Secondary ^{18}O and Primary ^{13}C Isotope Effects as a Probe of Transition-State Structure for Enzymatic Decarboxylation of Oxalacetate†

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ABSTRACT: A new method for directly measuring ^{18}O isotope effects on decarboxylation reactions has been developed. By running the reaction under high vacuum (10^{-5} torr), CO_2 leaves the solution before exchange with the oxygens of water to an extent greater than 2%. Thus, the method permits determination of ^{18}O isotope effects with the precision of the isotope ratio mass spectrometer, and without the necessity of resorting to the remote label method and its attendant required syntheses. The method is used to determine ^{18}O isotope effects for decarboxylation of oxalacetate (OAA) by Mg^{2+}, and enzymatically by OAA decarboxylase from *Pseudomonas putida*; ^{13}C isotope effects are also reported for this enzyme, as well as for decarboxylation of OAA by pyruvate kinase. Initial velocity patterns and pH profiles are reported for the *P. putida* enzyme, and all available data are used to discuss the kinetic and chemical mechanism of decarboxylation.

Very useful mechanistic information can be obtained from accurately determined secondary isotope effects on enzyme-catalyzed reactions (Cleland, 1987a–c). For example, secondary ^{18}O isotope effects on decarboxylation of formate by formate dehydrogenase indicate formate is bound in the enzyme–nucleotide complex in such a way that it is desolvated but not bound as a tight ion-pair (Hermes et al., 1984a). Unfortunately, in order to determine these secondary ^{18}O isotope effects, a significant amount of synthetic chemistry was required. Paneth and O'Leary (1985), however, found that CO_2 could be isolated under high vacuum (10^{-5} torr) without ^{16}O–^{18}O solvent exchange during their studies of the dehydration of bicarbonate by carbonic anhydrase. In this report, the nonenzymatic and enzymatic decarboxylation of oxalacetate (OAA)[1] was used as a model system to test the method of Paneth and O'Leary (1985) for measuring secondary ^{18}O isotope effects. Thus, CO_2 isolation under high vacuum was applied to decarboxylation reactions for the purpose of directly determining the secondary ^{18}O isotopoe effects using the natural abundance of ^{18}O as the label.

Detailed studies of divalent metal cation-dependent enzyme-catalyzed oxalacetate (OAA) decarboxylation have been accomplished for very few enzymes. Pyruvate kinase can catalyze the divalent metal cation-dependent decarboxylation of OAA (Creighton & Rose, 1976), as can malic enzyme, one of the few enzyme systems for which the decarboxylation of OAA has been extensively investigated (Park et al., 1986; Grissom & Cleland, 1988). While studies of OAA decarboxylation by pyruvate kinase and malic enzyme have been quite informative, *in vivo* OAA is not the natural substrate for these enzymes. OAA decarboxylase (EC 4.1.1.3) from *Pseudomonas putida*, however, does catalyze the decarboxylation of OAA to pyruvate and CO_2 *in vivo*. The only known requirement for *P. putida* enzyme activity is that of a divalent metal cation, either Mg^{2+} or Mn^{2+} (Piccirilli et al., 1987). The metal cation is thought to form a complex with the α-keto acid moiety of OAA, acting as an electron sink stabilizing the enolate intermediate (Westheimer & Steinberger, 1951). The role the enzyme plays in further stabilizing the transition state for the reaction, as well as the catalytic mechanism of the *P. putida* enzyme which has no other known catalytic activity or function, has not been directly addressed.

The present study was initiated in order to define the role the *P. putida* enzyme plays in decarboxylating OAA and also to determine the usefulness of secondary ^{18}O isotope effects in describing the transition state for the reaction. The studies that follow report on the pH dependence of the kinetic parameters for oxalacetate decarboxylase, as well as the secondary ^{18}O and primary ^{13}C isotope effects on the divalent metal cation-catalyzed and enzyme-catalyzed decarboxylation of OAA; in conjunction with the pH dependence of the kinetic parameters, these data are used to develop a catalytic mechanism for the enzyme. The ^{13}C isotope effect for the decarboxylation of OAA by pyruvate kinase is also reported and discussed in context with the proposed mechanisms and transition state for the reactions.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes. The oxalacetate and oxalate used in this study were obtained from Sigma. The NADH was purchased from Boehringer Mannheim. The Good's buffers were from Research Organics. All other reagents and chemicals obtained from commercial sources were of the highest quality available.

Oxalacetate decarboxylase from *P. putida* was obtained from Sigma as a lyophilized powder. The enzyme was reconstituted in 20 mM Hepes, pH 8.0, at an activity of 540 units/mg. This source of enzyme is free of pyruvate kinase,

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[1] Abbreviations: Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; LDH, lactate dehydrogenase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; OAA, oxalacetate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Taps, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid.

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as no activity was found for dephosphorylation of PEP in the presence of ADP under conditions where both substrates for pyruvate kinase were saturating. Aliquots of the enzyme solution were kept frozen until the day of use. Lactate dehydrogenase from hog muscle purchased from Boehringer-Mannheim was a glycerol preparation with an activity of 350 units/mL.

^{13}C Isotope Effect Studies. The ^{13}C isotope effects were determined using the methodology outlined by O'Leary (1980). The natural abundance of ^{13}C was used as the label in the substrate, and the isotope effects were determined by conducting isotope ratio mass spectral analysis of the CO_2 produced from the enzymatic reaction samples. Either the oxalacetate present in the reaction mixtures was totally decarboxylated to CO_2 and pyruvate (high-conversion samples) or the reaction mixture was quenched after 10–20% of the oxalacetate was decarboxylated (low-conversion samples). The carbon mass ratio in the substrate was determined from the high-conversion samples, while the low-conversion samples measure the isotopic discrimination by the enzyme.

(A) Oxalacetate Decarboxylase and Mg^{2+}-Catalyzed Decarboxylation of OAA. A CO_2-free stock solution of OAA was prepared by dissolving OAA in ice-cold H_2O and adjusting the pH to 5.0 with saturated KOH. The OAA solution was sparged for 3 h with N_2 that had been passed through both an ascarite column to remove CO_2 and acidic water to hydrate the N_2 to prevent water loss from the OAA solution. After sparging, aliquots of the OAA solution were stored at −135 °C. In order to conserve enzyme for the high-conversion samples, 2.4 mM OAA was decarboxylated in the presence of 100 mM $MgOAc_2$, pH 5.5, in a 10-mL reaction volume; this solution was sparged in the reaction vessel for 3 h to remove CO_2 before OAA was added. After an overnight incubation at room temperature, 1 mL of 18 N H_2SO_4 was added, and the CO_2 was isolated by high-vacuum distillation.

The low-conversion samples at pH 5.5 contained 45 mM OAA, 10 mM Mes, 1 mM $MgCl_2$, and 5 units of OAA decarboxylase in a 10-mL volume. The Mes/$MgCl_2$ solution was also sparged for 3 h prior to addition of enzyme and OAA. To prepare a stock solution of enzyme (1 unit/μL), the enzyme was dissolved in 1 mL of the sparged reaction buffer. The reaction was initiated by addition of OAA. After a 3-min incubation at room temperature, 1 mL of ice-cold 18 N H_2SO_4 was added to quench the reaction. The CO_2 was isolated immediately by high-vacuum distillation. The low-conversion samples at pH 9.45 contained 10 mM Ches, 0.0032 mM $MgCl_2$, and 25 units of OAA decarboxylase. The Ches/$MgCl_2$ solution was sparged at pH 5.5 for 3 h to remove CO_2, and then the pH was raised to 9.45 with saturated KOH and sparging continued for another hour.

Low-conversion samples for decarboxylation of OAA catalyzed by Mg^{2+} contained 50 mM $MgOAc_2$, pH 5.5, and 45 mM OAA in a 10-mL volume. The $MgOAc_2$ solution was sparged in the reaction vessel for 3 h to remove CO_2 prior to addition of OAA. After a 2.5-min incubation at room temperature, 1 mL of ice-cold 18 N H_2SO_4 was added to fully protonate the OAA to the neutral species which decarboxylates 100-fold slower than the dianion at 25 °C (Grissom & Cleland, 1986). Immediately after the addition of acid, the reaction vessel was put on ice to further reduce the rate of decarboxylation, and the CO_2 was isolated by high-vacuum distillation.

The fraction of reaction for both the Mg^{2+}- and enzyme-catalyzed reactions was determined by dividing the amount of CO_2 isolated from the low-conversion samples by the total amount of OAA added to the reaction. Malate dehydrogenase enzymatic end-point analysis was used to determine the stock solution concentration of OAA.

(B) Pyruvate Kinase-Catalyzed Decarboxylation of OAA. Pyruvate kinase was dissolved in ice-cold, CO_2-free buffer (100 mM Hepes, pH 7.5, containing 0.5 mM EDTA) and dialyzed overnight at 4 °C versus 2 L of the same buffer with continuous sparging with CO_2-free N_2 until the enzyme was added to the reaction mixture. A stock oxalacetate solution was made by dissolving OAA in ice-cold H_2O, adjusting the pH to ~6 with saturated NaOH, and sparging the solution with CO_2-free N_2 on ice for 2–3 h before adding the other components to initiate the reaction. During sparging, the pH of the stock OAA solution was periodically monitored and readjusted to pH 6.0, if necessary, with saturated NaOH. All other components of the reaction mixtures (pH 6) were sparged at 23–25 °C for 3 h after which the pH was raised to 7.5 with saturated NaOH and sparging continued for 2 more h. Pyruvate kinase was then added to the reaction mixtures (minus OAA). The concentration of the stock OAA solution was quickly determined by end-point analysis with malate dehydrogenase and excess NADH just before OAA addition to the reaction flask in order that the appropriate amount of OAA could be added. Addition of OAA started the reactions. The low-conversion reaction mixtures contained, after addition of OAA, 100 mM Hepes, 0.5 mM EDTA, 2.0 mM $MgSO_4$, and 20 mM OAA, pH 7.5, in 20 mL. The high-conversion mixtures contained 50 mM $MgSO_4$, 2.0 mM OAA, and no enzyme with the same concentrations of Hepes and EDTA as the low-conversion mixtures. The low-conversion reactions were allowed to proceed for a short time so that ca. 0.04 mmol of CO_2 was produced. The high-conversion reactions were also allowed to proceed until all the OAA was decarboxylated (also 0.04 mmol). The low-conversion reactions were stopped by addition of 1.0 mL of cold, 18 N H_2SO_4, and placed on ice until the CO_2 could be isolated (<3 h maximum). In a similar fashion, the high-conversion reaction mixtures were acidified before isolation of the CO_2. No CO_2 could be isolated from the low-conversion control reaction mixtures which were acidified immediately after addition of OAA.

^{18}O Isotope Effect Studies. For measurement of ^{18}O isotope effects, the low-conversion samples were run under conditions that were exactly the same as described above for the ^{13}C isotope effects, except a high vacuum (10^{-5} torr) was applied to the samples in order to minimize ^{16}O–^{18}O exchange of the isolated CO_2 with solvent. Isolation of CO_2 without ^{16}O–^{18}O solvent exchange was necessary to preserve the $^{18}O/^{16}O$ ratio generated by isotopic discrimination during the decarboxylation reactions. Paneth and O'Leary (1985) found CO_2 could be isolated under high vacuum without ^{16}O–^{18}O solvent exchange during their investigation of dehydration of bicarbonate by carbonic anhydrase. In order to determine the optimal conditions for isolating CO_2 with minimal ^{16}O–^{18}O exchange, parallel decarboxylation reactions with a small aliquot (0.025 mL) of 33% $H_2{}^{18}O$ added to the solvent were run either under vacuum or under N_2 where the CO_2 could freely and completely exchange with solvent. The degree to which the oxygens of the CO_2 isolated under vacuum had exchanged with the solvent oxygens was assessed by taking the ratio of the ^{18}O Δ value for CO_2 isolated under vacuum to the ^{18}O Δ value for CO_2 isolated under nitrogen where the CO_2 was allowed to equilibrate with solvent before isolation. The ^{18}O Δ values for the solvent water and the substrate OAA were 3.5 and −7.5, respectively.

The following conditions gave only 2% exchange of the ^{16}O–^{18}O of the isolated CO_2 with solvent. This small amount of exchange affected the resultant isotope effect only within

the error of the measurement. Reactions were run in a 250-mL round-bottom flask in a volume of 10 mL with vigorous stirring of the reaction mixture using a large, circular (1.6-cm diameter) stir bar which was found to be crucial for preventing solvent exchange. Moreover, in order to achieve minimal ^{16}O–^{18}O exchange with solvent, reactions had to be run at pH 6 or below. All reactions were conducted at room temperature. The reactions were stopped by simply closing the stopcock that isolates the reaction vessel from the high-vacuum line. Reaction times of 2–5 min were sufficient to achieve fractional reactions of 10–20%.

The high-conversion samples, which determined the initial $^{18}O/^{16}O$ ratio on the β-carboxyl group of OAA, contained 10 mM Mes, pH 5.5, 1 mM $MgCl_2$, 2.4 mM OAA, and 100 units of OAA decarboxylase. The Mes/$MgCl_2$ solution was sparged for 3 h in the reaction vessel before addition of enzyme. The reaction was initiated with the addition of OAA and run at room temperature. Complete decarboxylation of OAA was accomplished in ~20 min.

Initial Velocity Studies. Oxalacetate decarboxylase was assayed spectrophotometrically on either a Gilford 250 or an OLIS modernized Cary 14 monochromator interfaced to a Compaq 386S computer using an OLIS 4300S Operating System for data acquisition. All assays were carried out at 25 °C. Reaction rates were calculated from the observed changes in absorbance at 340 nm which were measured by coupling the pyruvate produced to the oxidation of NADH (E_{340} = 6.22 mM^{-1} cm^{-1}) in the presence of excess LDH. The cuvettes were 1 cm in path length, and 1 mL in volume.

A typical 1-mL reaction mixture contained 100 mM buffer, 1 mM DTT, 0.2 mM NADH, 7 units/mL LDH, and varying concentrations of OAA (0.1–1 mM), $MgCl_2$ (0.025–1 mM), and oxalate (0.01–0.08 mM). Prior to addition of oxalacetate decarboxylase, a blank rate which was due to the Mg^{2+}-catalyzed decarboxylation of OAA was measured for each cuvette. At pH 6.5, this rate was as much as 50% of the rate measured after enzyme addition. As the pH increased, the percent blank rate dropped to less than 5% at pH 9.5.

pH Studies. Determination of the kinetic parameters for OAA, V and V/K, and the K_i for $MgCl_2$ was carried out by measuring initial velocities at various OAA concentrations at three or four fixed $MgCl_2$ concentrations. The oxalate $K_{is.}$ values, as well as V and V/K_{OAA}, were determined by measuring initial velocities at 0.1 mM $MgCl_2$, various OAA concentrations, and concentrations of oxalate equal to 0, $K_{is.}$, and $2K_{is.}$. The $MgCl_2$ concentration was held at 0.1 mM to minimize blank rate contributions during the oxalate inhibition studies.

Buffers used to obtain the pH profile were the following: Pipes, pH 6.5–7.0; Hepes, pH 7.5; Taps, pH 8.0–8.5; Ches, pH 9.0–9.55. All buffers were titrated to pH with KOH. Data were not collected below pH 6.5 due to the large blank rates of the Mg^{2+}-catalyzed decarboxylation of OAA. Above pH 10, formation of $Mg(OH)_2$ caused the solutions to become turbid and therefore unusable.

Data Analysis. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations, and all double-reciprocal plots were linear. Data were analyzed using the FORTRAN programs of Cleland (1979) written for the following equations. Initial velocity data in the absence of inhibitors were fitted to the rate equation for an equilibrium-ordered mechanism (eq 1)in which $MgCl_2$ binds first. In eq

$$v = VAB/(K_{ia}K_b + K_bA + AB) \quad (1)$$

1, A is the $MgCl_2$ concentration, B is the concentration of OAA, K_{ia} is the dissociation constant for $MgCl_2$, K_b is the Michaelis constant for OAA, and V is the maximum velocity extrapolated to infinite substrate concentration.

Data for linear competitive inhibition were fitted to eq 2. A is the concentration of OAA, I is the concentration of oxalate, $K_{is.}$ is the slope inhibition constant for oxalate, K_a is

$$v = VA/[K_a(1 + I/K_{is.}) + A] \quad (2)$$

the apparent Michaelis constant for OAA, and V is the maximum velocity extrapolated to infinite substrate concentration. Values of V/K obtained from the fit of inhibition data to eq 2 are apparent values since $MgCl_2$ is subsaturating. True V/K values were obtained by multiplying the apparent V/K values by a correction factor which takes into account both the concentration and the dissociation constant of $MgCl_2$ at each pH.

Data for pH profiles that decreased with a slope of 1 at high pH were fitted to

$$\log Y = \log[C/(1 + K_1/H)] \quad (3)$$

In eq 3, K_1 is the dissociation constant for an enzyme group, Y is the value of the parameter observed as a function of pH, and C is the pH-independent value of Y.

The carbon isotope effects were calculated using the equation (Bigeleisen, 1958):

$$^{13}(V/K) = \log(1 - f)/[\log(1 - fR_p/R_0)] \quad (4)$$

In eq 4, R_p is the $^{13}C/^{12}C$ isotope ratio of CO_2 product at fractional reaction, f, while R_0 is the initial $^{13}C/^{12}C$ isotope ratio of OAA determined by measuring the carbon mass ratio of the CO_2 product at 100% completion.

The secondary ^{18}O isotope effects were calculated using eq 4, except that R_p is the $^{18}O/^{16}O$ ratio in the CO_2 product at fractional reaction f and R_0 is the $^{18}O/^{16}O$ in the CO_2 collected after complete decarboxylation of an OAA sample. All $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios were determined using a Finnigan Delta E isotope ratio mass spectrometer. The $^{13}C/^{12}C$ ratio in the CO_2 was calculated from the 45/44 mass ratio after correction for ^{17}O (Santrock et al., 1985; Craig, 1957). The $^{18}O/^{16}O$ ratio was derived from the 46/44 mass ratio.

RESULTS AND DISCUSSION

Direct Determination of ^{18}O Isotope Effects on Decarboxylation Reactions. Previous studies of ^{18}O isotope effects on decarboxylation reactions used the remote label method which involves the synthesis of the $^{12}C/^{16}O$ and $^{13}C/^{18}O$ forms of the substrate (Hermes et al., 1984a). These syntheses can sometimes be expensive and tedious. To obviate the need for synthesis of labeled substrates, we have found that the ^{18}O isotope effect on a decarboxylation reaction can be measured directly by using the natural-abundance label of ^{18}O, running the reaction under high vacuum and subsequently isolating the CO_2 before it exchanges with solvent. This method was tested on both the nonenzymatic and the enzymatic decarboxylation of oxalacetate. In the case of the Mg^{2+}-catalyzed reaction, for which a 4.9% (Table 1) ^{13}C isotope effect indicates that the chemistry of the reaction is completely rate-limiting, a small inverse ^{18}O isotope effect is expected, and indeed a 0.4% inverse isotope effect is observed (Table 1). This value is comparable to the 0.5% inverse ^{18}O isotope effect previously measured for decarboxylation reactions with the remote label method (Hermes et al., 1984a; Headly & O'Leary, 1990). Thus, this direct determination of the ^{18}O isotope effect for a decarboxylation reaction does appear to be feasible with the only shortcoming being that the reaction must be run at pH

Table 1: Isotope Effects on Nonenzymatic and Enzymatic Decarboxylation of OAA

catalyst	pH	$^{13}(V/K)$	$^{18}(V/K)$	no.[b]
Mg^{2+}	5.5	1.0485 ± 0.0007		4
		1.0477 ± 0.0009[a]	0.9966 ± 0.0002[a]	4
OAA decarboxylase	5.5	1.0227 ± 0.0007		4
		1.0220 ± 0.0005[a]	0.9993 ± 0.0003[a]	4
	9.45	1.0231 ± 0.0005		4
pyruvate kinase	7.5	1.0546 ± 0.0001		3

[a] Experiments were performed under a vacuum; otherwise, procedures are as described under Experimental Procedures. [b] Number of determinations.

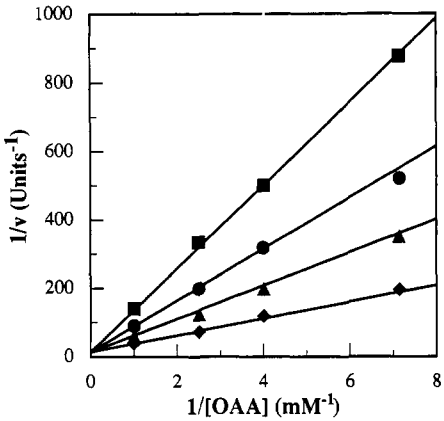

FIGURE 1: Double-reciprocal plot of initial velocity vs OAA concentration at varying $MgCl_2$ concentrations. The lines result from a fit of the entire data set to eq 1. The solutions were buffered with 0.1 mM Pipes, pH 6.96. $MgCl_2$ concentrations were 0.04 (■), 0.06 (●), 0.1 (▲), and 0.25 mM (♦).

6 or less. The method should also prove useful for measuring ^{18}O isotope effects on other types of enzymatic reactions which generate CO_2, such as biotin-dependent enzymes, where the transferred carboxyl group can be isolated as CO_2 by decarboxylation of carboxybiotin. The application of this method for characterization of the transition states of the nonenzymatic and enzymatic decarboxylation of oxalacetate is described below.

Initial Velocity Studies. Initial velocity patterns for the decarboxylation of OAA by OAA decarboxylase, such as the one shown in Figure 1, are indicative of a rapid-equilibrium ordered kinetic mechanism with Mg^{2+} adding to the enzyme prior to OAA. Thus, the kinetic mechanism for OAA decarboxylase is identical to that for the pyruvate kinase-catalyzed decarboxylation of OAA (Kiick & Cleland, 1989; Dougherty & Cleland, 1985). It is interesting to note that for the NAD-dependent malic enzyme, which can catalyze OAA decarboxylation, the kinetic mechanism is also rapid-equilibrium ordered with Mg^{2+} adding prior to malate (Park et al., 1984). Initial velocity patterns such as those shown in Figure 1, when obtained over the pH range 6.6–9.5 (see pH Dependence of Kinetic Parameters), indicate that the kinetic mechanism is pH-independent, again similar to pyruvate kinase.

pH Dependence of Kinetic Parameters. The pH dependence of the kinetic parameters, V and V/K, for *P. putida* OAA decarboxylase is shown in Figure 2. The maximum velocity is pH-independent throughout the pH range measured while V/K for OAA decreases above a pK value of 8.5 ± 0.1. On the basis of data (not shown) obtained in the UT–Memphis laboratory from both native and SDS–PAGE, as well as gel filtration of the decarboxylase enzyme vs known standards, the protein was identified to be a 64-kDa monomer; thus, the turnover number for OAA decarboxylase was calculated to

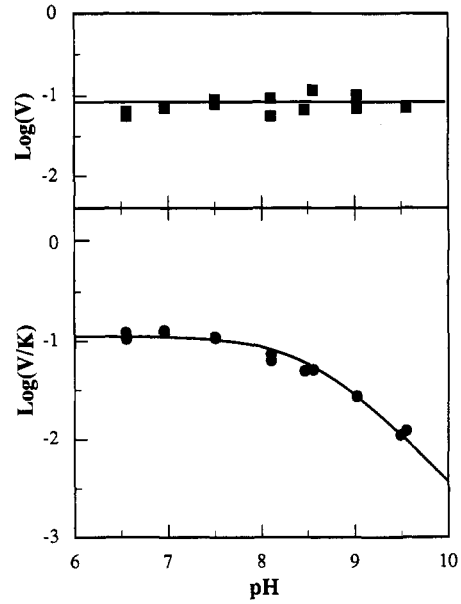

FIGURE 2: pH dependence of V and V/K_{OAA} for *Pseudomonas* OAA decarboxylase. The points represent the experimental values from fit of the initial velocity data to eq 1 if $[MgCl_2]$ was varied or to eq 2 if [OAA] was varied at fixed $[MgCl_2]$. The curve for V/K_{OAA} was determined by a fit of the data to eq 3.

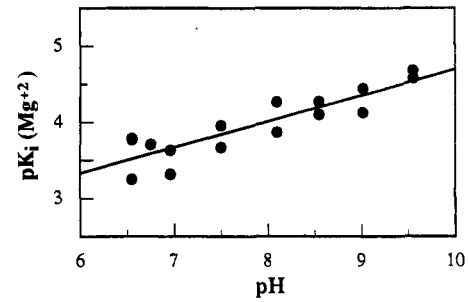

FIGURE 3: pH dependence of the dissociation constant of Mg^{2+} for *Pseudomonas* OAA decarboxylase. The K_i for Mg^{2+} was calculated either from initial velocity data (varying [OAA] at fixed levels of $MgCl_2$) fitted to eq 1 or from initial velocity data (varying $[MgCl_2]$ at 0.25 mM OAA) fitted to $K_{ia} = K_{app}([B] + K_b)/K_b$.

be 21.8 × 10^3 min^{-1}. The pH-independent values of the V/K for OAA is calculated to be 32.5 × 10^6 M^{-1} min^{-1}.

A pH–rate profile for V_{max} that is pH-independent is indicative of a catalytic mechanism where substrate binds only to the correctly protonated form of the enzyme. The results shown in Figure 2 indicate this to be the case for OAA decarboxylase. Also, for this type of mechanism, the pK values observed in the V/K pH profile are predicted to be the true ones; i.e., there should be no perturbation of the pK due to an external commitment factor (Cleland, 1977). Thus, the pK of 8.5 observed in the V/K pH profile for OAA is a true pK. Since there are no ionizable groups on OAA in the pH range studied, we can conclude this pK must be for an enzyme group. More will be said below about the role of this group in the discussion of the catalytic mechanism.

The pH dependence of the K_i for the divalent cation Mg^{2+} is shown in Figure 3. The Mg^{2+} pK_i appears to vary linearly with pH, exhibiting a slope of 0.34; thus, the K_i varies from 0.3 mM at pH 6.5 to 0.03 mM at pH 9.5. In contrast to pyruvate kinase, where an enzyme group exhibiting a pK of 7 must be protonated for the divalent cation to bind (Kiick & Cleland, 1989; Dougherty & Cleland, 1985), OAA decarboxylase apparently has no specific enzyme residue that affects Mg^{2+} binding in the pH range of 6–10.

Inhibition Data. Since the variable substrate concentration is extrapolated to zero in calculating a competitive inhibitor

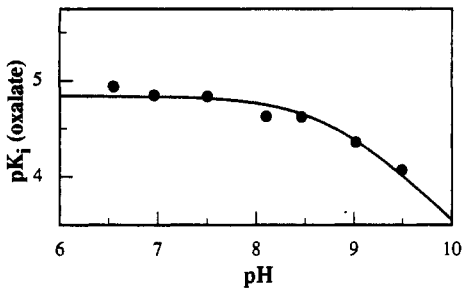

FIGURE 4: pH dependence of the dissociation constant of the competitive inhibitor oxalate for *Pseudomonas* OAA decarboxylase. The oxalate K_i for each pH was determined by fit of initial velocity data (varying [OAA] at fixed levels of oxalate and 0.1 mM $MgCl_2$) to eq 2. The points are the experimental values, while the curve drawn is from a fit of the pK_i values to eq 3.

dissociation constant, K_{is}, true pK values are obtained from the pH dependence of K_{is} for a competitive inhibitor. Thus, a comparison of the observed pK values in the V/K and pK_i pH profiles should be a valid test of the proposed catalytic mechanism for OAA decarboxylase. Oxalate can be considered a structural analog of the enolate of pyruvate and therefore should be a good competitive inhibitor vs OAA for the OAA decarboxylase reaction.

The pH dependence of the dissociation constant, K_{is}, for oxalate was determined from inhibition patterns obtained over the pH range 6.5–9.5. In all patterns, oxalate was competitive vs oxalacetate (data not shown but fitted to eq 2); the pK_i profile for oxalate is shown in Figure 4. The K_{is} for oxalate increases (pK_i decreases) above a pK value of 8.7 ± 0.1 which was calculated from a fit of the data to eq 3. The pH-independent value of the K_{is} was 0.014 ± 0.001 mM. A comparison of the pK values obtained in the pK_{is} and V/K pH profiles for oxalate and oxalacetate indicates that they are identical within experimental error. This is in agreement with the postulated mechanism for OAA decarboxylase were substrate binds only to the correctly protonated form of the enzyme.

Catalytic Mechanism. There are a number of possible roles a protonated enzyme group could play in facilitating catalysis. The mechanism for most α-keto acid decarboxylation reactions involves formation of an enolate intermediate, stabilization of which could be accomplished by an enzyme group protonating the carbonyl oxygen of the carbanion intermediate; however, this is most likely accomplished via metal ion complexation as has been shown to be the case not only in solution (Westheimer & Sternberg, 1951) but also for pyruvate kinase- and malic enzyme-catalyzed decarboxylation of OAA (Kiick & Cleland, 1989; Park et al., 1986). Another possibility is that the protonation state of an enzyme group directly affects the catalytic conformation of the enzyme but is not involved in the chemistry of the reaction. A third possible role is that the enzyme group donates a proton to the enolate of pyruvate before release from the enzyme. Lastly, a protonated active-site residue could be involved in binding the negatively charged carboxyl group. A final determination of the role of this residue in catalysis will have to await solution of the crystal structure of the enzyme with oxalate bound.

The V/K for an enzyme-catalyzed reaction includes all steps up to and including the first irreversible step of the overall mechanism. Thus, for the decarboxylation of OAA by the decarboxylase enzyme, the protonation state of an enzyme group interacting with the enolate of pyruvate would not be expected to show up in the V/K pH–rate profile for the enzyme. However, since OAA binds only to the correctly protonated form of the enzyme, the enzyme residue that protonates the enolate must be in the correctly protonated form. In this case, the group will be observed in the V/K pH–rate profile for the enzyme. The pH dependence of oxalate binding to the enzyme is consistent with this assignment for the enzyme residue. It should be noted that the pH dependence of both V and V/K for OAA decarboxylation by NAD–malic enzyme (Kiick et al., 1986; Park et al., 1986) exhibits the same characteristics as OAA decarboxylase.

Carbon Isotope Effects. From an internal discrimination experiment, the *P. putida* OAA decarboxylase ^{13}C isotope effect on V/K for OAA, $^{13}(V/K_{OAA})$, was determined in quadruplicate at pH 5.5 and 9.45; the calculated values of $^{13}(V/K_{OAA})$ are 1.0227 ± 0.0007 and 1.0231 ± 0.0005, respectively. Within experimental error, $^{13}(V/K_{OAA})$ appears to be pH-independent. The results are listed in Table 1 along with $^{13}(V/K_{OAA})$ for pyruvate kinase, which was determined to be 1.0546 ± 0.0001 at pH 7.5.

The rather large (5%) isotope effects on the metal ion- and pyruvate kinase-catalyzed reactions indicate that there is considerable motion of the carbon atom in the transition state. The carbon atom with a mass of 12 must move into collinear arrangement with two oxygens of mass 32. The effect for OAA decarboxylase is much smaller (2%). While it is very unlikely that this decrease in the observed isotope effect could be indicative of an early transition state for OAA decarboxylase, as is the case for prephenate dehydrogenase where the isotope effect is 1.5% (Hermes et al., 1984b), a more likely possibility is that the observed effect is smaller due to an internal commitment within the system. This internal commitment presumably masks a larger intrinsic isotope for OAA decarboxylase that is similar to the metal ion- and pyruvate kinase-catalyzed reactions. Equation 5allows for

$$^{13}(V/K) = (^{13}k + c_f)/(1 + c_f) \quad (5)$$

determination of the presence and estimation of the size of the commitment factor (Cleland, 1982, 1987; Cook & Cleland, 1981a). ^{13}k is the intrinsic isotope effect, and c_f is the forward commitment. Since dissociation of CO_2 is most likely irreversible, there is no reverse commitment. If we assume the intrinsic isotope effect is equal to that seen in the chemical reaction, ^{13}k = 1.0485, the forward commitment factor is 1.12.

Commitment factors are composed of internal and external partition ratios (Cleland, 1982). The internal commitment excludes steps involved with substrate binding and release. Conversely, the external portion contains the rate constants for substrate binding and release. Alterations in conditions, such as pH and substrate concentration, affect the magnitude of the external commitment but not the magnitude of the internal commitment. Moreover, commitments are related to the stickiness of the substrate and can perturb the pK of a V/K profile, such as that in Figure 2, above the true pK (Cleland, 1982; Cook & Cleland, 1981b,c). For OAA decarboxylase, the pK values determined for V/K OAA and for the pK_i of oxalate are identical. Thus, we conclude there is no external commitment.

The presence or absence of an external commitment factor can also be deduced by determining the $^{13}(V/K)$ effect at pH values above and below the pK observed in the V/K pH profile for the substrate (Cook & Cleland, 1981b,c). In an enzyme system with an external commitment, at pH values above the pK where V/K decreases rapidly, the external commitment is reduced to zero, since the chemical reaction itself becomes rate-limiting. Thus, an increase in the isotope effect indicates the presence of a external commitment. However, for OAA decarboxylase, isotope effect values of 1.0227 and 1.023

(measured at pH 5.5 and 9.45, respectively) are within experimental error equal, indicating again that there is no external forward commitment (Cook & Cleland, 1981b,c). Therefore, the value of 1.12 for c_f (calculated using eq 5) must represent an internal forward commitment. The nature of the internal commitment will be discussed below.

Secondary Oxygen Isotope Effects. Using Mg^{2+} as the divalent cation, inverse secondary ^{18}O isotope effects were determined for both the metal cation-catalyzed and OAA decarboxylase-catalyzed reactions. For the divalent metal cation-catalyzed reaction, a value of 0.9966 ± 0.0002 was determined while the value for the OAA decarboxylase-catalyzed reaction was determined to be 0.9993 ± 0.0003.

Secondary ^{18}O effects on the oxygens of the liberated CO_2 are unlike the primary ^{13}C effects in that they are not a measure of bond breaking in the transition state. Instead, ^{18}O effects stem from (1) changes in bond order, (2) changes in degree of solvation, and (3) changes in protonation state (Hermes et al., 1984a; Headley & O'Leary, 1990). Studies of such effects on decarboxylations have been performed for formate dehydrogenase (Hermes et al., 1984a; O'Leary, 1988) and the thermal decarboxylation of 4-pyridylacetic acid (Headley & O'Leary, 1990). For decarboxylations in which ^{13}k is 1.05–1.07, the contributions to the observed ^{18}O effect are (1) the increase in bond order from 1.5 to 2 which gives rise to an inverse value that may approach 0.98–0.99, (2) desolvation during substrate binding to the enzyme which can result in values of 1.01–1.02, and (3) protonation of the substrate, yielding an effect of 1.01.

The ^{18}O equilibrium isotope effect for decarboxylation ($-COO^- \rightarrow CO_2$) is estimated to be 0.984 (Hermes et al., 1984a). However, no secondary ^{18}O isotope effect has approached this value. The increase in bond order to the oxygen cannot really take place until the O–C–O system is nearly linear. Thus, the value of 0.996 for the Mg^{2+}-catalyzed reaction suggests that at the transition state the system is still nonlinear. The large ^{13}C isotope effect which demonstrates that the carbon is in motion supports this interpretation.

The value of 0.9993 for OAA decarboxylase indicates an altered transition state in the presence of enzyme. Since the primary ^{13}C isotope effects suggests the presence of an internal commitment, we must consider the effect of the commitment on the ^{18}O effect. Using eq 5 and substituting $^{18}(V/K)$ and ^{18}k for the analogous carbon effects, one can calculate the secondary ^{18}O effects. If the presence of the enzyme alters the reaction solely due to an internal commitment, using the Mg^{2+}-catalyzed value of 0.9966 for ^{18}k and 1.12 for c_f, a value of 0.9984 for $^{18}(V/K)$ results. Since the predicted value is more inverse than the observed value in Table 1, the enzyme must influence the reaction in ways not reflected by the commitment factor. The most likely cause is an isotope effect of 1.01 resulting from desolvation induced by binding of OAA to the enzyme since a change in environment is the most apparent difference between the Mg^{2+}- and OAA decarboxylase-catalyzed reactions.[2] This effect is an order of magnitude less than that estimated for the formate dehydrogenase reaction (Hermes et al., 1984a).

OAA decarboxylase appears to accelerate the reaction rate by 4.5 orders of magnitude over the Mg^{2+}-catalyzed reaction. O'Leary (1977, 1992) has suggested that this activation results from conformation changes in OAA that occur upon binding to enzyme which ensure that the carboxyl group is above the plane of the forming double bond, thus making it a good leaving group. This conformational control may very well be the root of the internal commitment factor for the reaction catalyzed by OAA decarboxylase.

[2] If desolvation of the carboxyl group of OAA does contribute to the ^{18}O isotope effect, then the calculated isotope effect from eq 5 needs to be multiplied by the ^{18}O equilibrium isotope effect on the binding step to give the observed value. However, the above analysis using eq 5 is valid as long as the binding step is at equilibrium, i.e., the substrate is not sticky, which is the case for OAA decarboxylase.

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