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RATE ENHANCEMENT OF PYRUVATE ALDOLIZATION BY DIVALENT CATIONS: A MODEL FOR CLASS II ALDOLASES

ANTHONY A. GALLO AND HENRY Z. SABLE*

Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106 (U.S.A.)

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SUMMARY

Zn^{2+} catalysis of pyruvate aldolization is approx. 10^5 times more effective than water at pH 6.4. The reaction is first order in pyruvate and general base catalyzed, suggesting that enolization of pyruvate is rate limiting. As a result of the high efficiency of Zn^{2+} catalysis, the half life of a 1.0 M pyruvate solution containing 24 mM Zn^{2+}, pH 6.4, is only 37 min. Mg^{2+} and Ca^{2+} are much less effective than Cu^{2+}, Zn^{2+}, Co^{2+} or Ni^{2+}.

The great enhancement of pyruvate aldolization by Zn^{2+} lends support to a previously suggested model of Class II (metallo) aldolase reactions in which coordination of metal ions to the carbonyl oxygen of the substrate labilizes the α-carbon–hydrogen bond.

Metal ions present in stock solutions of pyruvate at concentrations high enough to catalyze significant amounts of aldolization may lead to complications when pyruvate-containing assay mixtures are used in enzyme studies, since the aldol dimer of pyruvate is a potent inhibitor of the citric acid cycle.

INTRODUCTION

Many enzymes which catalyze reactions of pyruvate require a divalent metal ion for activity[1–9]. The role of the metal ion in these enzymes may be to form an enzyme–metal ion–substrate bridge[10]. Pyruvate resembles 2-pyridine aldehyde[11], *p*-nitrophenyl picolinate[12] and oxalocacetate[13] in the proximity and juxtaposition of a carbonyl group and a strong metal ion-binding site. In each case, a metal ion has been proposed to catalyze the appropriate reaction by binding to the substrate and then either polarizing the carbonyl group or participating in the direct transfer of water or OH^- to the carbonyl group.

Pyruvate aldolase, an enzyme catalyzing the aldol dimerization of pyruvate,

Abbreviation: DSS, sodium 2,2-dimethyl-2-*sila*pentane-5-sulfonate.

* Author to whom correspondence and requests for reprints should be addressed.

is a Class II metalloaldolase[15]. Enzymatic aldolizations fall into two classes, those involving tightly bound metal ions (Class II) and those in which a Schiff base functions as the electrophile (Class I). Pyruvate aldolase is a relatively poorly characterized enzyme isolated from germinating peanut cotyledons. The enzyme shows a strong requirement for a thiol reagent and an absolute requirement for metal ions[15]. The fundamental mechanistic features of the Class II aldolase reaction have been based mainly on the well characterized zinc metalloenzyme, yeast Fru-1,6-P_2 aldolase[14]. The tightly bound Zn^{2+} in yeast aldolase may be replaced by Mn^{2+} or Co^{2+} to yield an active preparation. An NMR experiment[10,14] comparing the effect of the Zn-enzyme, Co-enzyme, and Mn-enzyme on the relaxation rates of the C-1 protons of fructose diphosphate and C-3 protons of dihydroxyacetone phosphate suggests that the metal ion forms a bridge (aldolase–metal ion–substrate) for binding the substrate. The structural similarity between the Zn^{2+} complex of dihydroxyacetone phosphate[14] and the Zn^{2+} complex of pyruvate[18] in the proximity of a carbonyl group to the Zn^{2+} binding site, and the fact that these complexes are important in their respective enzymatic reactions suggested that a study of the kinetics of non-enzymatic aldolization of pyruvate might give useful insight into the mechanism of Class II aldolases.

The non-enzymatic aldol condensation of pyruvate has long been known to be promoted by strong bases, cyanide ion, and divalent metal ions[17–19]. The rate enhancements reported for metal ion catalysis over the water catalyzed reaction are not dramatic; *e.g.* a rate enhancement of 250–1000 has been reported for pyruvate aldolization in the presence of 1–20-fold molar excess of Zn^{2+} to pyruvate, at pH 4.0 (ref. 19). The present report deals with a reinvestigation of the aldol condensation of pyruvate. Enhancement of at least 10^5 by catalytic amounts of Zn^{2+} and significant additional enhancement due to general base catalysis by buffer dianion is also reported.

EXPERIMENTAL SECTION

Materials

Sodium pyruvate type II, dimer-free, was purchased from Sigma Chemical Company. NMR analysis indicated $< 0.5\%$ dimer present in a sample subjected to pH values no greater than 6.5. $ZnCl_2$, A.C.S. reagent and $CuCl_2$, reagent was purchased from Matheson, Coleman, and Bell; $CoCl_2$, A.C.S. certified, was purchased from Fisher Scientific Co.; $NiCl_2$ and $MgCl_2$, analyzed reagent, from Baker Chemical Co., and $CaCl_2$, analytical reagent, was purchased from Mallinckrodt Chemical Co. All solutions were prepared with double-distilled water and used without further purification. Maleic acid was purchased from Eastman Chemical Co. The lactone of the aldol dimer of pyruvic acid, 4-carboxy-2-keto-γ-valerolactone, Fig. 1, (Compd 3), mp. 117–119 °C, was prepared by the method of deJong[23]. The aldol of pyruvate Fig. 1 (Compd 2) was prepared from the lactone by the method of Webb[21]. The crystalline lactone was dissolved in water and the pH adjusted to 7.0 and allowed to stand overnight. The resulting solution (pH approx. 5) was readjusted to pH 7.0 and allowed to stand overnight again. An NMR spectrum of the final solution indicated that no residual lactone was present.

Methods

Kinetic measurements were performed with a Varian Associates A-60A NMR Spectrometer at ambient probe temperature, approx. 38 ± 2 °C. Chemical shift, δ, is expressed in Hz downfield from internal sodium 2,2-dimethyl-2-*sila*pentane-5-sulfonate (DSS) at 60 MHz. The NMR signals assigned to pyruvate and hydrated pyruvate were based on the report of Wadso[24]. Kinetic experiments were carried out as follows: the integrals of the CH_3-signal of pyruvate and the CH_3-signal of the dimer Fig. 1 (Compd 2) were determined at intervals after addition of the metal ion. The sum of the pyruvate CH_3-integral *plus* twice the dimer CH_3-integral remained constant throughout each kinetic experiment. The ratio of the pyruvate methyl integral to this integral sum was taken as the fraction of pyruvate present. This fraction was then multiplied by the initial pyruvate concentration to yield $[\text{pyruvate}]_t$, *i.e.* the concentration of pyruvate at time t. This method gave more consistent results than taking the pyruvate methyl integral as $[\text{pyruvate}]_t$ since any instrumental variation in integral intensity was effectively obviated. The equilibrium pyruvate concentration $[\text{pyruvate}]_\infty$, was determined by measuring [pyruvate] for at least 10 half lives for solutions prepared with pure pyruvate, buffer, and metal ions. The value, $[\text{pyruvate}]_\infty$, was also determined by the Zn^{2+}-catalyzed equilibration of essentially pure pyruvate aldol. A semilogarithmic plot of $[\text{pyruvate}]_t - [\text{pyruvate}]_\infty$ against time was made and the half time ($t_{\frac{1}{2}}$), *i.e.* the time required for [pyruvate] to reach the value $([\text{pyruvate}]_0 - [\text{pyruvate}]_\infty)/2$, was determined directly from the graph[25]. The observed first order rate constant (k_{obsd}) was calculated from the equation:

$$k_{obsd} = \frac{0.693}{t_{\frac{1}{2}}}$$

Pyruvate solutions were prepared just before use. Stock solutions of metal ons, 1.0 M, were prepared and small portions of such solutions were added to the pyruvate solutions in NMR tubes with either Microcaps disposable pipettes (Drummond Scientific Co.) or Eppendorf microliter pipettes. The pH change caused by the addition of these metal ions was negligible.

Acetoin was measured by the procedure of Westerfeld[26] using a molar absorption coefficient of $2.01 \cdot 10^4$ at 525 nm[27].

In each buffered solution of pyruvate and metal ions the concentrations of buffer anion (HA^- and A^{2-}), buffer-complexed metal cation, and OH^- must be known. If one of these concentrations is changed, the other two are also altered. Therefore, it was necessary to calculate the concentration of each species in solution from the known pK of the buffer and the stability constant of the complex of metal ion and buffer anion. As an approximation, the extent of complexation of metal ions with the buffer monoanion is considered small in comparison with the dianion. For the following equilibria

$$HA^- \rightleftarrows H^+ + A^{2-} \quad (1)$$

$$Zn^{2+} + A^{2-} \rightleftarrows Zn^{2+} : A^{2-} \quad (2)$$

one can derive an equation,

$$[A^{2-}]\ \{\text{antilog}\ (pK - pH) + 1\} + \left\{\frac{[Zn]_{total}}{1 + \frac{1}{(K_{assoc})\ [A^{2-}]}}\right\} = [A^{2-}]_{total} \quad (3)$$

which gives $[A^{2-}]$ at any condition of pH, total metal ion concentration and total buffer concentration for which the sample is prepared. The $[Zn^{2+}]$ is subsequently calculated from Eqn 4.

$$\frac{[Zn^{2+}]_{total}}{(K_{assoc})\,[A^{2-}] + 1} = [Zn^{2+}] \tag{4}$$

The remaining concentrations, $[Zn^{2+}:A^{2-}]$ and $[HA^{-}]$, can be calculated by subtraction from the known total concentrations of metal ion and buffer.

RESULTS

White and Drago[28] reported NMR evidence which suggested that a metal ion–thiamine pyrophosphate complex is a more efficient catalyst for the decarboxylation of pyruvate than thiamine pyrophosphate alone. As the beginning of the present study, we repeated their experiments and assayed the reaction by measuring the formation of acetoin[27]. There was no evidence that appreciable amounts of decarboxylation were occurring; instead, the NMR changes showed that the pyruvate was undergoing aldol condensation. The aldol condensation product, Fig. 1 (Compd 2), of pyruvate, prepared from its lactone by hydrolysis at pH 7, gave an NMR spectrum identical to that assigned to the aldol formed after addition of metal ions to a pyruvate solution. Furthermore, when Zn^{2+} was added to an essentially pure solution of Compd 2, an NMR spectrum was generated which was identical to that of the equilibrium mixture of Compds 1 and 2 obtained by the addition of Zn^{2+} to a pure solution of pyruvate (Fig. 1). The equilibrium of Compds 1 and 2 at pH 7.0 is not complicated

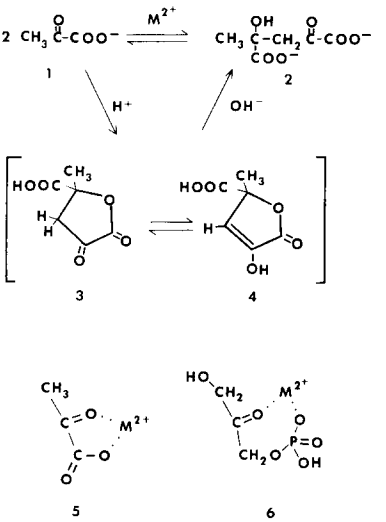

Fig. 1. Reactions of pyruvate and its aldol dimer. Addition of Zn^{2+} to either compd 1 or 2 results in the rapid attainment of the equilibrium compd 1 (20%) $\rightleftarrows$ compd 2 (80%) at pH 7. The equilibrium compd 3 (42%) $\rightleftarrows$ compd 4 (58%) is rapidly attained at pH 1. Suggested complexes of metal ions with pyruvate (compd 5) and dihydroxyacetone phosphate (compd 6) are also shown.

TABLE I

NMR CHEMICAL SHIFT DATA FOR PYRUVATE AND RELATED COMPOUNDS

	*Chemical shift (δ)**		
	CH_3	CH_2**	$C{=}C{-}H$
Pyruvate	139.9		
Pyruvate hydrate	88.3		
4-carboxy-2-keto-γ-valero-lactone, keto form (compd 3)	101.7	162.0	
Enol form (compd 4)	101.7		386.0
4-hydroxy-4-methyl--2-ketoglutarate (compd 2)	80.8	191.8	

* Chemical shift, δ, is expressed in Hz at 60 MHz downfield from internal DSS in water, pH 6.4.

** The coupling constant (J_{CH_2}) and chemical shift difference ($\nu_0\delta_{CH_2}$) between the non-equivalent methylene protons of compd 2 and compd 3 are: J_{CH_2}, 17.7; $\nu_0\delta_{CH_2}$, 7.2 and J_{CH_2}, 13.9; $\nu_0\delta_{CH_2}$, 27.2 Hz respectively[50].

by the presence of the lactone (Compd 3), since no lactone could be detected in the NMR spectra of Compd 2 at pH 7.0, whether the latter was prepared by hydrolysis of the lactone or by metal ion catalyzed aldolization of Compd 1. This result is in contrast to that of Montgomery and Webb[21] who report 24% lactone in equilibrium with Compd 2 at pH 7. Montgomery and Webb[21] however approached the equilibrium only from the lactone and allowed it to equilibrate at pH 7 for 24 h without further addition of NaOH. Addition of base is necessary to maintain a constant pH since OH^- is consumed by lactone hydrolysis. Confirming the report of Montgomery and Webb[21], we found the enol content of the lactone of pyruvate aldol to be 58% at pH 1.0. Both the enol and keto forms, Compds 3 and 4, of the lactone of pyruvate aldol, the open chain dimer, Compd 2, pyruvate hydrate, and pyruvate were easily distinguished by their unique NMR spectra (Table I).

Catalytic amounts of metal ion greatly accelerated the rate of pyruvate

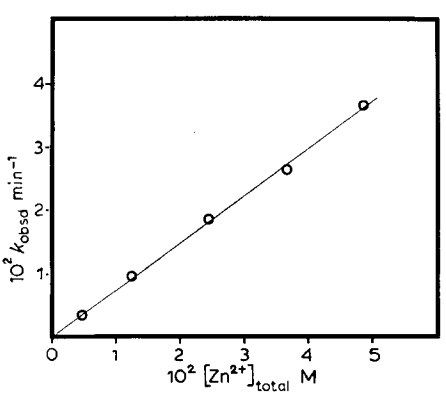

Fig. 2. Observed rate constant of pyruvate aldolization at varying total Zn^{2+} concentration Solutions were 1.0 M pyruvate; 0.1 M maleate buffer, pH 6.4 at 38 °C.

TABLE II

OBSERVED RATE CONSTANTS FOR THE ALDOLIZATION OF PYRUVATE

All solutions were 1.0 M pyruvate, 24.4 mM metal ions, 0.1 M maleate buffer, pH 6.4. The second column is the observed first-order rate constant for the forward process (dimerization). The third column is obtained by dividing the second column by $[M^{2+}]$, in this case 24.4 mM. The last column is obtained by dividing k_c for any metal ion by $1.3 \cdot 10^{-5}$, the value in the absence of metal ion.

Metal ion	k_{obsd} *(min^{-1})*	k_c *($M^{-1} \cdot min^{-1}$)*	*Rate enhancement*
None	$1.3 \cdot 10^{-5}$		1
Ca^{2+}	$9.9 \cdot 10^{-5}$	$4.1 \cdot 10^{-3}$	$3 \cdot 10^2$
Mg^{2+}	$7.6 \cdot 10^{-4}$	$3.1 \cdot 10^{-2}$	$2 \cdot 10^3$
Cu^{2+}	$1.5 \cdot 10^{-2}$	$6.0 \cdot 10^{-1}$	$4 \cdot 10^4$
Zn^{2+}	$1.9 \cdot 10^{-2}$	$7.7 \cdot 10^{-1}$	$6 \cdot 10^4$
Ni^{2+}	$2.0 \cdot 10^{-2}$	$8.1 \cdot 10^{-1}$	$6 \cdot 10^4$
Co^{2+}	$2.5 \cdot 10^{-2}$	1.0	$8 \cdot 10^4$

aldolization. The rate of the reaction was directly proportional to the metal ion concentration (Fig. 2). The catalytic rate constants, k_c, for metal ions were determined either from the slope of a plot of k_{obsd}* *vs* $[M^{2+}]$ (Fig. 2) or from kinetic experiments at a single metal ion concentration. In the latter case, the observed rate constant divided by the metal ion concentration was taken as k_c. The catalytic rate coefficients and the rate enhancements of various metal ion are given in Table II. The alkaline earth metal ions, Mg^{2+} and Ca^{2+}, were much less effective catalysts than Zn^{2+} and the transition metal ions, Cu^{2+}, Ni^{2+} and Co^{2+}. An enhancement for the Zn-catalyzed reaction over the water-catalyzed reaction can be conservatively estimated at 10^5. It is a conservative estimate since only the Zn^{2+} not complexed to buffer are likely to be catalytically active[13]. Furthermore, small amounts of contaminating metal ions may be present in the sample to which no metal ions were added (Table II),

TABLE III

COMPARISON OF THE RATES OF PYRUVATE ALDOLIZATION IN SEVERAL BUFFERS

All solutions were 1.0 M pyruvate, pH 6.4 ± 0.1 prepared with double distilled water. EDTA was present only in the first solution. All experiments were performed at ambient temperature.

Solution	$t_{\frac{1}{2}}$	k_{obsd} *(min^{-1})*
0.02 M EDTA	179 days	$2.4 \cdot 10^{-6}$*
0.10 M phosphate	90 days	$5.4 \cdot 10^{-6}$
0.10 M imidazole	61 days	$7.9 \cdot 10^{-6}$
0.10 M maleate	36 days	$1.3 \cdot 10^{-5}$
24 mM Zn^{2+} in 0.10 M maleate	37 min	$1.9 \cdot 10^{-2}$

* This value may be compared to that for the enolization of uncomplexed pyruvate[51], $5.6 \cdot 10^{-6}$ min^{-1}.

* We have followed the general procedure of presenting the kinetic data for strictly reversible reactions in terms of the experimental rate coefficient for equilibrium, k_{obsd}, which is actually the sum of forward and reverse rate coefficients, $k_{obsd} = k_f + k_r$. Since the equilibrium constant is equal to the ratio of k_f and k_r, $k_{eq} = k_f/k_r$, the forward and reverse rate coefficients can be calculated if necessary[25].

since a pyruvate solution containing 0.02 M EDTA had a greatly increased half life over that observed in solutions containing common buffers and no EDTA (Table III).

The aldolization of pyruvate is first order in pyruvate at more than 0.7 M pyruvate and showed positive deviations from a first-order rate law at concentrations below 0.7 M (Fig. 3), indicating a change in kinetic order at the lower concentrations[29]. The result is similar to that for the aldol condensation of acetaldehyde[30], in which the reaction changes from first to second order at concentrations less than 0.5 M. This positive deviation from linearity of the first-order plot is probably not due to an incorrect endpoint, $[\text{pyruvate}]_\infty$ (ref. 25), since the endpoint was determined on three separate samples of pyruvate of different buffer composition and metal ion

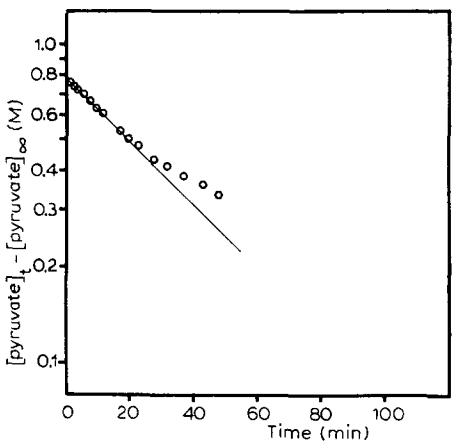

Fig. 3. Kinetics of pyruvate aldolization. Kinetic zero is the time of mixing 24.4 mM Zn^{2+} with 1.0 M pyruvate in 0.1 M maleate buffer, pH 7.1 at 38 °C. At completion (equilibrium), 79.5% of the pyruvate is converted to the dimer.

concentration and confirmed by measuring the equilibrium for the reverse reaction, aldol → ketone. The endpoint was determined at $t > 10\, t_{\frac{1}{2}}$ when the reaction had proceeded 99.9% to completion. When the solution was initially 1.0 M in pyruvate, pH 6.4, the concentration of pyruvate at equilibrium was 0.205 ± 0.005 M and that of the dimer was 0.398 ± 0.005 M.

$$k_{eq} = \frac{[\text{aldol dimer}]}{[\text{pyruvate monomer}]^2} = 9.4 \pm 0.7\ \text{M}^{-1}$$

The same equilibrium pyruvate concentration, 0.205 ± 0.005 M, was obtained in the presence of either 12 or 24 mM Zn^{2+}, suggesting that the equilibrium constant was not dependent on metal ion concentration. The value of k_{eq} is in reasonable agreement with values of 6.0 (ref. 17) and 6.5 (ref. 18) reported for cyanide-catalyzed aldolization of pyruvate.

In the presence of buffer, the rate of the reaction was dependent on $[OH^-]$ in a non-linear manner (Fig. 4a), but depended linearly on the calculated concentration of the dianion of the maleate buffer (Fig. 4b). This suggests that the aldolization is general base-catalyzed and that catalysis by OH^- is small compared to A^{2-} catalysis.

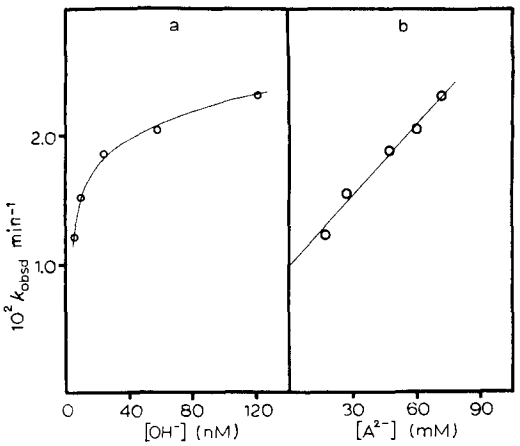

Fig. 4. (a) Effect of OH^- on the rate of pyruvate aldolization. The observed rate constant was measured as the pH of a buffered solution of pyruvate was varied. Solutions were 1.0 M pyruvate; 24.4 mM $[Zn^{2+}]_{total}$; 0.1 M maleate buffer at 38 °C. (b) Dependence of the rate of pyruvate aldolization on the concentration of the maleate dianion (A^{2-}). The $[A^{2-}]$ was calculated for each of the experiments at various pH values shown in (a) as described in Experimental.

i.e. $k'_{OH}[OH^-] \ll k'_A[A^{2-}]$. The observed rate constant may be represented by a sum of Zn^{2+}-catalyzed and water-catalyzed reactions (Eqn 5)[29].

$$k_{obsd} = k_0 + k_{OH}[OH^-] + k_{HA}[HA^-] + k_A[A^{2-}] + k'_0[Zn^{2+}] + k'_{OH}[Zn^{2+}][OH^-] + \\ + k'_{HA}[Zn^{2+}][HA^-] + k'_A[Zn^{2+}][A^{2-}] \quad (5)$$

The sum of the terms, $k_0 + k_{OH}[OH^-] + k_{HA}[HA^-] + k_A[A^{2-}]$, is negligible since the rate of the reaction is negligible with no metal ion added (Fig. 2). Catalysis by the mono-anionic buffer component, HA^-, is expected to be small compared to that of the more basic di-anionic buffer component, A^{2-} (ref. 31), and the term $k'_{HA}[Zn^{2+}][HA^-]$ is incorporated into the water-catalysis term, $k'_0[Zn^{2+}]$. Eqn 5 now becomes:

$$k_{obs} = k''_0[Zn^{2+}] + k'_{OH}[Zn^{2+}][OH^-] + k'_A[Zn^{2+}][A^{2-}] \quad (6)$$

We have determined the rate constants pertinent to Eqn 6, assuming that only the Zn^{2+} not complexed to buffer is catalytically active. Negatively charged buffers, such as citrate, diminish the catalytic activity of metal ions in the decarboxylation of dimethyloxaloacetic acid[13] presumably because these ions compete with dimethyloxaloacetate for the metal ions, forming complexes which are inactive in decarboxylation. Also, in the decarboxylation of acetonedicarboxylic acid in acetate buffer, the species $Cu^{2+}(acetate^-)_2(A^{2-})$ is completely inactive[32]. These facts are in agreement with the hypothesis that any ligand reducing the effective charge of the metal ion–substrate complex reduces the catalytic effect of the metal ion[33]. In three separate sets of experiments only the total buffer concentration, or the total metal ion concentration, or the pH was varied, and the observed rate constants (k_{obsd}) determined (Table IV). The constant k'_A is obtained from the slope of

$$\frac{k_{obsd}}{[Zn^{2+}]} \text{ vs } [A^{2-}]$$

TABLE IV

OBSERVED RATE CONSTANTS OF Zn^{2+}-CATALYZED ALDOLIZATION OF PYRUVATE

In all experiments, k_{obsd} was measured. In Exp 1, $[Zn^{2+}]_{total}$ was varied while the total buffer concentration (maleate) and pH were kept constant. In Expt 2, the total buffer concentration was varied while $[Zn^{2+}]_{total}$ and pH were kept constant. In Expt 3, the pH was varied while the total buffer concentration and $[Zn^{2+}]_{total}$ were kept constant. In each case, the calculated concentrations of a buffer component (A^{2-}) and of the Zn^{2+} not complexed to buffer, $[Zn^{2+}]$, as described in the experimental section, is given. The calculated concentrations were determined using the pK of maleic acid ($pK_2 = 6.20$) and the association constant of Zn^{2+} with the dianion of maleate ($K_{assoc.} = 10^2$) [52].

	Total buffer (M)	*$[Zn^{2+}]_{total}$ (mM)*	*$[OH^-]$ (nM)*	*$[A^{2-}]$ (mM)*	*$[Zn^{2+}]$ (mM)*	*$10^2 k_{obsd}$ (min^{-1})*
Expt 1						
$[Zn^{2+}]_{total}$		4.88		56.0	0.74	0.346
varied		12.2		52.0	1.97	0.976
	0.1	24.4	23.4	47.0	4.28	1.87
		36.6		42.0	7.04	2.62
		48.8		35.0	10.06	3.65
Expt 2						
Total buffer	0.1			47.0	4.28	1.87
concentration	0.2			105	2.12	1.98
varied	0.3	24.4	23.4	170	1.36	2.27
	0.4			225	1.04	2.24
Expt 3						
pH varied			4.17	17.0	9.03	1.22
			8.32	27.0	6.60	1.54
	0.1	24.4	23.4	47.0	4.28	1.87
			56.2	60.0	3.49	2.04
			120	72.0	2.98	2.31

at constant $[Zn]_{total}$ and constant pH (Eqn 6, Fig. 5a). The constant k'_{OH} is obtained from the slope of a plot of

$$\frac{k_{obsd}}{[Zn^{2+}]} - k'_A[A^{2-}] \text{ vs } [OH^-]$$

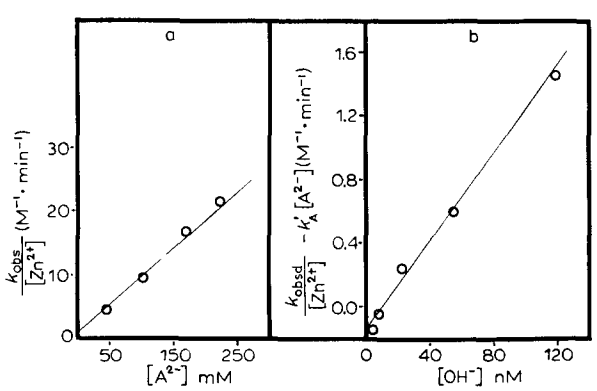

Fig. 5. (a) Determination of the catalytic rate coefficient k'_A (Eqn 6). The slope of a plot of $k_{obsd}/[Zn^{2+}]$ *vs* $[A^{2-}]$ is equal to k'_A at constant pH and $[Zn^{2+}]_{total}$. Solutions were 1.0 M pyruvate; 24.4 mM $[Zn^{2+}]_{total}$, pH 6.4 at 38 °C. (b) Secondary replot of Fig. 4a. Determination of the catalytic rate coefficient k'_{OH} (Eqn 6). The slope of a plot of $k_{obsd}/[Zn^{2+}] - k'_A[A^{2-}]$ *vs* $[OH^-]$ is equal to k'_{OH} at constant total buffer concentration and $[Zn^{2+}]_{total}$.

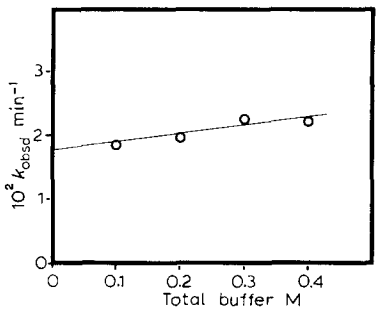

Fig. 6. Observed rate constant of pyruvate aldolization at various total buffer concentration. The data of this figure are the same as Fig. 5a. The catalytic rate coefficient k''_0 is calculated from the intercept at zero buffer concentration; $k_{obsd}/[Zn^{2+}]_{total} - k'_{OH}[OH^-] = k''_0$ (Eqn 6).

(Fig. 5b); and k''_{H_2O} is calculated from the intercept at zero buffer concentration of Fig. 6*. These values are summarized in Table V. The rate coefficient for the combined catalysis by Zn^{2+} and water (k_{Zn^{2+},H_2O}, Table V) substantiates the conclusion reached above that Zn^{2+} is 10^5 times as effective as water alone in catalyzing pyruvate aldolization. Also the combined catalysis of pyruvate aldolization by Zn^{2+} and maleate^{2-} ($k_{Zn^{2+},A^{2-}}$) is approx. 10^5 times that reported[34] for the acetate-catalyzed enolization of uncomplexed pyruvate, $3.0 \cdot 10^{-4}$ $M^{-1} \cdot min^{-1}$.

TABLE V

CATALYTIC RATE COEFFICIENTS (k_c) FOR ALDOLIZATION OF PYRUVATE

Catalyst	k_c ($M^{-1} \cdot min^{-1}$)	$\frac{k_c}{k_{H_2O}}$
Water*	$4.3 \cdot 10^{-8}$	1
Zn^{2+}, water**	$7.2 \cdot 10^{-3}$	$2 \cdot 10^5$
Zn^{2+}, A^{2-}	$8.8 \cdot 10^{1}$	$2 \cdot 10^9$
Zn^{2+}, OH^-	$1.4 \cdot 10^{7}$	$3 \cdot 10^{14}$

* The observed rate constant for an EDTA solution, pH 6.4 (Table III) divided by 55.5 M to obtain k_{H_2O} in $M^{-1} \cdot min^{-1}$. This value is an upper limit, because OH^- may contribute to the catalysis, at pH 6.4.

** The catalytic rate coefficients for Zn-catalyzed reactions correspond to those in Eqn 6. The rate constant k_{Zn,H_2O} may include a catalytic contribution by HA^- and the anion of pyruvate.

DISCUSSION

The general sequence of the pyruvate aldol condensation can be given as follows:

$$\text{Pyruvate} + B^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} BH + \text{pyruvate}^-$$

$$\text{Pyruvate}^- + \text{Pyruvate} \xrightarrow{k_3} \text{aldol}^-$$

$$\text{aldol}^- + BH \xrightarrow{k_4} \text{aldol} + B^-$$

* The value k''_{H_2O} can also be obtained from the intercept of Fig. 4b, assuming $k'_{OH}[OH^-]$ is negligible; in that case, $k''_{H_2O} = 7.0 \cdot 10^{-3}$ $M^{-1} \cdot min^{-1}$.

The last step is a type which is usually very rapid[29]. Two limiting cases which are of interest are those in which k_3[Pyr] is either much greater or much smaller than k_2[BH]. The rate equations for these two situations become Eqns 7 and 8 respectively[29]:

$$\text{rate} = k_1\,[\text{pyruvate}]\,[\text{B}^-] \tag{7}$$

$$\text{rate} = \frac{k_1 k_3}{k_2 K_B}\,[\text{pyruvate}]^2\,[\text{OH}^-] \tag{8}$$

K_B is usually designated[29] as the ionization constant of the base and is defined here by $K_B = [BH][OH^-]/[B^-]$. We find that the Zn^{2+}-catalyzed aldolization of pyruvate is first order in pyruvate (Fig. 3) and general base catalyzed (Table V). This is strong evidence that Eqn 7 represents the reaction and that enolization of pyruvate (k_1) is the rate-limiting step for non-enzymatic aldolization at the relatively high concentrations of pyruvate used in this study. In most aldol condensations of ketones (*e.g.* acetone), over a wide range of concentrations, the rate-limiting step is the one in which the new carbon–carbon bond is formed (k_3)[41]. If enolization of pyruvate (k_1) is rate-limiting for the conditions of this study, then metal ions may enhance the rate of the condensation step (k_3) relative to other ketones so that $k_3 > k_1$. The inductive effect of the metal ion-complexed carboxylate group of pyruvate probably enhances the rate of addition reactions at the α-carbonyl carbon relative to acetone[42]. Also, a metal ion complexed to the α-carbonyl oxygen of pyruvate, by increasing the polarization of the carbonyl group in the acceptor molecule, stabilizes a partial positive charge on the carbonyl carbon atom, thereby increasing its susceptibility to nucleophilic attack[37].

Metal ions can also enhance the enolization of pyruvate by polarizing the carbonyl group and thus stabilizing the incipient carbanion, or indirectly, by the transfer of OH^- to the vicinity of the proton to be removed. Because of the high acidity of water molecules bound to metal ions[45], these ions have the ability to carry appreciable amounts of potential OH^- at a pH where very little free OH^- could exist. The metal ion complex, containing OH^-, could be considered to be a bifunctional catalyst, the metal ion serving as a general acid and the OH^- as a base[11].

The enhancement by Zn^{2+} of the rate of non-enzymatic aldolization of pyruvate may have significance in understanding the mechanism of the Class II aldolase reaction. The comparison of kinetic data from the model reaction with enzymatic data is simplified by our choice of conditions which differ from those used in a previous study[19] using ultraviolet spectrophotometry. In that case, a pH of approx. 4 was used whereas for yeast Fru-1,6-P_2 the pH optima for the exchange reaction of Fru-1,6-P_2 and for the overall aldol cleavage are 6.0 and 7.2 respectively[20]. The pH range, 5.6–7.1, used in our study therefore allows a ready comparison. The low Zn^{2+}:pyruvate ratios, $5\cdot10^{-3}$:1 to $50\cdot10^{-3}$:1, used in this study, in contrast to the high ratios used in the previous study[19], allow a more valid comparison with enzyme systems since the usual condition for a catalyst (enzyme) is that it be present in small amounts relative to the reactants.

The mechanism of the reaction catalyzed by yeast Fru-1,6-P_2 aldolase, a Zn^{2+} metalloenzyme, is thought to involve the formation of a carbanionic form of dihydroxyacetone phosphate, which then adds to the polarized carbonyl of glyceral-

dehyde 3-phosphate, forming Fru-1,6-P_2 (refs 35, 36). The mechanistic role of metal ions in Fru-1,6-P_2 aldolase has been suggested to be polarization of the C-2 carbonyl of dihydroxyacetone phosphate and orientation of the C-1 phosphoryl group[14,36,37,46]. There is some doubt whether enolization of dihydroxyacetone phosphate is the rate-limiting step in overall synthesis[38–40]. A non-enzymatic model of this enzymic aldolization is the base-catalyzed addition of dihydroxyacetone to glyceraldehyde. The reaction is first order in total triose[43] and the hexoses formed contain no carbon-bound deuterium when the reaction proceeds in ^2H_2O (ref. 44). Therefore dihydroxyacetone, like pyruvate, has the unusual property of having the enolization step as the slow step of aldolization. The rate of yeast aldolase is of the order of 10^3 moles of Fru-1,6-P_2 cleaved per min per mole of enzyme[39]. The rate of the enzyme catalyzed reverse reaction must be even larger since the equilibrium constant does not favor Fru-1,6-P_2 cleavage. This may be compared with the rate of the base-catalyzed reaction of dihydroxyacetone and glyceraldehyde[16], which is 10^{-6} M·min^{-1} at pH 7. Thus, the enzymatic reaction of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate may be more rapid than the corresponding non enzymatic reaction by a factor of 10^9. The 10^5 rate enhancement of pyruvate aldolization by Zn^{2+} probably reflects electronic and entropic effects which may also be operative in Fru 1,6-P_2 aldolases. This may be true because the Zn^{2+}–pyruvate complex appears to be similar to the enzyme-bound Zn^{2+}–dihydroxyacetone phosphate complex with respect to the proximity of a carbonyl group to a binding site for metal ions[14] (Compds 5 and 6, Fig. 1).

A significant difference between the non enzymatic aldolization of pyruvate and the enzymatic aldolization of pyruvate and dihydroxyacetone phosphate is the relative activity of various metal ions. In non enzymatic pyruvate aldolization, Zn^{2+}, Co^{2+} and Ni^{2+} are nearly equally active (Table II) whereas Zn^{2+} and Co^{2+} but not Ni^{2+} are active in yeast Fru 1,6 P_2 aldolase[46]. In contrast, pyruvate aldolase from peanut cotyledon requires Mg^{2+} for activity but Zn^{2+} and Co^{2+} show no activity[15]. These differences almost certainly reflect the different ligand environments around the metal ion at the active sites of these enzymes and probably do not negate the mechanistic similarity between Fru-1,6-P_2 aldolase, pyruvate aldolase, and non-enzymatic pyruvate aldolization.

The purity and stability of pyruvate solutions and of radioactive pyruvate in particular, have been a source of concern to many investigators[47–49]. The aldolization of pyruvate appears to be the major source of difficulty. The aldol of pyruvate (Compd 2) is a potent inhibitor of the α-ketoglutarate dehydrogenase complex and interrupts citric acid cycle oxidations[21,22]. In the presence of Compd 2 the oxidation of pyruvate by rat heart mitochondria is blocked unless a dicarboxylic acid is also present, in which case α-ketoglutarate accumulates[21]. When 5 mM pyruvate is used in the assay, as little as 1% of the aldol in the pyruvate will produce 50% inhibition of this enzyme complex[22]. Mg^{2+}, although 25–30 times less effective than Zn^{2+}, Ni^{2+} and Co^{2+} in catalyzing the aldolization, may be a greater contaminant of assay mixtures used for study of the citric acid cycle and this may result in the production of significant amounts of aldol dimer. For example, preincubation of 24 mM Mg^{2+} with 1.0 M pyruvate for 12 min would result in the production of enough aldol dimer to inhibit severely the α-ketoglutarate dehydrogenase complex, even when the solution is subsequently diluted 100-fold for purposes of the assay. Cognizance should

therefore be taken of metal ion-catalyzed formation of pyruvate aldol and its complications in assays of the citric acid cycle.

The increase in stability of EDTA solutions of pyruvate over those of common buffers (Table III) suggests that adventitious metal ions were present in the buffered solutions. It is suggested that EDTA be added, when feasible, to all solutions of pyruvate that are being stored[47,48].

ADDENDUM

The purification of pyruvate aldolase from *Pseudomonas putida* has recently been reported[53]. It is a metalloenzyme requiring either Mg of Mn but no sulfhydryl reagent, in contrast to the partially purified pyruvate aldolase isolated from peanut cotyledon[15]. The non-enzymatic cleavage of pyruvate aldol by Co approached the rate of the enzyme-catalyzed reaction[53].

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