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Comparison of an Enzymatic Reaction and Its Model: Metal Ion Promoted Decarboxylation of Oxalacetate

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Decarboxylation rates of oxalacetate ($oxac^{2-}$) as catalyzed by pyruvate kinase (PK) in the presence of the activating metal ions Mg^{2+}, Mn^{2+}, and Zn^{2+} have been examined in detail, and quantitative comparisons have been made with rates obtained with these same metal ions in the absence of enzyme in aqueous and 50/50 H_2O/dioxane mixtures. The binding constants of $oxac^{2-}$ to the metalated enzymes lie close to those determined for the formation of the M(oxac) keto complexes in the semiaqueous solvent, suggesting that $oxac^{2-}$ is coordinated directly to the enzyme-bound divalent cation. In other important respects the enzyme and model systems differ. The enol/keto ratio of $oxac^{2-}$ bound to MnPK is considerably less than that determined for Mn(oxac) in 50% dioxane; and the rate constants for the decarboxylation of the ternary metal ion–PK–$oxac^{2-}$ complexes are higher than those of the models, showing the order $Mg^{2+} = Mn^{2+} > Zn^{2+}$, while the model rates are ordered $Mg^{2+} < Mn^{2+} < Zn^{2+}$. Other data are cited to show that the enzyme has an affinity for the α-keto group. It is concluded that the divalent metal ion aids the binding of $oxac^{2-}$ to the enzyme but other enzyme functional groups promote decarboxylation.

A long-standing goal of many chemists has been to understand how metal ions function in the reactions catalyzed by metalloenzymes. Owing to the complicated nature of enzymes, studies on simpler chemical "model" systems have been commonly made. While these models often do not closely resemble the enzymes, their study uncovers fundamental chemical behavior that is prerequisite to an understanding of the enzyme functions. One system, first described 40 years ago,[2] still holds particular interest: the metal ion catalyzed decarboxylation of oxalacetate (oxac).[3] Pronounced similarities found by Speck[4] between the rate–metal ion concentration profiles for the enzymatic (parsley root decarboxylase) and those for the nonenzymatic reactions suggested that the chemical system is a good model for the enzyme.

The reactions that give rise to rate maxima in the models are now well understood. Activation by divalent metal ion arises from the formation of 1:1 M(oxac) complexes,[5-11] and inhibition at higher metal ion concentrations is caused by the formation of dinuclear enolate complexes having the general formula $M_2(H_{-1}oxac)^+$.[10,11] It has been generally accepted that metal ion activation of the enzyme arises from the formation of an M(oxac) complex at the enzyme-active site similar to that formed in solution,[5] but the reactions giving rise to metal ion inhibition of the enzyme have remained unclarified. Also remaining unexplained is the high reactivity of the Mn-activated enzyme compared to lower activities shown by other metal ions and by the binary Mn(oxac) complex. To account for this Steinberger and Westheimer[5] have suggested that in the case of the enzyme, Mn^{3+} may be involved, but this hypothesis has never been tested. Observations[12-15] that the rates in the model systems increase dramatically in media of lower dielectric constant than H_2O have spurred further speculation that an important, if not the prime, function of the enzyme in this reaction is to surround the metal ion with a hydrophobic region.[15,16] Calculations employing predicted stabilities of the 1:1 and 2:1 metal ion–$oxac^{2-}$ complexes have shown[16] that simple electrostatic medium effects could quantitatively account for the differences between the enzymatic and nonenzymatic rates reported by Speck[4] if both the 1:1 and 2:1 metal ion–$oxac^{2-}$ complexes were formed at the enzyme-active site. In fact, it was shown that this medium effect operating solely on stabilities could account for the relatively high enzymic activity of Mn^{2+}.[16]

With the detailed study of the catalysis in semiaqueous media[17] together with the earlier studies, the model reactions are now well understood. Because many questions remain unanswered regarding the enzymatic reactions, we deemed it important to reexamine these latter systems. This goal was greatly facilitated by a recent report by Creighton and Rose[18] that codfish oxalacetate decarboxylase is really a well-known, readily available enzyme, pyruvate kinase (PK). In this paper we report on a detailed investigation of the decarboxylase activity of PK as influenced by Mg(II), Mn(II), and Zn(II), pH, and various inhibitors. A parallel study conducted in these laboratories on medium effects in the model reactions[17] provided important information that aided the interpretation of the enzymatic results. Mn(II) and Zn(II) were examined because they respectively induce high rates in the enzymatic and nonenzymatic reactions.[4] Mg(II) is important because it is the ion present in the native enzyme and yields maximum phosphotransferase activity.[19] Similar behavior between our observations with pyruvate kinase and those reported by Speck[4] suggests that parsley root decarboxylase may also be a pyruvate kinase.

Experimental Section

Materials. Pyruvate kinase from rabbit muscle (EC 2.7.1.40), phosphoenolpyruvate (PEP), ATP, and oxalacetic acid were purchased

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from Sigma Chemical Co., lactic acid dehydrogenase and 4-ethyloxalacetic acid were obtained from Nutritional Biochemicals Corp., and NADH and oxalic acid were obtained from Fisher Scientific Co. All other chemicals were reagent grade and were obtained from various sources. Concentrations of acids were determined by titration with standard base; NADH, ATP, and ADP were analyzed spectrophotometrically, PEP enzymatically, and $MgCl_2$ and $ZnCl_2$ stock solutions by titration with ethylenediaminetetraacetate. All stock solutions were made up with demineralized doubly distilled water and were kept in polyethylene bottles.

Enzyme Assay. Enzyme concentrations of buffered stock solutions were determined either by weight or from the absorbance of solutions at 280 nm by using a specific absorptivity of 0.54 mg^{-1} cm^{-2}.[20] Phosphotransferase activity was measured with a coupled assay system with lactate dehydrogenase. The rate of decrease of NADH absorbance at 340 nm was followed under the conditions 0.4 mM Mg^{2+}, 1.0 mM monocyclohexylammonium PEP, 2 mM ADP, 0.1 M KCl, 0.1 M imidazole buffer at pH 7.0, and 100 units of lactate dehydrogenase at 25 °C. Specific activities of the order of 200–220 units of PK were usually obtained [1 unit = 1 μmol of pyruvate min^{-1} (mg of protein)$^{-1}$], although activities as low as 100 units were occasionally observed. Creighton and Rose[18] cite specific activities of about 200 units for commercial preparations. Our low-activity preparations were usually contaminated by suspended denatured protein, which interfered with the absorbance measurements. The solid could be removed by centrifugation to yield solutions with high specific activities. Importantly, after this treatment the low-activity preparation showed decarboxylase rate behavior that agreed with high-activity solutions in regard to changes in solution compositions; i.e., the denatured material was inert and did not interfere with the reaction systems being investigated. Variations in activity from one series of experiments to another were normalized by correcting the observed decarboxylation rates to that observed with enzyme preparations having specific decarboxylase activities of 265 μmol of pyruvate/(mg min) in 3.1 mM $MnCl_2$, 0.3 mM oxac, 30 mM MES (pH 6.0), and 0.50 M KCl. Active-site concentrations of PK were calculated by assuming a molecular weight of 237 000 and four active sites/molecule.[20-23]

Kinetic Measurements. Pyruvate kinase requires both a monovalent cation, optimally 0.1 M K^+, and a divalent cation for activation of the phosphotransferase mode.[19-21] This dual metal ion dependence caused difficulties in maintaining a constant ionic strength in experiments over which the divalent cation was varied from 0 to 0.02 M. Rate determinations were therefore made at a constant but high KCl level of 0.50 M, with the aim of swamping out differences in electrostatic effects arising from changes in the concentration of the divalent metal salt. Since 0.030 M buffer was also present, the ionic strength varied from 0.53 to 0.59 over a series of runs. The buffers employed were acetate, 4-morpholineethanesulfonic acid (MES), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

Reactions were initiated by mixing solutions of divalent metal ion with or without enzyme with those of oxac. Both solutions contained the same amount of KCl and buffer and were thermally preequilibrated at 25 °C. After mixing, changes in oxac absorbance at 280 nm were monitored. The solution pH was determined with a Radiometer PHM26 pH meter after the completion of a run.

When an $oxac^{2-}$ solution is mixed with one containing a divalent metal ion, a biphasic absorbance change is observed.[8] A moderately fast (complete in about 20 s) absorbance increase results from the enolization of the $M(oxac)_{keto}$ complex, which is very rapidly formed.[10,11] A slow absorbance decrease (complete in about 600 s) results from decarboxylation to which is coupled the keto ⇌ enol equilibrium. The enol content of the solution decreases as the keto complex loses CO_2. The rate of the slow absorbance decrease has been found to be identical with the rate of CO_2 evolution.[10,11] The presence of pyruvate kinase has little influence on the faster rate but markedly increases the slower.

The wide separation in reaction rates permitted the two processes to be studied separately. A Durrum D-100 stopped-flow spectrophotometer was used to determine the faster absorbance changes and a Gilford 250 spectrophotometer was used for the slower. For the runs without enzyme, values of the pseudo-first-order rate constants for oxac disappearance were obtained by a least-squares fit of the acquired absorbance–time data points to the three-parameter equation

$$A_{obsd} = A_\infty + A_1 e^{-k_{obsd}t}$$

The enzymatic decarboxylation rates determined with initial concentrations of $oxac^{2-}$ at 3×10^{-4} M, which is well below the Michaelis constant, were found also to conform to simple first-order decay curves, and values of k_{obsd} were similarly calculated. The excellent adherence of the decay curves to first-order kinetics showed that the enzyme was stable to the completion of the decarboxylation reaction under our conditions. At higher concentrations of oxac, k_{obsd} was obtained from the initial rates of the absorbance change. Initial rates were also employed in the studies with Zn^{2+} at all $oxac^{2-}$ concentrations owing to the tendency of this metal ion (especially at higher concentrations) to slowly denature the enzyme. Values of k_{obsd} in these experiments were calculated by dividing the measured initial rate of disappearance of total oxac by the initial total oxac concentration.

Owing to the propensity of Mn(II) to be oxidized in solutions near neutral pH it has become routine in our laboratory when investigating Mn(II) reactions under these conditions to employ a N_2 atmosphere to perform spot checks of the rates. We found that the presence or absence of air had no effect on the rates, indicating that higher oxidation states of Mn are not important.

Table I. Equilibrium and Rate Constants for the Nonenzymatically Catalyzed Decarboxylation of Oxalacetate in Aqueous Medium[a]

reaction	log (equilibrium const) or rate const Mg(II)	Mn(II)	Zn(II)
$M^{2+} + oxac^{2-} \rightleftharpoons M(oxac)$	0.81	1.23,[b] 1.25[c]	1.99
$2M^{2+} + oxac^{2} \rightleftharpoons M_2(oxacH_{-1})^+ + H^+$	−6.4	−4.83,[b] −4.81[c]	−2.70
$M(oxac) \rightleftharpoons M(oxacH_{-1})^- + H^+$	−8.6	−7.9	
$M(oxac)_{keto} \rightleftharpoons M(oxac)_{enol}$	−0.5	−0.34	0.70
$M(oxac)_{keto} \rightarrow CO_2 + M(pyu)^+$	0.002 s^{-1}	0.006 s^{-1}	0.044 s^{-1}

[a] At 25 °C, 0.5 M KCl. [b] Kinetic value. [c] Spectrophotometric value.

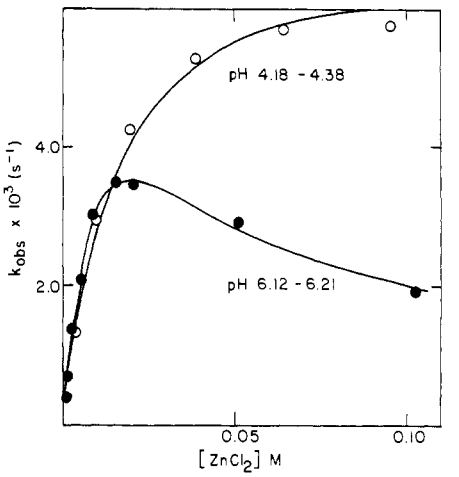

Figure 1. Rate constants for the Zn(II)-catalyzed decarboxylation of oxalacetate: (○) pH 4.18–4.38, acetate; (●) pH 6.12–6.21, MES (3.0×10^{-4} M $oxac^{2-}$, 0.030 M buffer, I = 0.55 (KCl)). The solid lines are theoretical.

Results

Nonenzymatic Decarboxylation. In order to determine the enzymatic rates of decarboxylation, it is absolutely necessary to know and correct for the bulk solution rates obtained under identical conditions. The rate parameters for the metal ions of interest here were redetermined for the 0.50 M KCl media. Excellent agreement with the reaction sequence found earlier

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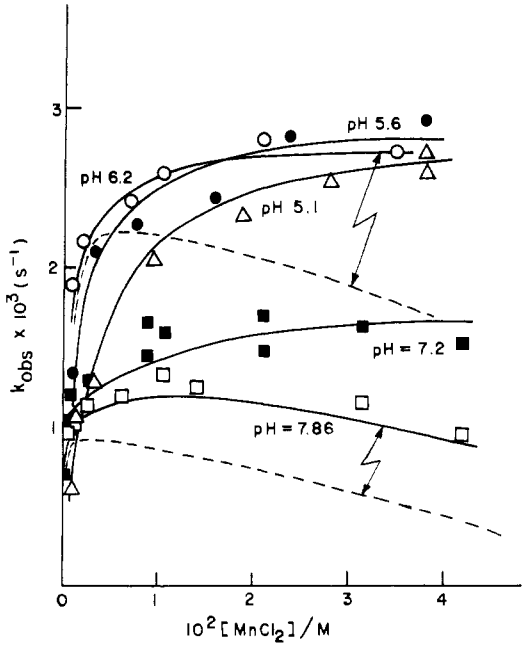

Figure 2. Influence of Mn(II) on the decarboxylation kinetics of $oxac^{2-}$ in the presence of pyruvate kinase. Pyruvate kinase = 2.4×10^{-6} M in active sites; $oxac^{2-} = 3.0 \times 10^{-4}$ M, except at pH 7.2, where it is 6.0×10^{-4} M; 0.030 M buffer; pH 5.1–6.2, MES; pH 7.2–7.86, HEPES; 0.50 M KCl. The solid lines are theoretical.

(see Scheme I of ref 17 was obtained, and the results are summarized in Table I.[24] Representative rate constants acquired for the Zn(II)-catalyzed decarboxylation are shown in Figure 1. The solid lines are theoretical, having been calculated by using the Table I values. Excellent agreement with the data points is seen to have been achieved.

In the case of Mn(II) catalysis, decarboxylation rates in aqueous media are sufficiently slow that it was also possible to determine the Mn(II)–$oxac^{2-}$ stability constant spectrophotometrically at 280 nm. Absorbance values were recorded 30–60 s after the metal ion and $oxac^{2-}$ solutions were mixed. After this time interval the same "preequilibrium" distribution of keto and enol species that prevails during slow decarboxylation had been attained. Excellent agreement between the kinetic and spectrophotometric evaluations of the stability constant was obtained, supporting the validity of the kinetic model (see Table I).

The true rate constants for the loss of CO_2 from the keto M(oxac) complexes were calculated by using the relationship[17]

$$k^{CO_2} = k_{obsd}(1 + K_{M(oxac)}{}^{enol})$$

where K^{enol} is the equilibrium ratio, $M(oxac)_{enol}/M(oxac)_{keto}$. The values of K^{enol} reported in Table I were obtained from UV absorbance measurements,[26,27] from ^1H NMR band areas,[28] or kinetically from the rate–concentration profiles of the tautomerization rates.[17]

The M(oxac) complexes have stabilities characteristic of those formed between divalent cations and dicarboxylate anions having 4–5 intervening carbon atoms between the carboxylate groups.[29] The usual order of stabilities is followed: $Mg^{2+} < Mn^{2+} < Zn^{2+}$. The 2:1 enolate complexes also follow this same order, but because two metal ions are involved, the increases in the stability constants in going from Mg^{2+} to Zn^{2+} are much greater than the increases in the stability constants for the mononuclear complexes. The low stability of $Mg_2(H_{-1}oxac)^+$ makes it possible to observe the formation of the 1:1 enolate, $Mg(H_{-1}oxac)^-$, whereas the high stability of $Zn_2(H_{-1}oxac)^+$ makes it difficult to detect the analogous Zn(II) complex. The pK_a of Mg(oxac) found here, 7.9 (I = 0.5), compares favorably with the titrimetric value, 7.75 (I = 0.1), reported by Tate and co-workers.[26]

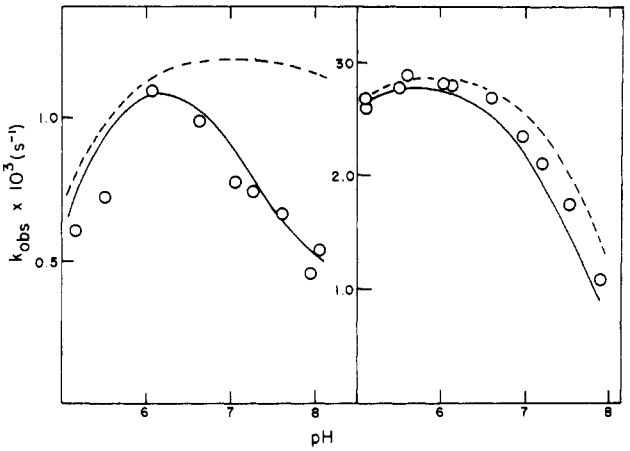

Figure 3. Influence of pH on the decarboxylation kinetics of $oxac^{2-}$ in the presence of Mn(II) pyruvate kinase. Left: 0.0032 M $MnCl_2$; 3.0×10^{-4} M $oxac^{2-}$; 0.030 M buffer, MES, or HEPES; 1.06×10^{-6} M pyruvate kinase active sites; 0.50 M KCl. Right: 0.032 M $MnCl_2$; 3.0×10^{-4} M $oxac^{2-}$; 0.030 M buffer, MES, or HEPES; 2.4×10^{-6} M pyruvate kinase active sites; 0.50 M KCl. The dashed lines indicate the behavior predicted from the best fit at low pH when Mn(PKH_{-1})oxac formation is ignored. The solid lines indicate the best fit obtained for all the data when Mn(PHK_{-1})oxac is included; see text.

Enzymatic Reaction Rates. The influence of the Mn(II) concentration and pH on values of k_{obsd} obtained at low $oxac^{2-}$ concentrations in the presence of PK is illustrated in Figure 2. The data points themselves have not been corrected for the contributions of nonenzymatic pathways, but these corrections have been made for the data at pH 6.20 and 7.86. The enzymatic components of k_{obsd} are indicated by the dashed lines. The pseudo-first-order enzymatic rate constants (hereafter termed k_{ENZ}) are obtained by subtracting the bulk solution rate from the k_{obsd}.

Qualitatively, the enzymatic rate profiles found here agree with those determined at the single pH of 5.0 by Speck:[4] activation in the presence of enzyme occurs at a lower metal ion concentration than in its absence, and the enzymatic rate–metal ion concentration profiles tend to exhibit maxima. Our results shown in Figures 2 and 3 for Mn^{2+} activation reveal that at a constant metal ion concentration enzymatic rates also exhibit pH maxima. In Figure 2 it is seen that the limbs obtained at low Mn(II) concentrations and below pH 6 progressively shift to the right with increasing acidity. This indicates that a proton competes with the activating Mn^{2+} ion for an enzyme donor group. Deactivation is also seen in Figures 2 and 3 to occur above pH 6. From the data analysis described below it was shown that this high pH inhibition arises from two sources: the complexation of $oxac^{2-}$ by metal ions in the bulk solution as the more stable enolate complexes are formed, and the ionization of protons from MnPK and MnPK(oxac) to give inactive species. Figure 4 shows the rate dependence of the initial velocities on the $oxac^{2-}$ concentration.

(24) A nonlinear least-squares curve-fitting program developed in our laboratories was employed in this task. The program is based on an earlier version[25] which was modified to fit equilibrium as well as rate constants.
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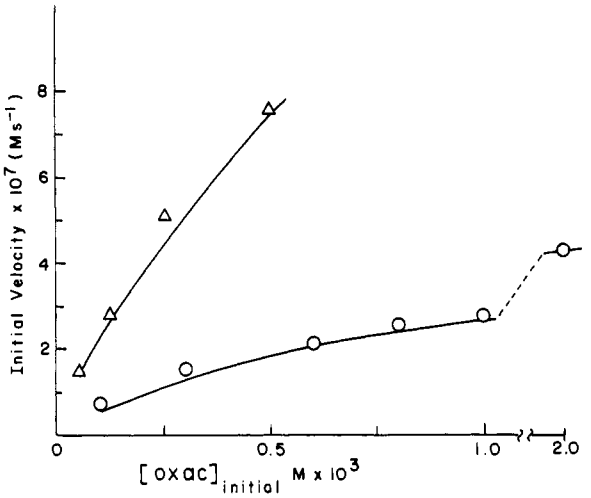

Figure 4. Influence of $oxac^{2-}$ on the initial velocities of oxac decarboxylation in the presence of Mn(II) pyruvate kinase: (○) 3.2 × 10^{-3} M $MnCl_2$, pH 7.8, HEPES, 1.1 × 10^{-6} M pyruvate kinase active sites; (△) 1.7 × 10^{-3} M $MnCl_2$, pH 6.2, MES, 0.50 M KCl, 2.4 × 10^{-6} M pyruvate kinase active sites. The solid lines are theoretical.

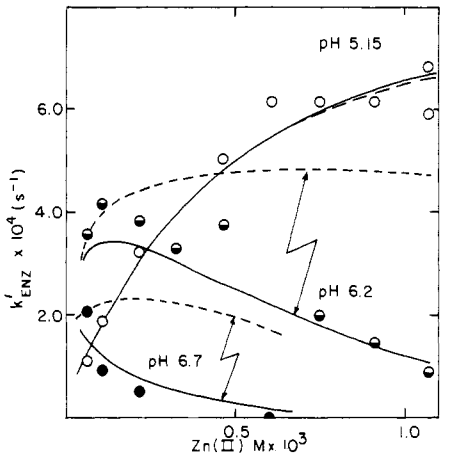

Figure 5. Influence of Zn(II) on the enzymic rates of oxac decarboxylation: 3.0 × 10^{-4} M $oxac^{2-}$; 2.4 × 10^{-6} M pyruvate kinase active sites; 0.030 M buffer, pH 5.15, acetate; pH 6.2 and 6.7, MES; 0.50 M KCl. The dashed lines indicate the behavior predicted from the low-pH results when $Zn_2H_{-2}PK$ is not included in the calculations. The solid lines are theoretical, with use of the full reaction model; see text.

The solid lines, which have been calculated with use of the rate constants and equilibrium constants reported here, are seen to describe the observed concentration dependence of these rates.

Similar sets of experiments were also run with Zn(II) and Mg(II) as the activating metal ions. Some of the Zn(II) rate constants are shown in Figure 5. A comparison of these values with those obtained for Mn(II) activation show that at a given pH, a lower Zn(II) concentration is required to activate the enzyme, but at the maxima higher rates are found with Mn(II). Zn(II) is able to effect enzyme activation at a lower pH than is required for Mn(II), but also deactivation, which occurs in alkaline regions, also sets in at a lower pH with Zn(II). The pH effects are very important and must be accurately defined before it is possible to make valid comparisons of the intrinsic reactivity of one metal ion with another. For example, the relatively slow rates observed by Speck[4] with Mg(II) may simply have resulted from the inability of this metal ion to displace a proton from the enzyme at pH 5 (see below).

Values of k_{obsd} as a function of Mg(II) concentration are shown in Figure 6. Owing to the relatively weak binding of

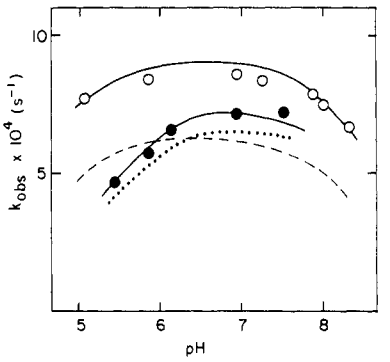

Figure 6. Influence of pH on the rates of decarboxylation of $oxac^{2-}$ in the presence of Mg(II) and pyruvate kinase: (○) 0.027 M $MgCl_2$; (●) 0.00435 M $MgCl_2$ (3.0 × 10^{-4} M $oxac^{2-}$, 4.2 × 10^{-6} M enzyme-active sites, 0.030 M buffer). The values of k'_{ENZ} (see text) fall along the dashed (0.027 M $MgCl_2$) and dotted (0.00435 M $MgCl_2$) lines. The solid line is theoretical.

this metal ion to the enzyme, a higher concentration is required to achieve a given level of activity than is necessary for Mn(II) and Zn(II). It is important to note that at Mg(II) concentrations sufficiently high to nearly saturate the enzyme, reaction rates similar to those attained by the other metal ions are observed. Maximum activity also exists over a broad pH range, and even at pH 8 the Mg(II) enzyme possesses significant activity.

Although the kinetic behavior of a ternary metal ion–$oxac^{2-}$–PK reaction system appears to be complicated, it was found possible to describe the enzyme rate behavior by using a relatively simple reaction model. The data suggest that proton competition for a metal ion is important at low pH, while proton loss from the metalated enzyme occurs at high pH. On this basis a preliminary rate model was devised and tested to see how well it could accommodate the observations. The reactions assumed are shown in eq 1–5.

$$HPK \underset{K_{1a}}{\rightleftharpoons} H^+ + PK \quad (1)$$

$$M^{2+} + PK \underset{\beta_{MPK}}{\rightleftharpoons} MPK \quad (2)$$

$$MPK \underset{K_{2a}}{\rightleftharpoons} MPKH_{-1} + H^+ \quad (3)$$

$$MPK + oxac^{2-} \underset{K_{MPK(oxac)}}{\rightleftharpoons} MPK(oxac) \quad (4)$$

$$MPK(oxac) \xrightarrow[k_{MPK(oxac)}]{} CO_2 + \text{other products} \quad (5)$$

Fortunately, it was possible to simplify the nonlinear curve-fitting calculations because some of these reactions had been studied earlier and values of the equilibrium constants reported. Reuben and Cohn[22] using EPR found that the average binding constant of Mn^{2+} to each of the four subunits of the apoenzyme is $10^{4.19}$ M^{-1} (0.1 M KCl, 25 °C). Mildvan and Cohn[30] studied the pH dependence of the Mn^{2+} binding using NMR and found evidence for two proton-ionization processes corresponding to pK_a values of 6.8 and 7.8. Competition between Mn^{2+} and Mg^{2+} was used to obtain the Mg^{2+} binding constant to PK.[30]

To fully account for the observed behavior it was also found necessary to incorporate into the reaction scheme the interactions and rate processes that occur between the metal ions and $oxac^{2-}$ in the bulk of the solution. These reactions are defined in detail in Table I.

A test of the model was first performed on the Mn(II) data. The literature[22,30] values of the constants defined by eq 2 and 3 were assumed, and the values of constants of eq 1, 4, and

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Table II. Equilibrium and Rate Constants for the Pyruvate Kinase (PK) Catalyzed Decarboxylation of Oxalacetate and a Comparison with Values for $M(oxac)_{keto}$ in 50% Dioxane

reactn	log (equilibrium const) or rate const Mg(II)	Mn(II)	Zn(II)
MPK + $oxac^{2-}$ ⇌ MPK(oxac)	2.4	3.3	3.9
M^{2+} + $oxac^{2-}_{keto}$ ⇌ $M(oxac)_{keto}$ [a]	2.3	2.4	3.0
M^{2+} + PK ⇌ MPK	3.42 [b]	4.16 [c]	4.32
H^+ + PK ⇌ HPK	6.3	7.0, 6.8 [b]	6.5
MPK ⇌ $MPKH_{-1}$ + H^+	−8.8	−7.8 [b]	−6.1
MPK(oxac) ⇌ MPK($oxacH_{-1}$) + H^+	−8.1	−7.1	−6.2
$2M^{2+}$ + PK ⇌ M_2PKH_{-2} + $2H^+$			−3.9
MPK(oxac) → CO_2 + ...	0.82 s^{-1}	0.88 s^{-1}	0.18 s^{-1}
$M(oxac)_{keto}$ → CO_2 + ... [a]	0.014 s^{-1}	0.069 s^{-1}	0.22 s^{-1}

[a] 50/50 (v/v) dioxane/water, ref 17. [b] Reference 30. [c] Reference 22.

5 were obtained by least-squares curve fitting.[24] It was found that for the low-pH data this model described the Mn(II) dependence of the rates very well, but it failed at high pH. This behavior can be seen in Figure 3 where the theoretical rate curves calculated for the preliminary "best fit" are indicated by the dashed lines.

To obtain a satisfactory fit to all of the Mn(II) data it was found necessary to incorporate only one more reaction into the scheme: the deprotonation of the ternary enzyme complex to give an inactive form.

$$MPK(oxac) \underset{K_{3a}}{\rightleftharpoons} MPK(oxacH_{-1}) + H^+ \qquad (6)$$

The best values for the equilibrium constants and decarboxylation rate constant for Mn(II) pyruvate kinase calculated with use of the final model are given in Table II. The solid lines drawn in Figures 2–4 are the theoretical curves predicted by these values, and it is seen that a very good fit to all of the data has been obtained. Strong support for the validity of this proposed scheme lies in the very good agreement between the value for the pK_a of HPK obtained by fitting our kinetic data (7.0) and that reported by Mildvan and Cohn (6.8).[30]

The data for Mg^{2+} activation of pyruvate kinase were also analyzed according to the extended reaction model and by using the literature value of $10^{3.42}$ M^{-1} for the binding of Mg^{2+} to the enzyme.[30] The results are also listed in Table II. The solid lines drawn in Figure 6 are theoretical, and once again an excellent fit to the data points is seen to have been achieved.

Preliminary calculations using our data obtained for Zn^{2+} activation suggested that the values previously reported[23,31] for the binding of Zn^{2+} to a PK subunit are too high, owing to the neglect of both the ionization of ZnPK and the binding of a second Zn^{2+} ion to each subunit under slightly alkaline conditions (see below). To obtain an independent value of the binding constant of Zn^{2+} to pyruvate kinase we performed spectrophotometric titrations at 295 nm of solutions of PK that were about 2.5×10^{-5} M in active-site concentrations. Two sets of titrations, one buffered at pH 5.1 and the other at pH 5.7 ($I = 0.55$) were made. The final Zn(II) concentrations attained a level of 3.5×10^{-4} M. A value of $10^{4.32}$ M^{-1} for the binding of Zn^{2+} to PK was found to be highly consistent with the decarboxylation rate data. To account for the rates determined above pH 6, it was found necessary to add another deprotonated species, Zn_2PKH_{-2}, to the reaction model. Under conditions where this species becomes appreciable, the enzyme slowly denatures.

It becomes clear from an inspection of Table I that after all the various factors are sorted from the observed rates, the behavior of Mn^{2+} is consistent with that of the other metal ions investigated here. Its enzymatic association constants follow the normal order of complex stabilities and lie between those of Mg^{2+} and Zn^{2+} and the rate constant for the decarboxylation of its enzyme complex lies in the range of those obtained with Mg^{2+} and Zn^{2+}. Clearly, there is no need to invoke Mn^{3+} as the catalytically active species.[5] The absence of any effect from exclusion of air from the reaction medium supports this conclusion.

Table III. Binding and Ionization Constants for 4-Ethyloxalacetate (25 °C, 0.5 M KCl)

reactn	log K
4-Et-$oxac^-$ ⇌ 4-Et-$oxacH_{-1}^{2-}$ + H^+	−9.4
4-Et-$oxac^-$ + Mg^{2+} ⇌ Mg(4-Et-oxac)$^+$	1.06
4-Et-$oxac^-$ + Mn^{2+} ⇌ Mn(4-Et-oxac)$^+$	1.12
4-Et-$oxacH_{-1}^{2-}$ + Mg^{2+} ⇌ Mg(4-Et-$oxacH_{-1}$)	3.7
4-Et-$oxacH_{-1}^{2-}$ + Mn^{2+} ⇌ Mn(4-Et-$oxacH_{-1}$)	4.5
4-Et-$oxac^-$ + MgPK ⇌ MgPK(4-Et-oxac)	3.16
4-Et-$oxac^-$ + MnPK ⇌ MnPK(4-Et-oxac)	3.80

Enol Content of MnPK(oxac). The values of the decarboxylation rate constants, which were obtained as described in the preceding section, have not been corrected for the distribution of the enzyme-bound oxac between keto and enol forms. While we have not been able to obtain the value of the enzymic enol/keto ratio, we have been able to assign an upper limit to it for the Mn enzyme. An experiment was designed in which the concentration of PK was sufficiently high to cause the decarboxylation of oxac to proceed at the same rate as that shown for oxac enolization in the bulk of the solution. The rate of pyruvate formation was followed in a stopped-flow apparatus using a coupled assay employing NADH and a large excess of lactic acid dehydrogenase. Under these conditions the reduction of pyruvate to lactate is fast relative to pyruvate generation. Only a single-exponential decay curve was observed for the disappearance of NADH. Numerical integrations employing the Gear algorithm[32] were made on a mathematical rate model in which it was assumed that both keto and enol forms of oxac were bound to the enzyme. Successive calculations using various values for the enzymic K^{enol} showed that a biphasic curve by pyruvate formation would be observed if K^{enol} were about 1 or greater. The absence of this behavior indicates that the enzymic $K^{enol} < 1$.

Inhibition. 4-Ethyloxalacetate (4-Et-$oxac^-$) is a potent competitive inhibitor of both the phosphotransferase and oxac decarboxylase functions of PK. About the same inhibition constant is found for both reactions.[18] We have examined the pH dependence of this inhibition to determine to what extent the enolate form of the ligand accounts for the strong binding.

The proton ionization and complexing of 4-Et-$oxac^-$ and its enolate by Mn^{2+} and Mg^{2+} were studied, and the results are given in Table III. As expected, the pK_a of 4-Et-$oxac^-$ (9.4) is considerably lower than that of $oxac^{2-}$ (13).[26] Mg^{2+} and Mn^{2+} react with both 4-Et-$oxac^-$ and the enolate, 4-Et-$oxacH_{-1}^{2-}$. Because the stabilities of the enolate complexes are much higher than those of the monoanion, the pK_a values of the M(II)–4-Et-$oxac^-$ complexes lie below 7. No polynuclear complexes (as are easily formed with $oxac^{2-}$ [10,11]) were found, because one of the necessary coordinating groups of the ligand is blocked by esterification.

Inhibition studies were performed in buffers at pH 5, 6, and 7 in the presence of Mg^{2+} or Mn^{2+}. Over this pH range large changes in the solution concentrations of the enolate complexes occur. Analysis of the data revealed that even under conditions

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where the enolate complexes comprise a high percentage of the total oxac, it is only 4-Et-oxac$^-$ that is bound to the metalated enzymes. The binding constants are seen in Table II to be greater for the enzymes than for the metal ions in the bulk of the solution and to vary with the enzyme-bound metal ion in such a way as to suggest that 4-Et-oxac$^-$ is coordinated to the metal ion. The drastic inversion of the stabilities of the 4-Et-oxac$^-$ and 4-Et-oxacH$_{-1}^{2-}$ complexes in the enzyme relative to solution cannot be explained on the basis of a simple solvent model for the enzyme-active site. Clearly, other enzyme functions must be present at the active site that are able to discriminate between 4-Et-oxac$^-$ and its enolate.

Further inhibition studies showed that acetate, tartrate, and glutarate are poor inhibitors of the decarboxylation reaction, but pyruvate and α-ketoglutarate are strong inhibitors. The α-keto group is obviously a preferred feature for a substrate. Oxalate, which is a strong inhibitor of the phosphoryl-transfer function,[35] is also a strong inhibitor of decarboxylation. The constants for oxalate binding were found here from decarboxylase inhibition to be $10^{5.14}$ M^{-1} for MgPK and $10^{5.96}$ M^{-1} for MnPK. The good agreement of these values with those found earlier[35] provides additional support to the contention of Creighton and Rose[18] that phosphoryl transfer and oxac^{2-} decarboxylation occur at the same or overlapping sites in the enzyme.

Discussion

The concentration–rate profiles found here for the decarboxylase mode of pyruvate kinase activated by divalent metal ions are very similar to those reported by Speck[4] for parsley root decarboxylase. The respective maxima obtained with Zn^{2+} and Mn^{2+} lie at about the same concentrations for both enzymes and the specific activities at the maxima are also about the same. With each enzyme higher concentrations of Mg^{2+} than Mn^{2+} or Zn^{2+} are required to initiate activation. Certainly, with respect to decarboxylation parsley root decarboxylase strongly resembles pyruvate kinase.

Although the pyruvate kinase catalyzed reactions show rather complicated behavior with respect to changes in pH and metal ion concentration, some of the reactions that influence our results have already been characterized in other studies.[22,23,30,31] After inclusion of these in the reaction model, it was gratifying to find that only a relatively small number of additional reactions involving the enzyme and oxac^{2-} needed to be invoked to account for the enzyme-catalyzed decarboxylation rates.

At the lower end of the pH range, at which activation by divalent metal ions is observed, a proton competes with the metal ion for an enzyme site. In our curve-fitting procedures[24] pK_{1a} for this proton was treated as an unknown for each metal ion and the "best" value that fits a particular data set was obtained. In Table II it is seen that the resulting values are roughly independent of the metal ion, as is required by the model. The average of 6.6 is in excellent agreement with the value of 6.8 determined by Mildvan and Cohn[30] from NMR measurements on MnPK. Because the value obtained for pK_{1a} is sensitive to the stability constant employed for the M(II)–PK complex, the differences shown in Table II cannot safely be considered to arise from any specific phenomena since the literature values for Mg^{2+} and Mn^{2+} binding to PK were determined[22,30] under slightly different conditions than employed here.

Proton ionization of the metalated enzyme is also observed at higher pH. The pK_{2a} values in Table II are seen to be highly dependent on the divalent metal ion, indicating that the conjugate base is coordinated after the proton is lost. The source of the proton could be either one of the enzyme functional groups near the metal ion or an H_2O molecule that is already bound to it. When oxac^{2-} is also bound to the enzyme, the second proton ionization tends to shift to slightly lower pH values. It is not clear whether proton abstraction occurs from the same group that ionizes in the absence of oxac^{2-} or whether oxac^{2-} itself is converted to an enolate, as is observed in the models. This latter possibility, however, is not consistent with the observation that 4-Et-oxac$^-$ shows no tendency to form an enolate when bound to the Mg^{2+} or Mn^{2+} enzymes. In either case the deprotonated oxac–enzyme complex is inactive.

The equilibrium constants found for the binding of oxac^{2-} to the metalated enzymes, MPK + oxac^{2-} $\rightleftharpoons$ MPK(oxac), show particularly revealing behavior (Table II). The values are not only highly dependent on the metal ion and increase with increasing coordinating ability of the metal ion, but they are only slightly higher than the formation constants determined for the respective M(II)–oxac^{2-} complexes in 50% dioxane.[17] If the function of the enzyme is to serve as a milieu of low dielectric constant, the active-site region appears from this to behave as a solvent that is slightly less polar than 50% dioxane.

The close parallel in oxac$^{2-}_{keto}$ binding constants between the enzyme and models suggests that the oxac^{2-} is bound to the enzyme in the same way as it is in the models, i.e., within the primary coordination sphere of the divalent metal ion. This conclusion differs somewhat from that drawn from the results of NMR studies on the MnPK complexes with pyruvate. In the presence of bound phosphate, pyruvate has been found to lie outside the primary coordination sphere of the Mn^{2+} ion,[36] and in the presence of ATP, pyruvate is located in the vicinity of the K^+ ion while ATP is found in the secondary coordination sphere of the Mn^{2+} [37–39] ion. Both of these cases represent a more complicated situation than exists here where oxac^{2-} is the sole ligand. In the NMR studies a stronger complexing agent than the keto acid was also present at the active site. It has also been suggested that ATP is bound as a complex involving a second divalent metal ion.[37–39] Competition by the second metal ion would certainly account for the failure of ATP to coordinate directly to the enzyme-bound Mn^{2+} ion. Thus, the present conclusions regarding the binding of oxac^{2-} are not in conflict with those reported earlier.

In several important respects, the enzyme behavior cannot be explained simply in terms of a low dielectric constant solvent. For one, in the model systems inhibition was found to arise from the formation of 2:1 metal ion–enolate complexes. The formation constants for these species are higher in semiaqueous media than in water, and their formation at the enzyme-active site could quite easily account for the inhibition observed at high metal ion concentrations in the enzymic rates.[16] However, the evidence obtained in this study shows that the enzyme has little tendency to bind the enolate, and the decrease in enzymic rates at high metal ion concentrations actually arises from competitive bulk solution complexation of oxac^{2-}, e.g., MPK(oxac) + M^{2+} $\rightleftharpoons$ MPK + M(oxac), etc. At a relatively low pH, as employed by Speck,[4] this inhibition mode occurs at lower concentrations of Zn^{2+} than of Mn^{2+} owing to the higher bulk solution stability of Zn(oxac).

In two other important respects the enzymic decarboxylation rates do not follow their model counterparts: (i) the decarboxylation rate constants in the models are ordered Mg^{2+} < Mn^{2+} < Zn^{2+}, which is the order of the metal ion complexing ability, while in the metalated enzymes the order is Mg^{2+} =

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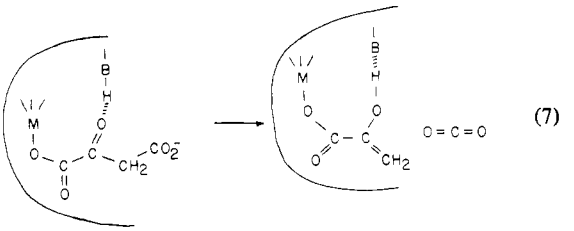
$Mn^{2+} > Zn^{2+}$; (ii) the rate constants for the Mg^{2+} and Mn^{2+} enzymes have much higher values than can be explained in terms of known effects observed in the model reactions.[41] It appears that although the $oxac^{2-}$ is bound to the divalent metal ion in the enzyme, other enzyme functional groups assist in the CO_2-loss mechanism.

In contrast, Zn(II) is a poor activator of the catalytic mechanism. The low activity of Zn(II) pyruvate kinase is not unique to oxac decarboxylation since this form of the enzyme also shows low activity in the phosphoryl transfer function.[40] Zn(II) appears to be too strong a Lewis acid for the enzyme and possibly binds additional groups that need to be free to achieve maximum activity.

While it is not at this time possible to say with certainty in what way the enzyme might promote decarboxylation, a likely mode is through proton donation. It should be noted that a proton is required as pyruvate kinase catalyzes the transfer of a phosphoryl group from phosphoenol pyruvate. The same proton donor might also be involved in promoting oxac decarboxylation, and it is significant that enol pyruvate is formed in both reactions. A possible mechanism might involve interactions and reactions such as (7).



(7)

According to this picture the metal ion chelate of pyruvate enolate is replaced by one involving the metal ion and another enzyme group. The advantage gained by the enzyme is a favorable prereaction geometry and the formation of a strong O-H bond, which maintains the chelate structure.

Some evidence that proton donation can indeed assist metal ion catalyzed decarboxylation has been obtained in studies on the models in 50% dioxane. There the pathway Mg(oxac) + HOAc $\rightleftharpoons$ CO_2 + $Mg(pyu)^+$ + Oac^- was found.[17] These studies are being pursued to uncover more details of how coordinated oxac may be activated by other components of a reaction mixture.

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Registry No. Oxalacetate, 328-42-7; Mg, 7439-95-4; Mn, 7439-96-5; Zn, 7440-66-6; PK, 9001-59-6.

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Intra- and Intermolecular Interactions in $[Ni_2(ND_2C_2H_4ND_2)_4Br_2]Br_2$. A Study by Inelastic Neutron Scattering and Magnetic Measurements

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$[Ni_2(ND_2C_2H_4ND_2)_4Br_2]Br_2$ was studied by inelastic neutron scattering (INS) and magnetic measurements. Intramolecular energy splitting due to exchange interactions and magnetic anisotropy were directly determined by INS. The exchange coupling is ferromagnetic with $J = 3.55 \pm 0.20$ cm^{-1}; the anisotropy is axial with $D = -6.8 \pm 0.8$ cm^{-1} and $E = 0 \pm 0.8$ cm^{-1}. Information on the intermolecular coupling was obtained from the low-temperature magnetization. The coupling is antiferromagnetic with $J' = -0.25 \pm 0.05$ cm^{-1}.

1. Introduction

Complexes of the type $[Ni_2(en)_4X_2]^{2+}$, where X = Cl^-, Br^-, or SCN^- (Figure 1), are well-known dimers with ferromagnetic intramolecular coupling.[2a] The ferromagnetic nature of the exchange coupling was deduced from the magnetic susceptibility. A number of linear chain compounds with very similar $Ni(\mu\text{-}X)_2Ni$ bridging geometries also exhibit ferromagnetic behavior.[2b] The principal difficulty in properly describing the electronic ground-state properties of a compound like $[Ni_2(en)_4Br_2]Br_2$ arises from the single-ion anisotropy energies having the same order of magnitude, that is, a few wavenumbers, as the exchange interaction energies. The two effects compete, and as a result, the energy splitting pattern may not be simple. Furthermore, even though the shortest intermolecular Ni-Ni distance is 7.8 Å in the bromide,[3] interdimer interactions cannot a priori be excluded due to the ferromagnetic intradimer interaction. They are usually incorporated in the effective Hamiltonian by a molecular field term.[2a] In the least-squares procedures used to extract parameters from the magnetic susceptibility data, the molecular field parameter J' is highly correlated with the intramolecular parameters J

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