic Resonance Measurements. Measurements of the longitudinal relaxation time, T_1, of water protons were made at 24.3 MHz using a pulsed nuclear magnetic resonance (nmr) spectrometer as described in previous papers (Reed and Cohn, 1973; Cohn and Leigh, 1962). PRR titrations were analyzed by curve fitting to the observation equation[2] for the enhancement as described by Reed *et al.* (1970). Epr spectra were recorded at ~9.1 GHz using a Varian E-3 spectrometer with a standard accessory for temperature control. Samples were contained in high purity quartz capillary tubing as described previously (Reed and Cohn, 1973).

Results

Inhibition Studies. Oxalate is a potent inhibitor of the pyruvate kinase reaction at micromolar concentrations. Results of initial velocity measurements in the presence of oxalate are shown in the form of a Lineweaver–Burk plot in Figure 1 where Mn(II) is the divalent cation activator. A similar pattern of inhibition is observed with Mg(II) ion as the activator. In both cases, the inhibition by oxalate is competitive with respect to P-enolpyruvate.[3] Replots of the slopes *vs.* oxlate concentra-

[2] The observation equation for the enhancement is $\epsilon^* = \Sigma_i([M-i]/[M]_T)\epsilon_i$ where ϵ_i is the enhancement for the ith species containing paramagnetic metal ion at a concentration [Mi] and $[M]_T$ is the total analytical concentration of metal ion. The equilibria which are considered are: K_1 = [M][S]/[MS]; K_D = [M][E]/[EM]; K_s = [E][S]/[ES]; K_3 = [EM][S]/[EMS]. Related equilibria are: K_2 = [E][MS]/[EMS] = K_3K_D/K_1; K_A' = [ES][M]/[EMS] = K_3K_D/K_S. Values for K_1 (Gelles and Hay, 1958), K_D, and ϵ_b (Reuben and Cohn, 1970) were taken from the literature. ϵ_a was measured for a solution containing 50 μM $MnCl_2$ and 1 mM oxalate. No value for K_s was available in the literature; however, a simultaneous fit of the data points for two different concentrations of enzyme led to a reasonably unique pair of constants K_s and K_3 (Price *et al.*, 1973).

[3] Oxalate also inhibits the proton exchange reaction catalyzed by pyruvate kinase. D_2O solutions containing pyruvate, P_i, $MgCl_2$, KCl, and enzyme show no decrease in the intensity of the methyl peak of pyruvate (measured by nmr) over a period of 24 hr when 2 mM oxalate is present. An ~70% decrease in the methyl peak is observed during this period for a similar sample without oxalate.

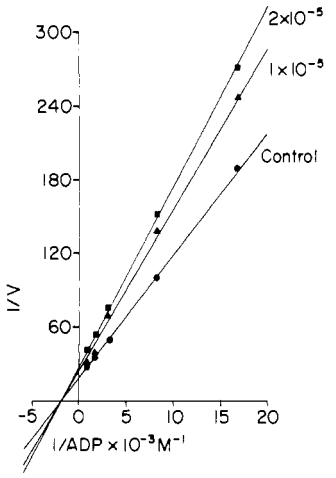

FIGURE 2: Double reciprocal plot of the influence of oxalate on the initial velocity of the Mg(II) activated pyruvate kinase reaction with ADP as the variable substrate. Velocities are in units of micromoles of product formed per minute with 0.33 μg of protein. Other conditions are given under Materials and Methods.

TABLE I: Inhibitor Constants and Dissociation Constants for Oxalate Interaction with Pyruvate Kinase.

	Mg^{2+}	Mn^{2+}
K_i(apparent)	6.5 ± 1.6 μM	6.0 ± 1.5 μM
K_M(P-enolpyruvate)	~50 μM	~58 μM
	K^+	$(CH_3)_4N^+$
$K_3 = \frac{[E-Mn][Ox]}{[E-Mn-Ox]}$	1.2 ± 0.4 μM	1.3 ± 0.5 μM
K_3(P-enolpyruvate)	1.5 μM[a]	25 μM[a]
	Mg^{2+}	Mn^{2+}
$K_1 = \frac{[M^{2+}][Ox]}{[MOx]}$	1.7 mM[b]	0.13 mM[c]

[a] Values from James *et al.* (1973). [b] Values from Schwarzenbach and Anderegg (1958). [c] Values from Gelles and Hay (1958).

tion are linear over a concentration range five times the apparent inhibition constant ($K_i \sim 6$ μM) for both the Mg(II) and Mn(II) activation of the reaction. The constants obtained from the initial velocity measurements are collected in Table I. Figure 2 shows the results of similar experiments with ADP as the variable substrate. Here the inhibition appears to be noncompetitive with respect to ADP.

The structural similarities of oxamate and of acrylate to oxalate prompted an investigation of their interactions with pyruvate kinase. However, no inhibition was observed with freshly prepared solutions of either oxamate or acrylate at concentrations as high as 5 mM.

PRR Titrations. The PRR of water was measured for solutions of Mn(II) pyruvate kinase at varying concentrations of oxalate. Binding of Mn(II) to pyruvate kinase produces an ~24-fold enhancement in the relaxation rate of water protons at 24.3 MHz and 25° (Reuben and Cohn, 1970). Addition of oxalate to solutions of enzyme and Mn(II) reduces the observed enhancement. To confirm that the change in enhancement is not due to the trivial competition between oxalate and the enzyme for Mn(II), the titration curves were fitted to the observation equation with a computer routine to solve the coupled binding equilibria (Reed *et al.*, 1970). The analysis shows that decreases in the observed enhancement with increasing concentrations of oxalate do not result from the simple competition between enzyme and oxalate for the Mn(II). Representative titration curves taken in the presence of $(CH_3)_4NCl$ or of KCl are shown in Figures 3A and 3B, respectively. The solid curves are the theoretical plots drawn with the constants listed in the legends. The analysis gives a value of 1.2 μM for the dissociation constant, K_3, of oxalate from the enzyme–Mn complex in the presence of KCl with the enhancement, ϵ_t, of 9. In the presence of $(CH_3)_4NCl$, K_3 is 1.3 μM with the same enhancement of 9 for the ternary complex.

Although the titration curves are not especially sensitive to the value of K_s used in the analysis, it was possible to arrive at a reasonable fit only with $K_s > K_3$. Since the ratio of the effective dissociation constant for Mn(II) from the enzyme in the presence and absence of oxalate is determined[2] by $K_3/K_s < 1$, titration curves indicate that there is a synergism in Mn(II) and oxalate binding to the enzyme. This synergism is also apparent if one monitors the concentration of free Mn(II) by epr

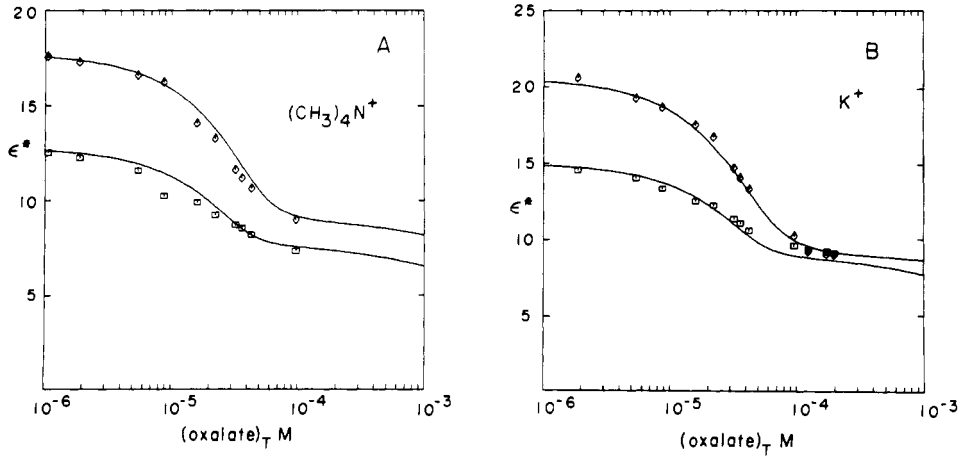

FIGURE 3: PRR titrations of Mn(II)–pyruvate kinase solutions with oxalate at 24.3 MHz and 25°. (A) Solutions contain 50 mM Hepes–$(CH_3)_4NOH$ (pH 7.5), 75 mM $(CH_3)_4N^+Cl^-$, 50 μM $MnCl_2$; (◇) data points for 81 μM enzyme sites; (□) data points for 50 μM enzyme sites. Solid curves were drawn with the following parameters: K_1 = 130 μM; K_s = 50 μM; K_D = 25 μM; K_3 = 1.3 μM; ϵ_a = 1.2; ϵ_b = 24; ϵ_t = 9. (B) Solutions contain 50 mM Hepes-KOH (pH 7.5), 75 mM KCl, 50 μM $MnCl_2$; (◇) data points for 200 μM enzyme sites; (□) data points for 90 μM enzyme sites. Solid curves were drawn with the following parameters: K_1 = 130 μM; K_s = 50 μM; K_D = 45 μM; K_3 = 1.2 μM; ϵ_a = 1.2; ϵ_b = 24; ϵ_t = 9.

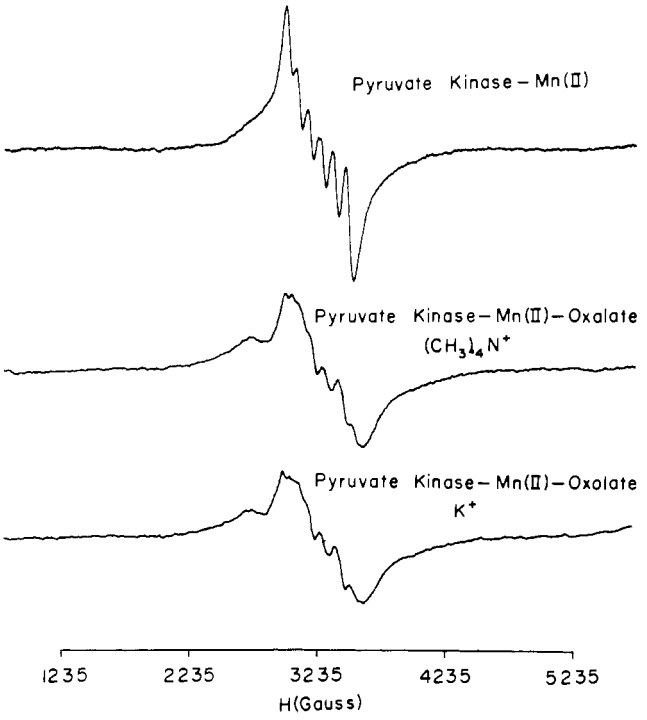

FIGURE 4: Epr spectra for Mn(II) pyruvate kinase complexes. All solutions contained 180 mg/ml of pyruvate kinase in 50 mM Hepes-$(CH_3)_4NOH$ (pH 7.5), 75 mM $(CH_3)_4NCl$, 1.1 mM $MnCl_2$. Additional components: 3 mM oxalate ($(CH_3)_4N^+$ salt) (middle spectrum), 40 mM KCl (bottom spectrum). Spectra were recorded at 5°.

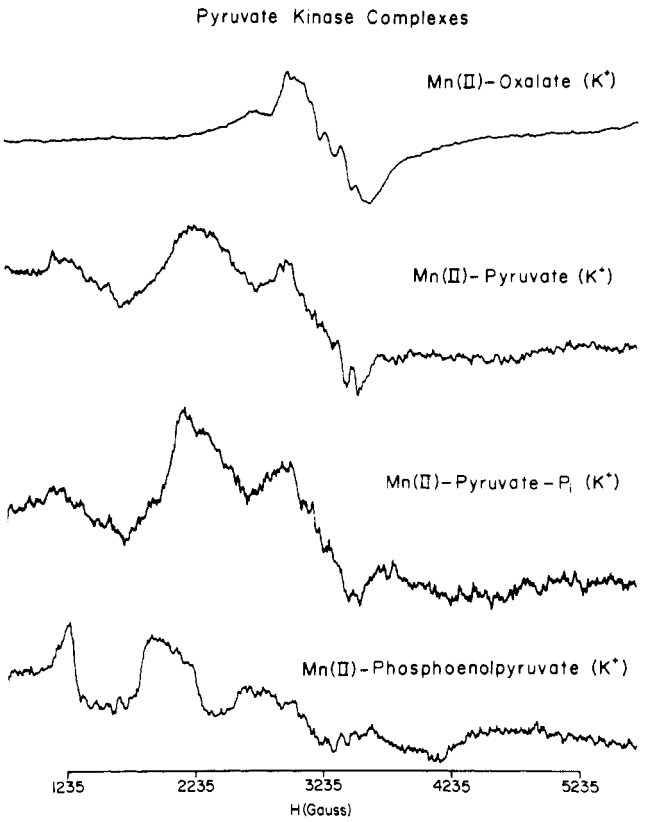

FIGURE 5: Comparison of epr spectra for ternary complexes of pyruvate kinase. All solutions contained 180 mg/ml of pyruvate kinase in 50 mM Hepes-KOH (pH 7.5), 75 mM KCl, and 1.1 mM $MnCl_2$. Additional components: 3 mM oxalate (K^+ salt), 20 mM PO_4^{3-} (K^+ salt at pH 7.5), 3 mM P-enolpyruvate (tricyclohexylammonium salt). Spectra were recorded at 5°.

in solutions containing both enzyme and oxalate. In this case the concentration of free Mn(II) is much lower than can be accounted for by binding of Mn(II) to enzyme and to oxalate individually.

Addition of ADP or of P_i to solutions containing enzyme, Mn(II), and oxalate (all components present in approximately stoichiometric amounts) does not alter the observed enhancement of the PRR. Thus, water relaxation measurements could not be used to detect the presence of higher complexes involving the nucleotide substrate ADP, or the enzymatic enolization cofactor, P_i.

Epr Spectra. Figure 4 shows epr spectra for the binary complex, enzyme–Mn, and for the ternary complex, enzyme–Mn–oxalate, in the presence of KCl or of $(CH_3)_4NCl$. Line shapes for the binary and ternary complexes differ appreciably. The spectrum for the ternary complex has a broad signal to the low-field side of the pattern for the binary complex. This low-field signal is part of the fine structure splitting which arises whenever there are asymmetries in the electronic environment of the bound Mn(II) (Reed and Ray, 1971). The change in the spectrum upon formation of the oxalate ternary complex can be ascribed either to a ligand substitution or to a geometrical distortion in the primary coordination sphere of Mn(II) which accompanies oxalate binding to the enzyme. It is also evident from the spectra in Figure 5 that the "K^+" and "$(CH_3)_4N^+$" forms of the oxalate ternary complex cannot be distinguished by epr. Thus, in the oxalate complex the electronic environment of the divalent cation, the dissociation constant for oxalate, and ϵ_t are not sensitive to the species of monovalent cation present in solution.

Spectra for ternary complexes of Mn–pyruvate kinase with pyruvate, P-enolpyruvate, and oxalate are compared in Figure 5. The oxalate ternary complex shows considerably less anisotropy than either of the other complexes. It is also noteworthy that the epr spectrum of the oxalate complex is unaffected by addition of the nucleotide substrates, ADP or ATP, by addition of ADP and nitrate, or by addition of an enzymatic enolization cofactor, P_i. Thus, the epr spectrum of the bound Mn(II) in the oxalate ternary complex does not respond to addition of compounds which may occupy the nucleotide or phosphate binding sites on the enzyme.

Discussion

The kinetic experiments presented here clearly show that oxalate is a potent inhibitor of rabbit muscle pyruvate kinase. The K_i for oxalate is lower than those obtained for various phosphorylated analogs of P-enolpyruvate (Nowak and Mildvan, 1970). The inhibition patterns indicate that oxalate binds at the P-enolpyruvate site on the enzyme and that there is a separate binding site for the nucleotide substrate, ADP. These observations are consistent with an equilibrium, random-order reaction mechanism which has been proposed for this enzyme (Ainsworth and Macfarlane, 1973; Kayne, 1973).

The high affinity of pyruvate kinase for oxalate is suggestive of a role for its structural analog, enolpyruvate, in the transition state of the phosphoryl transfer reaction of the enzyme (Wolfenden, 1972). However, the suggestion that the oxalate complex itself is an analog of the transition state of the enzyme must be tempered because other properties of the oxalate complex do not conform to expectations of a transition state analog. Thus, the epr and nmr parameters for the oxalate complex are not sensitive to the presence of the nucleotide substrate, ATP, or to the presence of the monovalent cation cofactor, K^+. Furthermore, the binding constant for oxalate does not change

upon substitution of the nonactivator, $(CH_3)_4N^+$, for K^+ in the solution. The transition state analog theory (Wolfenden, 1972) implies that factors which alter the enzymatic to nonenzymatic rate ratio should produce a parallel influence on the binding for an analog of the transition state, and this does not appear to hold true for the oxalate complex of pyruvate kinase. Nevertheless, the very potent interaction of oxalate with pyruvate kinase should not be overlooked in defining the structure of the transition state of this reaction.

Both Mg(II) and Mn(II) form complexes with oxalate (*cf.* Table I), and one possible mode of binding oxalate in the ternary complex would entail a "metal bridge" structure with oxalate bound to available coordination sites on the enzyme-bound metal ion. However, this possible mode of binding does not account for such a potent binding of oxalate. The apparent inhibitor constants for oxalate are tighter by factors of 260 and 20, respectively, than the dissociation constants for the binary Mg-oxalate and Mn-oxalate complexes (*cf.* Table I). Although the increased affinity could be attributed to differences in the local dielectric constant between free solution and the active site of the enzyme, such an explanation would not predict the virtual equivalence of the inhibitor constant for oxalate in the Mg(II) and Mn(II) complexes on the enzyme. Thus, a dielectric effect would be expected to influence the Mg(II) and Mn(II) constants by the same factor, and this is not the case. The possibility that protein ligands to the metal ion alter the relative affinities of Mn(II) and Mg(II) for oxalate cannot be refuted at this point. However, the value of K_s (*cf.* Table I) estimated from the titration curves argues for a substantial involvement of the protein in the binding of oxalate.

The agreement between the kinetic inhibitor constant for oxalate and the constant K_3 obtained from PRR titrations is only within a factor of ~five (*cf.* Table I). A similar discrepancy between K_i values and K_3 has been reported for analogs of P-enolpyruvate with pyruvate kinase (Nowak and Mildvan, 1970) where values for K_3 were consistently lower than the kinetically determined K_i. However, it is doubtful that any significance can be attached to this discrepancy which may originate from the accumulation of uncertainties in equilibrium constants required to analyze the PRR titration curves.

It is particularly significant that oxalate binds equally well to the enzyme with either the nonactivator, $(CH_3)_4N^+$, or the activator, K^+, present. This behavior is in marked contrast to the situation with binding of the substrate, P-enolpyruvate, where K^+ strengthens the binding constant by a factor of ~16 over that in the presence of $(CH_3)_4N^+$ (James *et al.*, 1973) and to the situation with pyruvate where a 3.5-fold increase in binding is reported when K^+ replaces $(CH_3)_4N^+$ (Nowak and Mildvan, 1972). Nowak and Mildvan have suggested that the monovalent cation, K^+, functions as a bridge between the carboxylate group of pyruvate and the enzyme (1972). ^{205}Tl nmr measurements of Reuben and Kayne (1971) and recent ^1H nmr experiments with $CH_3NH_3^+$ by Nowak (1973) indicate that the monovalent activator binds in close proximity to the divalent cation at the active site. The absence of a monovalent cation dependence in the binding of oxalate suggests either that the binding schemes for the carboxylate groups of oxalate and pyruvate differ or that the monovalent cation has a different role than to bridge the carboxylate group of the substrate. Nmr experiments with the oxalate complex of pyruvate kinase should help to resolve questions regarding the role of the monovalent cation at the active site.

Finally, oxamate and acrylate do not show an appreciable affinity for pyruvate kinase. The failure of these two compounds to interact with the enzyme probably reflects the enzyme's requirement for a highly polar or charged group at the position of the substrate molecule where phosphorylation occurs.

Acknowledgments

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