Oxaloacetate Decarboxylase from Cod. Catalysis of Hydrogen–Deuterium Exchange in Pyruvate*

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ABSTRACT: The mechanism for the decarboxylation of oxaloacetate by oxaloacetate decarboxylase proceeds by way of a metal ion–oxaloacetate–enzyme complex through the enol form of pyruvate to the keto form of pyruvate.

The reverse reaction should then lead to the enol from the keto form of pyruvate, so that the enzyme in the presence of metal ion should catalyze the exchange of hydrogen atoms of pyruvate with the solvent. This prediction has been realized; the rate of the reaction has been measured by nuclear magnetic resonance techniques. This catalyzed hydrogen exchange has been found to be both metal-ion dependent and inhibited by oxalate, as is the catalyzed decarboxylation of oxaloacetate and the catalyzed $NaBH_4$ reduction of pyruvate.

Oxaloacetate decarboxylase from codfish has been suggested to catalyze the decarboxylation of oxaloacetate by the mechanism shown in Scheme I (Steinberger and Westheimer, 1951; Kosicki and Westheimer, 1968).

Reactions analogous to those in eq 1–4 have been previously illustrated in model systems using dimethyl oxaloacetate (Steinberger and Westheimer, 1951). In the codfish enzyme the decarboxylation is Mn^{2+} or Mg^{2+} ion dependent (Schmitt *et al.*, 1966), as it is in the decarboxylase from *Micrococcus lysodeikticus* (Herbert, 1955) and from other sources (Utter, 1961). The binding of the pyruvate as the metal ion–enzyme complex was demonstrated by stereoselective $NaBH_4$ reduction (eq 5) of the pyruvate to D-lactate (Kosicki and Westheimer, 1968).

This paper is concerned with the enolpyruvate–metal ion–enzyme complex and the reversal of the reactions shown in eq 3 and 4 involved in forming the enol complex. We have demonstrated the reversal of the reactions shown in eq 3 and 4 and have shown them to be dependent upon divalent metal ions and inhibited by oxalate. In order to observe reactions 3 and 4, we allowed pyruvate to react with D_2O in buffered solution in the presence of enzyme and divalent metal ions, and have followed the loss of methyl protons from pyruvate by the decrease in the appropriate nuclear magnetic resonance signal as deuterium atoms were incorporated into the methyl groups of pyruvate. Similar techniques have been used to demonstrate enzymically catalyzed proton exchanges in reactions catalyzed by acetoacetate decarboxylase (Tagaki and Westheimer, 1968) and citrate synthase (Srere, 1967).

Reactions 3 and 4 were also observed in the reverse direction by allowing pyruvate-d_3 to react with H_2O in buffer solution in the presence of enzyme and divalent metal ions, and in this case, following the appearance of methyl protons in pyruvate.

Experimental Section

Methods. Oxaloacetate decarboxylase from codfish was prepared according to Kosicki (1968). The concentration of enzyme given in milligrams per milliliter is based on the 280 mμ to 260 mμ absorbance ratio (Warburg and Christian, 1942) and the units of enzyme activity are given in micromoles of oxaloacetate decarboxylated per minute at 30° (specific activity of crystalline enzyme is 1.8). Samples of oxaloacetate decarboxylase, here designated as B_1, were freed from residual calcium ions (from the calcium–phosphate column used in its preparation) by the following procedure. The enzyme from the calcium phosphate column was incubated for 15 min at room temperature with 0.1 M EDTA at pH 6.80 and then precipitated by the addition of $(NH_4)_2SO_4$ (472 g/l. of solution). The precipitated enzyme (77.7 mg) was redissolved in 3.70 ml of 0.02 M potassium phosphate buffer (pH 6.80). A sample of oxaloacetate decarboxylase, designated as B_2, was desalted on a 2.5× 30.0 cm column of Sephadex G-25; the enzyme was eluted from the column with the same buffer. The fractions containing the enzyme were combined. Another sample of enzyme, designated as C, was freed from residual calcium ions and phosphate ions by the procedure described for sample B_2, but replacing the phosphate buffer with 2×10^{-2} M *N,N,N',N'*-tetraethylethylenediamine-HCl buffer (pH 6.6). For use in D_2O systems the enzyme samples B_1, B_2, and C were precipitated by the addition of $(NH_4)_2SO_4$ (final concentration 472 g/l. of solution); the precipitate was

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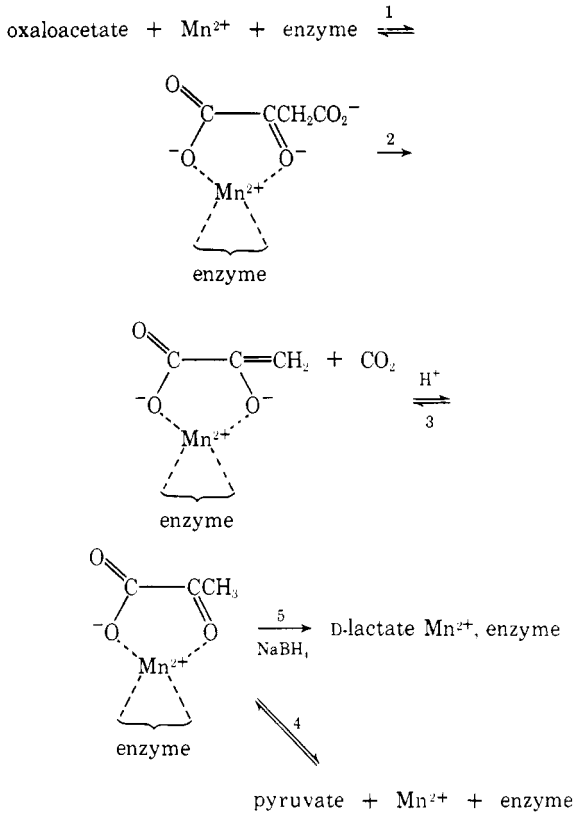
SCHEME I



collected by centrifugation and dissolved in D_2O which was 0.02 M in potassium phosphate or *N,N,N′,N′*-tetraethylethylenediamine-HCl buffer (pD 7.2).

The following materials were commercial preparations: oxaloacetic acid, sodium pyruvate (Calbiochem), *N,N,N′,N′*-tetraethylethylenediamine (freshly distilled), and *t*-butyl alcohol (Eastman). Other reagents were commercial preparations of analytical grade.

Sodium pyruvate-d_3 was prepared by dissolving 1.10 g of sodium pyruvate in 30 ml of D_2O (99.77%) and adding 8 units of oxaloacetate decarboxylase dissolved in D_2O and 350 μmoles of $MgCl_2$. The exchange reaction was allowed to proceed for 3 days at room temperature. The exchange of the methyl protons of pyruvate by this method was confirmed by the disappearance of the methyl proton peak of pyruvate (δ 2.37) from its nuclear magnetic resonance spectrum. When the reaction was complete the solution was acidified with concentrated HCl to below pH 1, evaporated to a syrupy residue on a rotory evaporator, and the pyruvic acid was dissolved in ether. The ether was evaporated and the syrupy acid neutralized by NaOH was dissolved in D_2O. The neutral solution was evaporated to dryness under vacuum.

In an alternative and simpler procedure for preparation of sodium pyruvate-d_3, 1.10 g of sodium pyruvate was dissolved in 30 ml of D_2O and allowed to incubate at 45° for 18 days to complete the exchange of methyl protons. The solution was then evaporated.

Assay of Oxaloacetate Decarboxylase. The assay (Kosicki, 1968) depends upon following the decrease in absorption of the enol form of the equilibrated tautomeric mixture of the oxaloacetate–Mn^{2+} complex at 260 mμ.

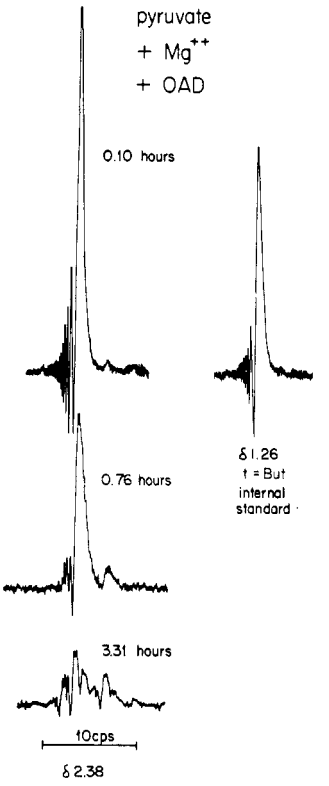


FIGURE 1: The nuclear magnetic resonance spectra, at various times, of pyruvate-h_3 and *t*-butyl alcohol in the presence of oxaloacetate decarboxylase and $MgCl_2$. The details of composition of the reaction mixture are presented in Figure 2.

For the assay of decarboxylation and exchange reactions of oxaloacetate in the presence of Mg^{2+}, Ca^{2+}, and higher concentrations of Mn^{2+}, the potassium phosphate buffer was replaced by *N,N,N′,N′*-tetraethylethylenediamine-HCl buffer (pH 6.60). For systems assayed in D_2O the components of the assay were dissolved in D_2O rather than H_2O. The *N,N,N′,N′*-tetraethylethylenediamine-HCl buffer in D_2O was titrated to a pH reading on a glass electrode of 6.20 using concentrated HCl (Glascoe and Long, 1960). The potassium phosphate buffer in D_2O was made from the solid salts dissolved in a ratio of 3 moles of KH_2PO_4 to 2 moles of K_2HPO_4 (pD 7.2).

Exchange Assays. The isotopic compositions of solutions of pyruvate-h_3 or pyruvate-d_3 were determined in potassium phosphate and *N,N,N′,N′*-tetraethylethylenediamine-HCl buffers by nuclear magnetic resonance spectroscopy with a Varian A-60 spectrometer. The signal from the methyl group of pyruvate appears in D_2O at δ 2.37. The methyl peaks of sodium 2,2-dimethyl-2-silapentane-5-sulphonate (δ 0.00 in D_2O) and *t*-butyl alcohol (δ 1.26 in D_2O) were used as internal standards for nuclear magnetic resonance measurements.

As the reaction with pyruvate in D_2O proceeds and hydrogen atoms are replaced by deuterium atoms, the area of the peak from methyl group of pyruvate diminishes, relative to that of *t*-butyl alcohol. Con-

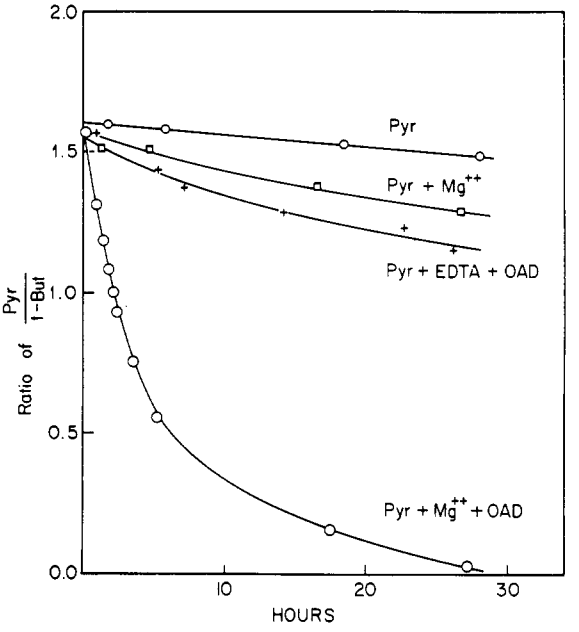


FIGURE 2: The change with time of the ratio of integrated peak areas of the methyl protons of pyruvate (δ 2.38) to the peak areas of the methyl protons of *t*-butyl alcohol (δ 1.26). Each reaction mixture contained 100 μmoles of potassium phosphate (in a ratio of 2 moles of K_2HPO_4 to 3 moles of KH_2PO_4, pD 7.2), 1% *t*-butyl alcohol, 200 μmoles of sodium pyruvate, and (as indicated) 10 μmoles of $MgCl_2$, oxaloacetate decarboxylase type B_2 in D_2O (0.8 unit), 1.0 μmole of the disodium salt of EDTA, and D_2O to make a final volume of 0.5 ml.

versely, in the measurements made with pyruvate-d_3 in H_2O, the area of the peak associated with the methyl group of pyruvate increases with time as the deuterium atoms are replaced by hydrogen atoms. The measurements in H_2O can be made despite the enormous peak from the solvent itself, which totally obscures the spectrum in the region around δ 5 and produces massive spinning side bands that may come as low as δ 3. Fortunately, the measurements were concerned only with the region between δ 1.1 and 2.5. In the presence of Mn^{2+} the resonance peaks are depressed because of the paramagnetic property of Mn^{2+} (Cohn, 1963) but the relative ratio of integrated peak areas of sample to internal standard remain the same.

Results

The enzyme catalyzes the exchange of hydrogen atoms between pyruvate and D_2O. Nuclear magnetic resonance spectra measured at various times for pyruvate in D_2O are shown in Figure 1. The area of the peak for the methyl group of pyruvate (δ 2.38 relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate) decreases relative to that of the internal standard, *t*-butyl alcohol (δ 1.26 relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate), to a small multiplet by 30 hr (Figure 2). The splitting in the methyl peak of pyruvate is similar to that reported for the exchange reaction of acetone by acetoacetate decarboxylase (Tagaki and Westheimer, 1968).

TABLE I: The Hydrogen–Deuterium Exchange Rates of Pyruvate-h_3.[a]

Reaction	Rate of Exchange (μmoles/min)
Pyruvate-h_3	0.0073
+ Mn^{2+}	0.012
+ Mg^{2+}	0.023
+ Oxaloacetate decarboxylase	0.125
+ EDTA + oxaloacetate decarboxylase	0.038
+ Mn^{2+} + oxaloacetate decarboxylase	0.187
+ Mg^{2+} + oxaloacetate decarboxylase	0.565

[a] The rate reported as the initial velocity observed in the reaction vessel is based on 200 μmoles of sodium pyruvate giving a peak area with a ratio of pyruvate to *t*-butyl alcohol of 1.60 in D_2O. Each reaction vessel contained the components as indicated in the concentrations given in the legend to Figure 2 and 0.01 μmole of $MnSO_4$.

The oxaloacetate decarboxylase catalyzed hydrogen–deuterium exchange of pyruvate is metal ion dependent as shown in Table I and Figure 2. The residual rate with oxaloacetate decarboxylase alone can be reduced by the addition of EDTA. Magnesium ion was found to be more effective than manganese ion in the exchange reaction. In Table II the data for the exchange reaction are compared with those for the enzymic decarboxylation, where Mn^{2+} is more effective.

The exchange reaction, like the decarboxylation of oxaloacetate and the stereoselective reduction of pyruvate by $NaBH_4$, is dependent upon oxaloacetate decarboxylase and metal ions and inhibited by oxalate, as shown in Table III. Addition of excess Mg^{2+} can stimulate the exchange rate in the presence of oxalate.

The enzyme does not exchange protons with D_2O solvent when pyruvate is replaced by acetone or dihydroxyacetone under conditions identical with those present in Figure 2 as measured by following the nuclear magnetic resonance signals from the respective methyl and hydroxy methyl groups.

The exchange reaction can also be followed by nuclear magnetic resonance using pyruvate-d_3 in H_2O (Table IVA). The optimum pH for this exchange is at least one unit higher (Table IVB) than the optimum (pH 6.6) for decarboxylation of oxaloacetate (Schmitt *et al.*, 1966). In addition to Mn^{2+} and Mg^{2+} ions, it was observed that Ca^{2+} also promotes the exchange (Table IVC), whereas Ca^{2+} inhibits the decarboxylation of oxaloacetate. In the decarboxylation assay using *N,N,N',N'*-tetraethylethylenediamine-HCl buffer (pH 6.6) to replace phosphate ions, 10^{-2} and 10^{-4} M $CaCl_2$ not only did not promote the enzymic decarboxylation

TABLE II: The Rates of Decarboxylation of Oxaloacetate Compared with the Rates of Exchange of Methyl Protons of Pyruvate Catalyzed by Oxaloacetate Decarboxylase.[a]

System	Activity of Oxaloacetate Decarboxylase (μmoles/min)	
	A. Decarboxylation of Oxaloacetate	B. Exchange of Pyruvate
1. pH 6.6, pyruvate-h_3 + oxaloacetate decarboxylase (B_2)		
+ Mn^{2+}	1.00	
+ Mg^{2+}	0.39	
2. pD 7.2, pyruvate-h_3 + oxaloacetate decarboxylase (B_2)		
+ Mn^{2+}	0.95	0.16
+ Mg^{2+}	0.44	0.76

[a] (A) The activity of oxaloacetate decarboxylase is reported as initial velocities for one unit of oxaloacetate decarboxylase (at pH 6.6) as measured by following the initial rate of decrease in absorbance at 260 mμ as oxaloacetate decarboxylates in H_2O with oxaloacetate decarboxylase (type B_2) and 10^{-4} M $MnSO_4$ in a potassium phosphate buffer (pH 6.60). The details of the assay are described in the Experimental Section for the phosphate system. The metal ion concentrations were 10^{-4} M $MnSO_4$ or 10^{-3} M $MgCl_2$. (B) The data (from Table I) are expressed as initial velocities for the same amount of oxaloacetate decarboxylase as in A.

TABLE III: The Inhibition of Enzymic Exchange of the Methyl Protons of Pyruvate by Oxalate.[a]

Reaction	Rel Rate of Exchange (μmoles/min)
Pyruvate-h_3	0.0073
+ Oxaloacetate decarboxylase + Mg^{2+}	0.678
+ Oxaloacetate decarboxylase	0.262
+ Oxaloacetate decarboxylase + oxalate	0.083
+ Oxaloacetate decarboxylase + oxalate + Mg^{2+}	0.209
+ Mg^{2+}	0.023

[a] Each reaction vessel contained the components as described in Figure 2 and 1.0 μmole of lithium oxalate.

TABLE IV: The Deuterium–Hydrogen Exchange Rates of Pyruvate-d_3.

Reaction	Rate of Exchange[a] (μmoles/min)
A. Pyruvate-d_3, pH 6.6	0.0034
+ Mg^{2+}	0.0035
+ Mg^{2+} + oxaloacetate decarboxylase (C)	0.099
+ Oxaloacetate decarboxylase (C)	0.081
+ Oxaloacetate decarboxylase (B_1)	0.033
B. Pyruvate-d_3 + Mg^{2+} + oxaloacetate decarboxylase (B_2)	
at pH 6.8	0.074
7.2	0.120
7.5	0.220
C. Pyruvate-d_3 + oxaloacetate decarboxylase (C), pH 6.6	Relative Rate
+ Mg^{2+}	100
+ Ca^{2+}	65
+ Mn^{2+}	42

[a] The rates of exchange of the deuterium atoms of the methyl groups of sodium pyruvate-d_3 are reported as initial velocities of the rate of appearance of methyl hydrogens of pyruvate (δ 2.38) calculated for 1.0 unit of oxaloacetate decarboxylase (pH 6.6 decarboxylation assay) and based on 200 μmoles of sodium pyruvate-d_3 giving a peak area with a ratio of the methyl hydrogens of pyruvate-h_3 to *t*-butyl alcohol (1%) of 1.69 in H_2O. (A and B) Each reaction tube contained 1% *t*-butyl alcohol and as indicated 100 μmoles of potassium phosphate (pH 6.6, 6.8, 7.2, or 7.5), 2×10^{-4} M $MgCl_2$ and oxaloacetate decarboxylase, and H_2O to make a final volume of 0.5 ml. (C) Each reaction vessel contained the components as described above using $MgCl_2$, $CaCl_2$, and $MnSO_4$ at 1×10^{-3} M.

of oxaloacetate, but also competitively inhibited the Mn^{2+}-promoted reaction. In the same system using *N,N,N',N'*-tetraethylethylenediamine-HCl buffer (pH 6.6), saturating levels of $MnSO_4$ (1×10^{-3} M) and $MgCl_2$ (1×10^{-2} M) gave relative rates of 62 and 38, respectively.

Discussion

Oxaloacetate decarboxylase catalyzed the exchange of the methyl protons of pyruvate, the product of oxaloacetate decarboxylation, as proposed in eq 3 and 4. Like the decarboxylation of oxaloacetate the initial rate of exchange is zero order (Figure 2), metal ion dependent (Table I), and inhibited by oxalate (Table III). Unlike the decarboxylation reaction, however, the

exchange reaction has an increased pH optimum (Table IVB) and can be promoted by Ca^{2+} in addition to Mg^{2+} and Mn^{2+} (Table IVC).

The measured rate of exchange of the methyl protons of pyruvate is slower than the rate of decarboxylation not only because of the deuterium isotope effects using $D_3CCOCO_2^-$, but because the exchange measures the rate of an unfavorable process. The exchange reaction is a measure of the rate of enolization of the keto form of pyruvate, while the decarboxylation of oxaloacetate forms enolpyruvate, which ketonizes quickly. Furthermore, a different pH optimum (Table IVB) indicates that other dissociable groups are involved in the catalysis.

The difference in the metal ion dependency of the exchange reaction may reflect the difference in binding. Calcium ion competitively inhibits the Mn^{2+}-promoted decarboxylation of oxaloacetate but promotes the exchange of the hydrogen atoms of the methyl group of pyruvate more effectively than Mn^{2+}. This system is a suitable tool for further studies of the role of metal ions in the enzymic decarboxylation.

The metal ion, in model systems, can promote the exchange of enolizable hydrogen atoms in two ways: it can increase the acidity of the hydrogen atoms by an electrostatic effect, and it can stabilize the enol that is formed in the reaction. But perhaps the metal ion in the enzymic system can do more. Does the protein moiety in the enzyme affect the catalytic effectiveness of the metal, or does it merely serve to improve the binding of cation and substrate?

The enzymic exchange of the methyl protons of pyruvate further confirms the model for decarboxylation of oxaloacetate proposed by Steinberger and Westheimer (1951).

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