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Decarboxylation of Oxalacetate by Pyruvate Carboxylase[†]

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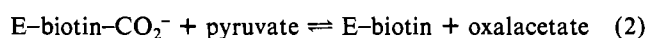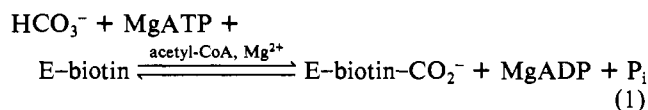*Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706*

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ABSTRACT: The decarboxylation of oxalacetate by pyruvate carboxylase in the absence of ADP and P_i is stimulated 400-fold by the presence of oxamate, which is an inhibitory analogue of pyruvate. The observation of substrate inhibition when either oxamate or oxalacetate is varied at a fixed concentration of the other indicates that both molecules bind at the same site on the enzyme. The pH profiles for this reaction show no evidence of the involvement of an enzymic acid–base catalyst, suggesting that the proton and CO_2 units may be exchanged directly between the reactants (although CO_2 sequestered in the active site may be an intermediate in the process). The pH profiles of the full reverse reaction of pyruvate carboxylase in which oxalacetate decarboxylation is coupled to ATP formation and where P_i is the variable substrate do, however, indicate that such an acid–base catalyst is involved in the other partial reaction of the enzyme in proton transfer to and from biotin. The enzyme also displays two oxamate-independent oxalacetate decarboxylating activities, one of which is biotin-dependent and the other is independent of biotin.

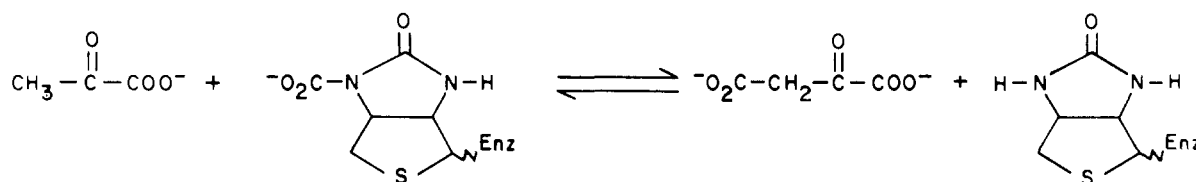Pyruvate carboxylase (EC 6.4.1.1) catalyzes the following reactions:

$$HCO_3^- + MgATP +$$
$$E\text{–}biotin \underset{}{\overset{acetyl\text{-}CoA,\ Mg^{2+}}{\rightleftharpoons}} E\text{–}biotin\text{–}CO_2^- + MgADP + P_i \quad (1)$$

$$E\text{–}biotin\text{–}CO_2^- + pyruvate \rightleftharpoons E\text{–}biotin + oxalacetate \quad (2)$$

These partial reactions are thought to occur at spatially

Scheme I

distinct subsites within the active site of the enzyme, with the biotin prosthetic group acting as a mobile carboxyl group carrier between the subsites. Isotopic exchange reactions between ATP and [^{32}P]P$_i$ (Utter & Keech, 1963a) and ATP and [^{14}C]ADP (Utter & Keech, 1963b) occur in the absence of oxalacetate or pyruvate. The exchange reaction between oxalacetate and [^{14}C]pyruvate also occurs in the absence of adenine nucleotides, bicarbonate, phosphate, and Mg^{2+} (Scrutton & Young, 1972). Thus, reactions 1 and 2 can occur independently. A structural representation of reaction 2 is shown in Scheme I. This scheme makes it clear that the overall reaction involves the exchange of a carboxyl group and a proton between pyruvate and biotin. We are interested in how and in what order these transfers occur.

Goodall et al. (1981) found that oxamate (H$_2$NCOCOO$^-$) stimulated the decarboxylation of the isolated enzyme–carboxybiotin complex. They suggested that the binding of oxamate acts as a signal for the movement of the carboxybiotin from the site of the first partial reaction to the site of the second partial reaction where it is labile. It therefore seemed likely that the presence of oxamate would greatly stimulate the enzymic decarboxylation of oxalacetate, since in the absence of oxamate the rate-limiting step in the reaction would be the very slow decarboxylation of carboxybiotin (Attwood et al., 1984). By using oxamate in this way, it is possible to study reaction 2 in isolation from reaction 1, and we report in this paper the kinetics of this reaction. In the following paper (Attwood et al., 1986) we report isotope effect studies that suggest possible chemical mechanisms for the reaction.

MATERIALS AND METHODS

Materials. Avidin was obtained from Sigma, while all other materials were high-purity preparations from commercial suppliers, except for chicken liver pyruvate carboxylase, which was purified as described by Goss et al. (1979). The enzyme was purified to specific activities of 18–22 units (mg of protein)$^{-1}$ (1 unit of enzymic activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of oxalacetate min^{-1} under saturating substrate conditions at 30 °C). This definition applies where units of pyruvate carboxylase are referred to in the text and figure legends.

Pyruvate Carboxylation Assay. The conditions for this assay were described by Duggleby et al. (1982). Reaction mixtures contained 2.5 mM ATP, 7 mM MgCl$_2$, 10 mM pyruvate, 20 mM NaHCO$_3$, 0.25 mM acetyl coenzyme A (acetyl-CoA), 0.24 mM NADH, and 11 units of malate dehydrogenase in 0.1 M *N*-[tris(hydroxymethyl)methyl]glycine (Tricine), pH 7.8.

Oxalacetate Decarboxylation Assays Linked to ATP Production. Reaction mixtures contained the following, unless otherwise stated: 2 mM ADP, 10–50 mM P$_i$, 0.1 mM oxalacetate, 0.25 mM acetyl-CoA, 3 mM MgCl$_2$, 0.24 mM NADH, 2 mM glucose, and 10 units of each of hexokinase and lactate dehydrogenase. The buffer used for the pH profiles was a mixture of 50 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris), 25 mM Tricine, and 25 mM glycine, which is a constant ionic strength buffer system (Ellis & Morrison, 1982). In all other experiments, 0.1 M Tricine,

pH 7.8, was the buffer. The reaction volume in all cases was 1 mL, and the experiments were performed at 25 °C. The levels of oxamate used did not inhibit the coupling enzymes.

Pyruvate release was monitored by its conversion to lactate by lactate dehydrogenase, and the consequent oxidation of NADH was observed by the change in absorbance at 340 nm. ATP was removed from the system by hexokinase, which converts it to ADP while phosphorylating the glucose. In all cases the reaction was started by the addition of pyruvate carboxylase, but prior to its addition, a background rate of oxalacetate decarboxylation was established, and this rate was subtracted from the rate in the presence of pyruvate carboxylase.

Oxalacetate Decarboxylation Assays in the Presence or Absence of Oxamate. Where oxalacetate was variable substrate, the reaction mixtures contained the following, unless otherwise stated: 24 μM NADH, 0.25 mM acetyl-CoA, 0.5 mM oxamate, 1–5 μM oxalacetate, and 10 units of lactate dehydrogenase. The buffers were as described above except at pH 5 where 0.025 M acetate was used with the addition of NaCl to maintain the ionic strength at the level of the normal buffer system. The reactions were performed as described for the ATP-linked assays except that a 10-cm pathlength cuvette was used with a reaction volume of 3 mL. In the case where oxamate was the variable substrate, the concentration of oxalacetate was 0.5 mM, and the buffer used was 0.1 M Tricine, pH 7.8. The reaction volume in the assays was 1 mL, and the path length of the cuvette used was 1 cm.

Biotin Dependence of Oxalacetate Decarboxylation in the Absence of Oxamate. Three solutions were prepared as follows in final volumes of 120 μl: (1) 1.1 mg of pyruvate carboxylase (0.078 mM biotin) + 3 mg of avidin (1.5 mM biotin-binding sites) [avidin is a protein that binds biotin very tightly and thus is a potent inhibitor of pyruvate carboxylase (Duggleby et al., 1982)]; (2) 1.1 mg of pyruvate carboxylase; (3) 3 mg of avidin. The solutions were incubated at pH 7.8 and 25 °C for 1.5 h, after which an aliquot of solution 1 was assayed for pyruvate carboxylating activity. The enzyme was found to be fully inhibited. Aliquots of 20 μL of each solution were then assayed for oxalacetate decarboxylating activity at pH 7.8 in the absence of oxamate at 1.25 μM, 0.1 mM, and 1 mM oxalacetate.

Data Analysis. All data were fitted by the least-squares method with a program written by Duggleby (1981). Individual saturation curves were fitted to eq 3, while the curves

$$v = VA/(K + A) \qquad (3)$$

for the decarboxylation of oxalacetate in the presence of oxamate in the range of concentrations of these molecules where substrate inhibition was evident were fitted to eq 4. The pH

$$v = VA/(A + K + A^2/K_i) \qquad (4)$$

profile that decreased both above pK_2 with a slope of -1 and below pK_1 with a slope of 1 was fitted to eq 5. The pH profile

$$\log Y = \log [C/(1 + H/K_1 + K_2/H)] \qquad (5)$$

that decreased with a slope of -1 above pK_3 and decreased with a slope of 2 at acid pHs was fitted to eq 6.

$$\log Y = \log (C/[1 + H/K_1 + K_3/H + H^2/(K_1 K_2)]) \qquad (6)$$

Table I: Oxalacetate Decarboxylating Activities of Pyruvate Carboxylase at pH 7.8

activity	V_{max} (μmol min^{-1} mL^{-1})	% of pyruvate carboxyl- ation
pyruvate carboxylation	265	(100)
OAA decarboxylation (ATP linked)	26.0	9.8
OAA decarboxylation (+oxamate)	3.2	1.2
OAA decarboxylation (−oxamate)	0.10[a]	0.039
	0.01[b]	0.003

[a]Oxalacetate concentration 1–10 mM. [b]Oxalacetate concentration 1–5 μM.

RESULTS AND DISCUSSION

Comparison of Oxalacetate Decarboxylating Activities to Pyruvate Carboxylation. Table I shows the various catalytic activities of pyruvate carboxylase, expressed as a percentage of the forward pyruvate carboxylation reaction. As can be seen, the oxamate-stimulated decarboxylation reaction has a V_{max} that is only about 12% of the reaction linked to ATP formation. This indicates that the rate-limiting step lies in the decarboxylation of carboxybiotin induced by oxamate. It is not clear whether oxamate is actually carboxylated by carboxybiotin and then decomposes or merely activates the changes in the enzyme that lead to decarboxylation of carboxybiotin.

In the absence of oxamate, the decarboxylation of oxalacetate occurs at different rates, which depend on the concentration range of oxalacetate used. At low concentration (1–5 μM), which correspond to the levels used in most of the oxamate-dependent assays, the oxamate-independent reaction is only 0.3% of the reaction in the presence of oxamate. At higher concentrations of oxalacetate, the reaction is about 3% of the reaction in the presence of oxamate.

Kinetics of Oxalacetate Decarboxylation in the Presence and Absence of Oxamate. Figure 1 shows the kinetics of the reaction in the presence of oxamate when either oxamate or oxalacetate is varied at a fixed concentration of the other. Both profiles show substrate inhibition at high concentrations of the varied component, and this indicates that oxamate and oxalacetate are competing for the same binding site on the enzyme. The apparent K_m for oxalacetate calculated from Figure 1B was 3 μM, thus explaining the necessity for the use of a 10-cm cuvette in the experiments where oxalacetate is the varied substrate. In subsequent experiments where oxalacetate was the varied substrate, a concentration range of 1–5 μM oxalacetate was used, since reciprocal plots were linear in this range. This low K_m for oxalacetate is the result of the oxamate-induced decarboxylation of carboxybiotin being much slower than the carboxylation of biotin by oxalacetate. The K_m for oxalacetate decarboxylation linked to ATP formation is 28 μM (Barden et al., 1972).

When acetyl-CoA was omitted from the reaction, the rate of oxalacetate decarboxylation was seen to decrease during the course of the assay. In the reaction mixture, the enzyme concentration was about 0.2 unit mL^{-1}, which is well below the 3–4 units mL^{-1} under which the phenomenon of dilution inactivation is manifested in the absence of acetyl-CoA (Ashman et al., 1972). It is probable that the observed decrease in rate during the assay in the absence of acetyl-CoA is due to dilution inactivation. This observation seems to be contrary to the unpublished data reported by Attwood and Keech (1984), which showed that dilution inactivation does not affect the oxalacetate-[^{14}C]pyruvate isotopic exchange reaction. In both types of reaction all the chemistry takes place
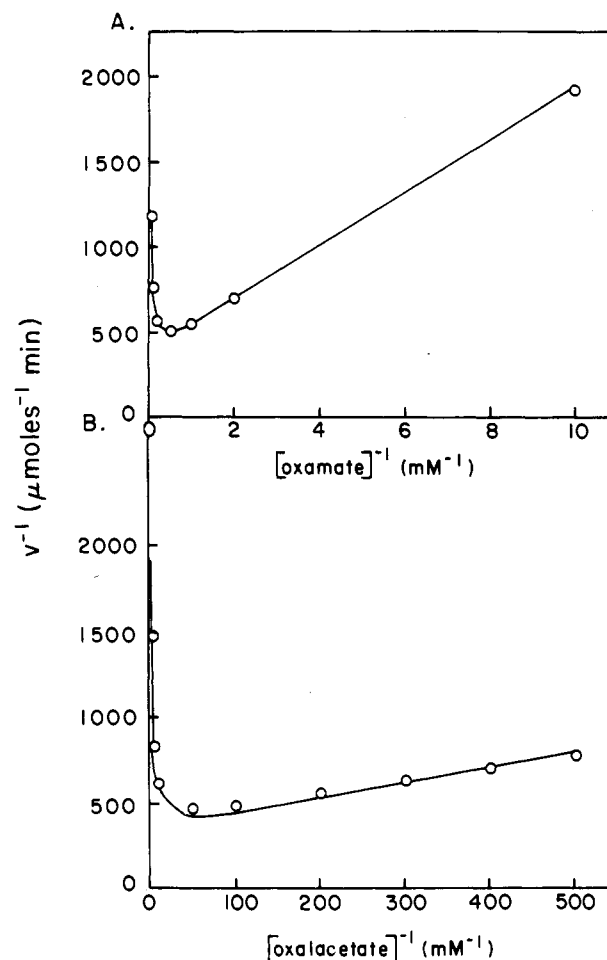

FIGURE 1: Reciprocal plots of oxalacetate decarboxylation reaction in the presence of oxamate, where either oxamate or oxalacetate was varied at a fixed concentration of the other. The reactions were carried out in 0.1 M Tricine, pH 7.8, at 25 °C with 0.24 unit of pyruvate carboxylase per assay. (A) Oxamate was the varied substrate at an oxalacetate level of 0.5 mM. From a fit to eq 4, K_m was 0.5 mM and K_I was 8.4 mM. (B) Oxalacetate was the varied substrate at an oxamate level of 0.5 mM. From a fit to eq 4, K_m was 3.1 μM and K_I was 95 μM.

at the enzymic subsite where reaction 2 occurs, and it seems likely that a factor that affects one reaction would have a similar effect on the other. Dilution inactivation has been suggested to result at least partially from a moving apart of the enzymic subsites so that the biotin can no longer interact with the subsite at which reaction 1 occurs (Attwood & Keech, 1984). The interaction of biotin with the subsite at which reaction 2 occurs may also be affected by dilution inactivation.

Biotin Dependence of Oxalacetate Decarboxylation in the Absence of Oxamate. The oxalacetate decarboxylating activity of pyruvate carboxylase is low in the absence of oxamate, and there are relatively large background rates of nonenzymic decarboxylation to be subtracted at high oxalacetate concentrations. This makes the quantitative interpretation of the velocity profiles difficult; however, it is evident that at low oxalacetate concentrations (1–50 μM) the velocity of the reaction increases with increasing substrate concentration. Above about 50 μM oxalacetate, the velocity decreases, but then above 1 mM oxalacetate, the velocity again increases with increasing substrate concentration.

Table II shows the biotin dependency of the oxalacetate decarboxylation reaction in the absence of oxamate at different concentrations of oxalacetate. At low oxalacetate concentrations, the reaction seems to be entirely dependent on biotin and presumably corresponds to the reaction in the presence

Scheme II

$^-O_2C-C(=O\cdots Mn^{2+})-CH(H)-H$ ... (chemical scheme structures)
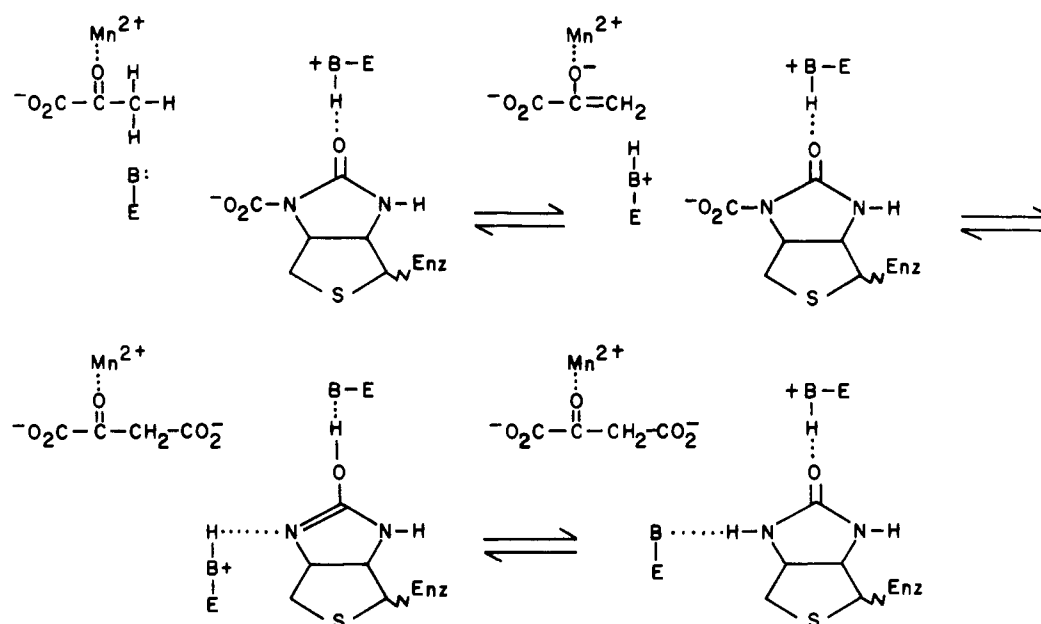
Table II: Biotin Dependence of Rate of Oxalacetate Decarboxylation in the Absence of Oxamate at Different Levels of Oxalacetate[a]

	addition		[oxalacetate] (μM)		
	pyruvate carboxylase (1.1 mg)	avidin (3.3 mg)	1000	100	1.25
v_1	+	+	6.75	0.83	0
v_2	+	–	7.94	10.60	4.29
v_3	–	+	0.99	0	0
% of reaction dependent on biotin[b]			27.5	92	100

[a] Velocities are expressed as μmol min^{-1} × 10^4. See Materials and Methods for experimental details. [b] Percent of reaction dependent on biotin was calculated as $100(v_2 - v_1 + v_3)/v_2$.

of oxamate exept that the rate of decarboxylation of carboxybiotin is much slower. The decarboxylation of the isolated enzyme–carboxybiotin complex has been shown by Attwood et al. (1984) to show similar first-order kinetics (except for the rate) and similar inhibition by high levels of Mg^{2+} in the presence or absence of excess pyruvate. These authors have thus proposed that carboxybiotin is decarboxylated in the absence of pyruvate at the subsite where reaction 2 normally occurs.

The decrease in reaction velocity observed at the intermediate range of oxalacetate concentrations appears caused by competitive substrate inhibition; as the concentration of oxalacetate increases, the catalytic site on the enzyme is more frequently occupied by oxalacetate. Since the decarboxylation of carboxybiotin appears to occur in this catalytic site, the presence of oxalacetate prevents this reaction and hence slows down the completion of the catalytic cycle. This effect presumably also causes the substrate inhibition seen at high oxalacetate concentrations in the presence of oxamate.

At 0.1 mM oxalacetate, the main decarboxylation activity is still biotin dependent, but at 1 mM oxalacetate only 28% of the activity is biotin-dependent. This suggests that the enzyme is catalyzing the decarboxylation of oxalacetate in a way that does not involve carboxybiotin formation. This phenomenon was observed previously by Scrutton and Mildvan (1968) at 3.5 mM oxalacetate. As this biotin-independent

reaction becomes faster (it has a high K_m of ∼10 mM), the decrease in velocity of the biotin-dependent reaction is overcome, and the net result is an increase in the rate of oxalacetate decarboxylation.

It is interesting that at high concentrations of oxalacetate avidin alone will catalyze the decarboxylation reaction, and it may be that lysine residues on both avidin and pyruvate carboxylase are catalyzing the reaction via Schiff base formation in the way described by O'Leary (1977). In pyruvate carboxylase, this residue or residues is (are) probably not in the active site, since the bulky avidin molecule would be expected to impose some restriction on the access of oxalacetate to the active site.

pH Profiles of Oxalacetate Decarboxylation in the Presence of Oxamate. Figure 2 shows the pH profiles of V and V/K for the reaction where oxalacetate was varied at a fixed concentration of oxamate. Neither profile shows evidence of the involvement of an ionizable group in the reaction with a pK within the range of pHs studied. The pH profiles for oxalacetate decarboxylation where oxamate was varied at a fixed concentration of oxalacetate also are pH-independent. In addition, the V/K profile for oxalacetate in which the decarboxylation was linked to ATP formation also shows no sign of the involvement of an ionizable group.

Two possible explanations for this apparent lack of an acid–base catalyst are, first, that there is such a catalyst but in the free enzyme its pK lies outside of the range of pHs studies. When oxalacetate or oxamate and biotin bind to that enzymic subsite, its pK would have to shift into the neutral pH range. In this case the reaction could proceed as shown in Scheme II, where we have not specified the chemistry involved in carboxyl transfer [the coordination of enzyme-bound Mn^{2+} by pyruvate and oxalacetate may be a second sphere rather than inner sphere interaction (Fung et al., 1974)].

The second explanation is that there is no such ionizable group present and that there is direct transfer of the proton between biotin and pyruvate, possibly as shown in Scheme III. In this scheme both carboxybiotin and oxalacetate undergo decarboxylation prior to proton transfer to form CO_2 and the enolate of either biotin or pyruvate. We will discuss this and other possible mechanisms for this reaction in the following paper.
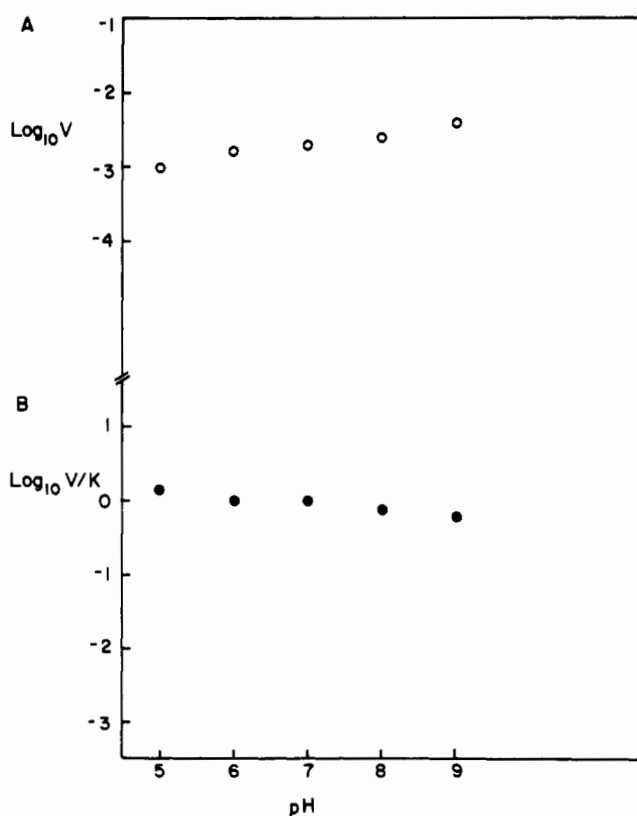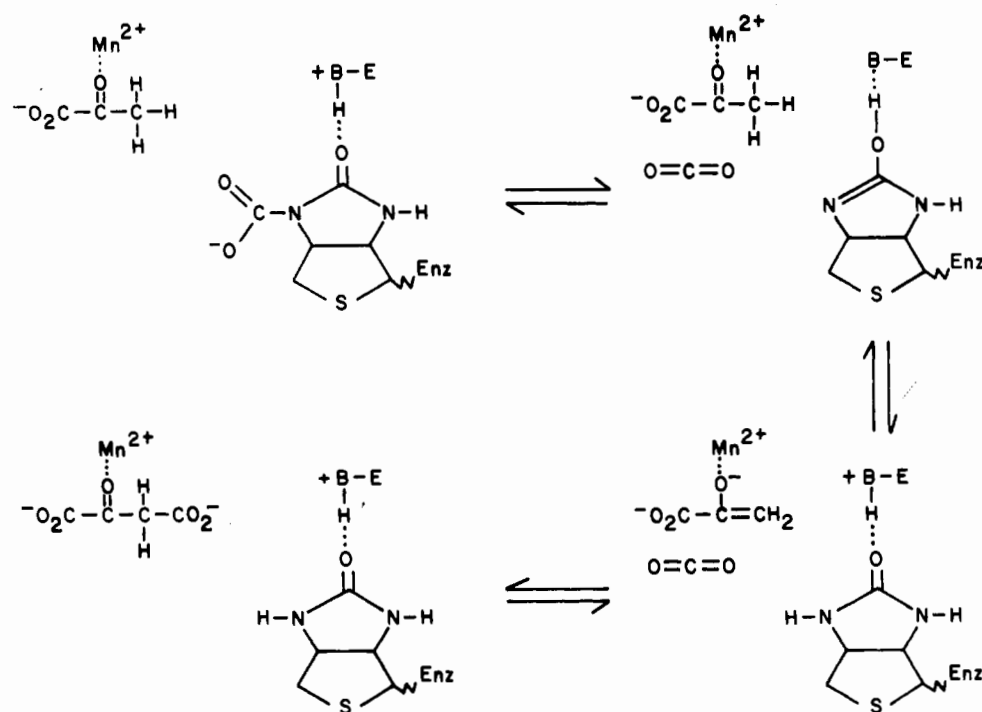
Scheme III

FIGURE 2: pH profiles for decarboxylation of oxalacetate in the presence of oxamate. The reactions were carried out in 0.05 M Bis-Tris, 0.025 M Tricine, and 0.025 M glycine at 25 °C in a reaction volume of 3 mL in a 10-cm path-length cuvette. A total of 0.3 unit of pyruvate carboxylase was used per assay.

pH Profiles of Oxalacetate Decarboxylation Reaction Coupled to ATP Formation. Figure 3 shows the pH profiles for the decarboxylation of oxalacetate where all substrates are at fixed concentrations except P_i. On the low pH side of the V/K profile, there is evidence of an ionizable group with a pK of 7.6, and in the V profile a pK of 7.0 is observed. These pK's
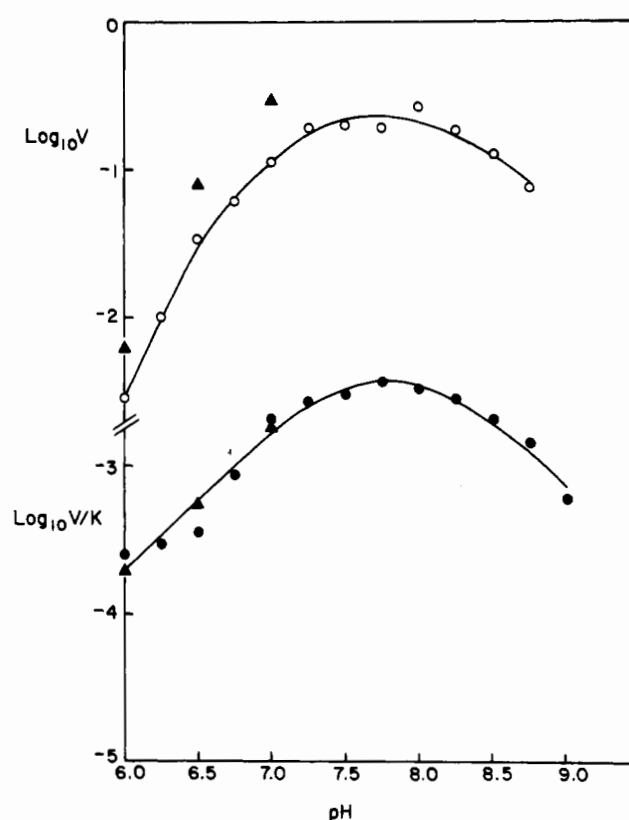
FIGURE 3: pH profiles for decarboxylation of oxalacetate coupled to ATP formation. The reactions were carried out in 0.05 M Bis-Tris, 0.025 M Tricine, and 0.025 M glycine containing 3 mM $MgCl_2$ at 25 °C in a reaction volume of 1 mL, with 0.3 unit of pyruvate carboxylase per assay with P_i as the variable substrate. (▲) Reaction was performed in the presence of 7 mM $MgCl_2$. From a fit of the V profile to eq 6, pK_1 = 7.0, pK_2 = 7.° and pK_3 = 8.3. From a fit of the V/K profile to eq 5, pK_1 = 7. nd pK_2 = 7.9.

could represent the ionization of P_i, which would indicate that protonation of the dianion does not affect binding but does interfere with catalysis. On the other hand, these pK's could represent an ionizable group(s) on the enzyme. The pK_I profile

of an inhibitor that is competitive with respect to P_i may help to resolve this question.

On the high pH side of the profiles there is evidence of an ionizable group with a pK of about 8 that is required to be protonated in this reaction. After the removal of the carboxyl group from carboxybiotin, probably to form carboxy phosphate (Attwood & Keech, 1984), the biotin must be reprotonated, and this may be the function of this protonated group. In the forward reaction this base would deprotonate biotin to form the enolate, which is strongly nucleophilic and could react with carboxyphosphate or (more likely) with CO_2 derived by decarboxylation of carboxyphosphate.

A second pK with a value of about 7 is evident on the acid side of the V profile but is not seen in the V/K profile. The reaction was carried out with the lactate dehydrogenase coupling system to monitor pyruvate release, but also in the presence of hexokinase and glucose to remove ATP. Since the steps between oxalacetate binding and pyruvate release have been shown to be pH-insensitive and the effect is on V alone, and not on V/K for P_i, the pH-sensitive step must involve either the release of the second product (probably MgATP) or the translocation of biotin or carboxybiotin between the two active sites.

To obtain the pH profiles, the reactions were carried out with a suboptimal concentration of Mg^{2+} because of solubility problems at the higher pHs with $MgHPO_4$. As shown in Figure 3, when higher Mg^{2+} concentrations were used at neutral and acid pHs, V was increased by a uniform degree across the pH range while V/K was essentially unaffected. The V profile still shows a slope of 2 at low pH, however. Since raising $[Mg^{2+}]$ only affects V, the Mg^{2+}-sensitive step must involve faster release of the second product, or the movement of biotin between the active sites. It is known that Mg^{2+} affects the translocation of carboxybiotin between the enzymic subsites (Attwood et al., 1984), and Goodall et al. (1981) suggested that Mg^{2+} enhances the binding of carboxybiotin to the site of the first partial reaction. It is therefore possible that the effect of increasing $[Mg^{2+}]$ on V is produced by the enhancement of the rate of carboxybiotin binding to the site of the first partial reaction, which is rate-limiting for V.

In this paper we have demonstrated that pyruvate carboxylase catalyzes an oxamate-stimulated decarboxylation of oxalacetate that involves chemistry only at the enzymic subsite at which pyruvate is normally carboxylated. This enables the study of this partial reacton without the complication of linking oxalacetate decarboxylation to the first partial reaction involving ATP formation. We have defined the kinetics of the reaction and have made some comparison with the first partial reaction in terms of pH profiles. The information obtained in this paper has been exploited in the following paper in a further analysis of the oxamate-stimulated oxalacetate decarboxylation reaction using kinetic isotope effects.

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