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Oxamate Is an Alternative Substrate for Pyruvate Carboxylase from *Rhizobium etli*

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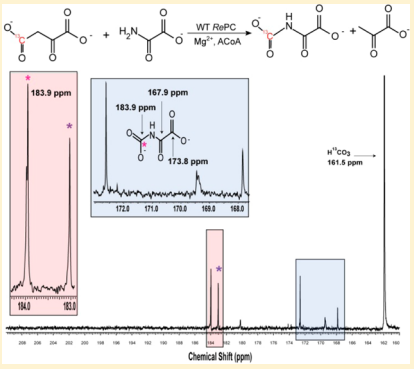
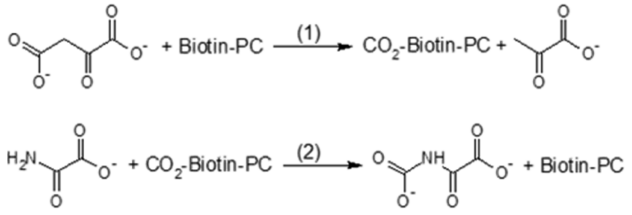
Supporting Information

ABSTRACT: Oxamate, an isosteric and isoelectronic inhibitory analogue of pyruvate, enhances the rate of enzymatic decarboxylation of oxaloacetate in the carboxyl transferase domain of pyruvate carboxylase (PC). It is unclear, though, how oxamate exerts a stimulatory effect on the enzymatic reaction. Herein, we report direct ^{13}C nuclear magnetic resonance (NMR) evidence that oxamate acts as a carboxyl acceptor, forming a carbamylated oxamate product and thereby accelerating the enzymatic decarboxylation reaction. ^{13}C NMR was used to monitor the PC-catalyzed formation of [4-^{13}C]oxaloacetate and subsequent transfer of $^{13}CO_2$ from oxaloacetate to oxamate. In the presence of oxamate, the apparent K_m for oxaloacetate is artificially suppressed (from 15 to 4−5 μM). Interestingly, the steady-state kinetic analysis of the initial rates determined at varying concentrations of oxaloacetate and fixed concentrations of oxamate revealed initial velocity patterns inconsistent with a simple ping-pong-type mechanism. Rather, the patterns suggest the existence of an alternate decarboxylation pathway in which an unstable intermediate is formed. The steady-state kinetic analysis coupled with the normal $^{13}(V/K)$ kinetic isotope effect observed on C-4 of oxaloacetate [$^{13}(V/K)$ = 1.0117 ± 0.0005] indicates that the transfer of CO_2 from carboxybiotin to oxamate is the partially rate-limiting step of the enzymatic reaction. The catalytic mechanism proposed for the carboxylation of oxamate is similar to that proposed for the carboxylation of pyruvate, which occurs via the formation of an enol intermediate.



Oxamate, an isosteric and isoelectronic analogue of pyruvate, has been shown to be both a noncompetitive inhibitor[1] and an uncompetitive inhibitor[2] with respect to pyruvate in the pyruvate carboxylase (PC)-catalyzed MgATP-dependent carboxylation reaction. Interestingly, oxamate stimulates the overall rate of oxaloacetate decarboxylation in the reverse reaction catalyzed by PC isolated from chicken liver[3−5] and *Rhizobium etli*.[6,7] Binding studies have indicated that oxamate competes with oxaloacetate for occupancy of the carboxyl transferase (CT) domain active site,[8] while additional studies suggest that the enzymatic decarboxylation of oxaloacetate in the presence of oxamate occurs solely in the CT domain of the enzyme.[4,5] Saturating concentrations of oxamate increased the rate of PC-catalyzed decarboxylation nearly 10-fold when compared to the rate of the overall reverse reaction in which MgADP and P_i generate MgATP from oxaloacetate.[4,5,7] As part of a ping-pong mechanism (Scheme 1), C-4 of oxaloacetate is initially transferred to the tethered biotin moiety to form carboxybiotin in the CT domain. In the second step of the reaction, carboxybiotin is decarboxylated in the presence of oxamate.[6] Even though early kinetic studies have indicated that the second step of the reaction is rate-limiting,[4,5,9,10] the mechanism by which oxamate induces the PC-catalyzed decarboxylation of oxaloacetate is still unclear.

Scheme 1. Transfer of a Carboxyl from Oxaloacetate to Oxamate via the Formation of Carboxybiotin



We have previously established that the PC-catalyzed carboxylation of pyruvate in the CT domain of *Re*PC occurs through the initial decarboxylation of carboxybiotin to form the biotin−enolate intermediate.[6] A concurrent abstraction of a proton from the methyl group of the Zn^{2+}-coordinated

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pyruvate substrate is facilitated by a strictly conserved Thr residue. Mutation of this Thr to Ala resulted in a complete loss of CT domain activity in both *Re*PC and in *Staphylococcus aureus* PC.[11] Reaction of the nucleophilic pyruvate enol with the liberated CO_2 generates oxaloacetate, and the abstracted proton is used to re-form biotin, thus completing the catalytic cycle.[6] It occurred to us that the amide ($CONH_2$) portion of oxamate could form a similar enolate-like intermediate that could react with CO_2 to give the carbamate product, thereby stimulating the enzymatic decarboxylation of oxaloacetate by promoting the decarboxylation of the carboxybiotin intermediate in the CT domain.

While this is an attractive mechanism, the carbamylated oxamate product had not been previously observed, most likely because of the strongly acidic conditions used in preceding experiments.[3−5] Herein, we report direct evidence that the stimulatory effect of oxamate on the PC-catalyzed decarboxylation of oxaloacetate arises from the ability of oxamate to act as a carboxyl acceptor. Using ^{13}C NMR, we observed the enzymatic transfer of ^{13}C-enriched CO_2 from [4-^{13}C]-oxaloacetate to oxamate in the presence of *Re*PC, forming the acid-labile carbamylated product. Steady-state kinetic analysis of the decarboxylation reaction in the absence and presence of oxamate indicates that the kinetic mechanism of the oxamate-induced decarboxylation of oxaloacetate is more complicated than expected. The $^{13}(V/K)$ kinetic isotope effect (KIE) observed on the transfer of CO_2 from oxaloacetate to oxamate is consistent with the second step of the reaction being rate-limiting. A mechanism for oxamate carboxylation in the CT domain of PC, which invokes the formation of an oxamate−enolate intermediate, is proposed.

EXPERIMENTAL PROCEDURES

Materials. IPTG, biotin, NADP, NADH, ampicillin, and chloramphenicol were purchased from Research Products International Corp. (RPI). Ni^{2+}-Profinity IMAC resin was obtained from Bio-Rad, and Q-Sepharose Fast Flow resin was purchased from GE Healthcare Life Sciences. Lactate dehydrogenase was purchased from Calzyme Laboratories, Inc. (San Luis Obispo, CA). All other materials were obtained from Sigma-Aldrich and of the highest purity available.

Methods. *Steady-State Kinetic Analysis of the PC-Catalyzed Decarboxylation of Oxaloacetate in the Presence and Absence of Oxamate.* Overexpression of wild-type PC from *R. etli* was essentially performed as previously described[6] with the exception that the pET-17b $(His)_8$ *Re*PC plasmid and pCY216 plasmid, containing biotin protein ligase, were used to cotransform BL21STAR(dE3) *Escherichia coli* cells (Invitrogen). Full details of the isolation and purification of wild-type *Re*PC are presented in the Supporting Information. The initial rates of the nonenzymatic and PC-catalyzed decarboxylation of oxaloacetate were determined in the presence and absence of oxamate by measuring the rate of pyruvate release using the lactate dehydrogenase coupled assay system.[6,7] While oxamate can affect the activity of lactate dehydrogenase,[12] numerous control experiments established the direct correlation between the rates of NADH oxidation by lactate dehydrogenase and the PC-catalyzed rate of oxaloacetate decarboxylation and subsequent pyruvate release, verifying the suitability of LDH as a coupling enzyme in this system.[6,7] Fresh stocks of oxaloacetate were made prior to use and stored on ice during all experiments. Concentrations of oxaloacetate stock solutions were determined via an end point assay using malate dehydrogenase and NADH.

Because of the low apparent K_m for oxaloacetate, 5 cm path length cells were required to monitor the reactions spectrophotometrically. The 7 mL reaction mixtures (25 °C) contained 100 mM Tricine (pH 7.5), 0.25 mM acetyl-CoA, 25 μM NADH, and 20 units of lactate dehydrogenase. The k_{cat} (inverse minutes) and apparent K_m (micromolar) were determined by varying the oxaloacetate concentration (1.0−46 μM) in the absence of oxamate. The rates of nonenzymatic decarboxylation of oxaloacetate were determined under identical conditions at each concentration of oxaloacetate and subtracted from the enzymatic rate. Enzymatic reactions were initiated with the addition of wild-type *Re*PC (0.9−1.5 mg). Reactions were also performed as described above in the presence of oxamate (0.25−1.0 mM). Rates of nonenzymatic decarboxylation of oxaloacetate in the presence of oxamate were similarly determined and subtracted from the enzymatic rates.

Determination of the $^{13}(V/K)$ Kinetic Isotope Effect (KIE) on C-4 of Oxaloacetate for the Enzymatic Decarboxylation of Oxaloacetate in the Presence of Oxamate. The $^{13}(V/K)$ KIE on C-4 of oxaloacetate for the *Re*PC-catalyzed oxamate-induced decarboxylation reaction was determined in a manner similar to that previously described.[3] Total reaction volumes of 20 mL were used, and all reactions were performed at 25 °C in Tricine buffer (100 mM, pH 7.5). Buffer solutions containing 0.25 mM acetyl-CoA were sparged with CO_2-free N_2 overnight in round-bottom flasks equipped with a side arm stop cock and rubber septum. After the mixtures had been sparged, oxaloacetate (final concentration of 6.0 mM), oxamate (final concentration of 60 mM), and NADH (final concentration of 7.0 mM) were added as solids under a N_2 stream; 200 μL of a 13.2 KU/mL solution of lactate dehydrogenase, made in previously sparged buffer, was added to the reaction mixture via a syringe. Prior to addition of *Re*PC, a 200 μL aliquot of the reaction mixture was removed and used to monitor the extent of the nonenzymatic decarboxylation of oxaloacetate during the course of the enzymatic reaction. Immediately after the removal of the aliquot, 3 mL of the wild-type *Re*PC (2.5−3.5 mg) solution was added to the reaction mixtures, and a second aliquot (200 μL) was removed to monitor the simultaneous enzymatic and nonenzymatic oxamate-stimulated decarboxylation of oxaloacetate. Control reactions indicated that the addition of the lactate dehydrogenase and *Re*PC enzyme solutions did not introduce significant amounts of endogenous CO_2 into the reaction mixtures. The extent of the enzymatic decarboxylation of oxalacetate was periodically determined spectrophotometrically using the lactate dehydrogenase coupled assay system. To estimate the amount of CO_2 produced by nonenzymatic decarboxylation, parallel reactions under identical conditions in which PC was omitted from the reaction mixture were conducted. Knowing the extent of nonenzymatic decarboxylation allowed for estimation of the extent of enzymatic decarboxylation in the isotope effect experiments. Using this and the known values of $^{13}(V/K)$ for the nonenzymatic decarboxylation of oxaloacetate, a reasonable calculation of the $^{13}(V/K)$ for the enzymatic decarboxylation was obtained (eq 3). To confirm that the concentration of pyruvate formed was directly proportional to the amount of CO_2 generated, the amount of CO_2 produced during the enzymatic and nonenzymatic reaction of one trial was determined manometrically.

Once the reaction had proceeded to a point where sufficient product had been formed (40−70% of total decarboxylation), 600 units of malic dehydrogenase was added to the reaction mixture, which immediately and irreversibly converted any residual oxaloacetate to malate to effectively quench the reaction. After quenching, 0.8 mL of concentrated H_2SO_4 was added to the reaction mixtures via a syringe to decompose the carbamylated oxamate product, thus liberating CO_2 for analysis using the isotopic ratio mass spectrometer (IRMS). Reaction vessels were attached to high-vacuum lines, and the contents were frozen using a dry ice/2-propanol bath. CO_2 and N_2 were distilled through two dry ice/2-propanol traps, and the CO_2 was collected in a liquid nitrogen trap. The CO_2 was further distilled under vacuum into a collection tube. The isotopic enrichment of the collected gas was then analyzed against a standard CO_2 sample using the IRMS. This entire procedure is essentially the same as that in the literature.[13] In a similar manner, the $^{13}(V/K)$ on the nonenzymatic decarboxylation of oxaloacetate in the presence of oxamate was also determined.

Monitoring the Wild-Type PC-Catalyzed Decarboxylation of Oxaloacetate in the Presence of Oxamate by ^{13}C NMR. ^{13}C NMR spectra were recorded on a Bruker 500 MHz NMR instrument. Optimal acquisition parameters for the proton-decoupled ^{13}C NMR experiments were determined to be 3072 scans with an acquisition time of 1.1 s and a delay of 4 s (D1) between each acquisition. Spectra were processed using an exponential window function and 1.5 Hz line broadening (ACD/NMR Processor, Academic Edition) and referenced to DMSO-d_6 [$^{13}C\{^1H\}$ NMR (125.8 MHz) δ 39.5], which was added (15 μL) as an internal standard.

[4-^{13}C]Oxaloacetate was generated *in situ* from the *Re*PC-catalyzed carboxylation of pyruvate by $H^{13}CO_3^-$ (98% ^{13}C incorporation, Aldrich). The 0.8 mL NMR reaction mixtures contained 50 mM phosphate buffer (pH 8.0, 35% D_2O), 20 mM pyruvate, 20 mM $H^{13}CO_3^-$, 3.0 mM $MgCl_2$, 2.5 mM MgATP, and 0.25 acetyl-CoA. Because of the substrate inhibition of the PC-catalyzed reaction by MgATP at concentrations greater than 3.0 mM,[7] 20 mM acetyl phosphate and 20 units of acetate kinase were added to regenerate MgATP during the catalytic reaction. The carboxylation reaction was initiated via the addition of 250 μL of purified wild-type *Re*PC that had been previously dialyzed against 500 mL of 50 mM phosphate buffer (pH 8.0) for 2 h. An identical control reaction mixture, from which *Re*PC was omitted, was used as a reference sample. After the addition of *Re*PC, the reaction mixtures were incubated at room temperature for approximately 5 h prior to the acquisition of the initial ^{13}C NMR spectra. These spectra confirmed the nearly complete conversion of pyruvate to [4-^{13}C]oxaloacetate (98%). Oxamate (20 mM) was added to both the enzymatic and control reaction mixtures, which were allowed to incubate at room temperature for an additional 5 h prior to the acquisition of the ^{13}C NMR spectra. The pH of both the enzymatic and control samples was then adjusted to ~3 with 18 wt % DCl in D_2O, and final ^{13}C NMR spectra were recorded.

Selected spectral data referenced to DMSO-d_6 in a 35% D_2O/65% H_2O mixture (pH 8.0): $^{13}C\{^1H\}$ NMR for pyruvate (125.8 MHz, DMSO-d_6) δ 206.6 (C-1, -CO_2), 173.8 [C-2, $CH_3C(O)$]; $^{13}C\{^1H\}$ NMR for $H^{13}CO_3^-/^{13}CO_2$ (125.8 MHz, DMSO-d_6) δ 154.4 (HCO_3^-), 125.6 (CO_2^-); $^{13}C\{^1H\}$ NMR for [4-^{13}C]oxaloacetate (125.8 MHz, DMSO-d_6) δ 180.3 [C-1, $C(O)CO_2$], 179.6 [C-2, $CH_2C(O)$], 176.4 (^{13}C-4, CH_2CO_2); $^{13}C\{^1H\}$ NMR for oxamate (125.8 MHz, DMSO-d_6) δ 172.6 (C-1, -CO_2), 167.2 [C-2, $H_2NC(O)$]; $^{13}C\{^1H\}$ NMR for carbamylated oxamate (125.8 MHz, DMSO-d_6) δ 183.9 (^{13}C-4, -$NHCO_2$), 173.8 [C-1, $C(O)CO_2$], 167.9 [C-2, NHC(O)].

Data Analysis. *Steady-State Kinetics of the PC-Catalyzed Decarboxylation of Oxaloacetate in the Presence and Absence of Oxamate.* Least-squares nonlinear regression analysis was performed, and best-fit lines were plotted using GraphPad Prism version 5.0. Standard errors reported for all kinetic parameters were determined from the fits of these data to the corresponding equation. In the absence of oxamate, k_{cat} (inverse minutes) and apparent K_m (micromolar) values were determined by fitting velocity versus substrate concentration data to eq 1 using least-squares nonlinear regression, where A is the concentration of oxaloacetate.

$$v = \frac{k_{cat}A}{K_m + A} \tag{1}$$

In the presence of fixed, varying concentrations oxamate, the sigmoidal velocity versus substrate concentration curves were individually fit to eq 2

$$v = \frac{k_{cat}A^n}{K_{0.5}{}^n + A^n} \tag{2}$$

where A is the concentration of oxaloacetate, k_{cat} (inverse minutes) is the rate of the oxamate-stimulated decarboxylation reaction, $K_{0.5}$ is the apparent Michaelis constant for oxaloacetate (micromolar), and n is the Hill coefficient. In the presence of oxamate, substrate inhibition was observed at oxaloacetate concentrations of >20 μM (Figure S1 of the Supporting Information). These data were not included in this analysis.

$^{13}(V/K)$ KIE for the Catalytic Carboxyl Transfer from Oxaloacetate to Oxamate. The $^{13}(V/K)_{obs}$ on C-4 of oxaloacetate in the PC-catalyzed oxamate-induced decarboxylation was determined using the internal competition method by measuring the ^{13}C enrichment of the carbamylated oxamate product. This procedure uses only natural abundance compounds. Individual values for the fraction of reactions, raw δ values, and the calculated enzymatic and nonenzymatic isotope effects are presented in the Supporting Information (Table S1). The $^{13}(V/K)_{obs}$ is a measure of both the nonenzymatic and enzymatic decarboxylation of oxaloacetate. Because CO_2 resulting from the enzymatic and nonenzymatic reactions cannot be separated and a large primary isotope effect is observed on the nonenzymatic decarboxylation, eq 3 is used to calculate the enzymatic $^{13}(V/K)$ of the PC-catalyzed reaction from the $^{13}(V/K)_{obs}$.

$$^{13}(V/K)_{obs} = \left(\frac{f_{non\text{-}enzy}}{f_{non\text{-}enzy+PC}}\right)[^{13}(V/K)_{non\text{-}enzy}] + \left(\frac{f_{PC}}{f_{non\text{-}enzy+PC}}\right)[^{13}(V/K)_{PC}] \tag{3}$$

In this equation, $^{13}(V/K)_{obs}$ is the isotope effect on both the enzymatic and nonenzymatic decarboxylation. $f_{non\text{-}enzy}$ is the fraction of the nonenzymatic decarboxylation reaction, while $f_{non\text{-}enzy+PC}$ is the fraction of the reaction for both the enzymatic and nonenzymatic reactions. f_{PC} is the fraction of the reaction for the PC-catalyzed decarboxylation and is calculated using the total and nonenzymatic fractions of reactions. $^{13}(V/K)_{non\text{-}enzy}$ is

the isotope effect for the nonenzymatic decarboxylation of oxaloacetate determined under conditions similar to those of the enzymatic reaction . Finally, $^{13}(V/K)_{PC}$ is the calculated isotope effect on the PC-catalyzed transfer of CO_2 from oxaloacetate to oxamate. Standard errors reported are the standard deviations of three separate determinations of both the enzymatic and nonenzymatic reactions.

RESULTS

Steady-State Kinetics of the *Re*PC-Catalyzed Decarboxylation of Oxaloacetate in the Presence and Absence of Oxamate. The initial rates of oxaloacetate decarboxylation were determined at varying concentrations of oxaloacetate by measuring the rate of pyruvate release using the lactate dehydrogenase coupled assay system.[6,7] In the absence of oxamate, the initial rate dependence on oxaoloacetate concentrations exhibited normal Michaelis–Menten kinetics (Figure 1). Fits of these data to eq 1 revealed a k_{cat} of 1.72 ± 0.07 min^{-1} and an apparent K_m of 15 ± 1 μM. In contrast, in the presence of fixed concentrations of oxamate, the dependence of the initial rates on oxaloacetate concentrations was decidedly sigmoidal (Figure 2) and substrate inhibition was observed at oxaloacetate concentrations of >20 μM (Figure S1A,B of the Supporting Information). Low levels of oxamate (0.25–1.0 mM) were used in the kinetic studies to prevent further complication of the kinetic mechanism. Concentrations of both oxaloacetate and oxamate used for the PC-catalyzed *in situ* formation of [4-^{13}C]oxaloacetate and carbamylated oxamate were well above saturating levels (20 mM), indicating that the substrate inhibition conveyed by either of the two substrates was only a partial inhibition. Double-reciprocal plots were nonlinear and are not consistent with the parallel patterns expected for a simple ping-pong-type mechanism. These data were individually fit to the Hill equation (eq 2), and kinetic parameters resulting from the nonlinear regression analysis are listed in Table 1. Increasing concentrations of oxamate had a marked effect on the enzymatic rate of oxaloacetate decarboxylation. Interestingly, the concentration of oxaloacetate that gives half the maximal velocity was approximately one-fifth to one-seventh of the K_m for oxaloacetate determined in the absence of oxamate. The artificial suppression of the apparent affinity of the enzyme for oxaloacetate in the presence of oxamate indicates that the carbamylation of oxamate by carboxybiotin is significantly slower than the initial decarboxylation of oxaloacetate to form carboxybiotin.

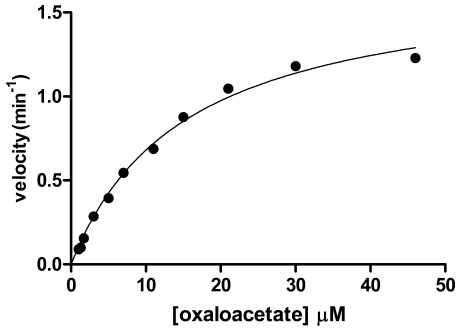

Figure 1. Initial rates of the PC-catalyzed decarboxylation of oxaloacetate (1.0–46 μM) in the absence of oxamate. Data were fit to eq 1, and the k_{cat} and apparent K_m for oxaloacetate were determined to be 1.72 ± 0.07 min^{-1} and 15 ± 1 μM, respectively.

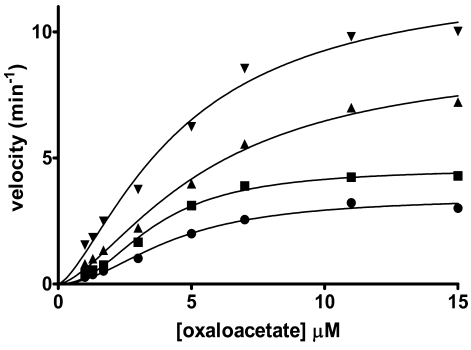

Figure 2. Initial rate vs substrate curves for the PC-catalyzed decarboxylation of oxaloacetate (1.0–25 μM) at fixed concentrations of oxamate: (●) 0.25, (■) 0.5, (▲) 0.75, and (▼) 1.0 mM. Kinetic parameters from individual fits of the data to eq 2 are listed in Table 1.

Table 1. Kinetic Parameters Determined for the PC-Catalyzed Oxamate-Stimulated Decarboxylation of Oxaloacetate[a]

	k_{cat} (min^{-1})[b]	$K_{0.5}$ (μM)[c]	n
0.25 mM oxamate	3.4 ± 0.2	2.1 ± 0.3	2.0 ± 0.3
0.5 mM oxamate	4.6 ± 0.2	1.8 ± 0.07	2.2 ± 0.2
0.75 mM oxamate	9.1 ± 0.8	3.0 ± 0.2	1.5 ± 0.2
1.0 mM oxamate	12 ± 1	3.0 ± 0.2	1.4 ± 0.4

[a]Reaction conditions: 25 °C, 100 mM Tricine (pH 7.5, 5 cm path length), oxaloacetate (1.0–46 μM), 0.25 mM acetyl-CoA, 0.24 mM NADH, 10 units of lactate dehydrogenase, and *Re*PC (500–1500 μg). [b]Data fit to eq 2. Initial rates corrected for the rate of nonenzymatic decarboxylation of oxaloacetate. [c]From eq 2, the units of $K_{0.5}$ are $(\mu M)^n$. These values have been corrected to micromolar for comparison with the apparent K_m.

$^{13}(V/K)$ KIE on the Transfer of CO_2 from Oxaloacetate to Oxamate. The $^{13}(V/K)$ for the nonenzymatic decarboxylation of oxaloacetate in the presence of oxamate (60 mM) was determined to be 1.0527, which is in good agreement with the large, normal effect previously observed.[5] The $^{13}(V/K)_{obs}$, which is the KIE for both the PC-catalyzed and nonenzymatic decarboxylation reactions, was determined in three separate trials [$^{13}(V/K)_{obs}$ = 1.0190 ± 0.004] (Table 2). The $^{13}(V/K)$ KIE on the PC-catalyzed decarboxylation and transfer of CO_2 from oxaloacetate to oxamate was calculated using eq 3, and a normal isotope effect of 1.2% [$^{13}(V/K)_{PC}$ = 1.0117 ± 0.005] was established.

Identification of the Carbamate Product in the Oxamate-Induced PC-Catalyzed Decarboxylation of Oxaloacetate. To

Table 2. $^{13}(V/K)$ Effects on the PC-Catalyzed Transfer of CO_2 from C-4 of Oxaloacetate to Oxamate[a]

	$^{13}(V/K)_{obs}$	$^{13}(V/K)_{PC}$[b]
determination 1	1.0187	1.0113
determination 2	1.0194	1.0121
determination 3	1.0189	1.0115
average ± standard deviation	1.0190 ± 0.0004	1.0117 ± 0.0005

[a]Reaction conditions: 25 °C, 100 mM Tricine (pH 7.5, total volume of 17 mL), 60 mM oxamate, 20 mM oxaloacetate, 0.25 mM acetyl-CoA, 0.24 mM NADH, 2.5 units of lactate dehydrogenase, PC (2.5–3.0 mg). [b]$^{13}(V/K)_{PC}$ values determined from eq 3.

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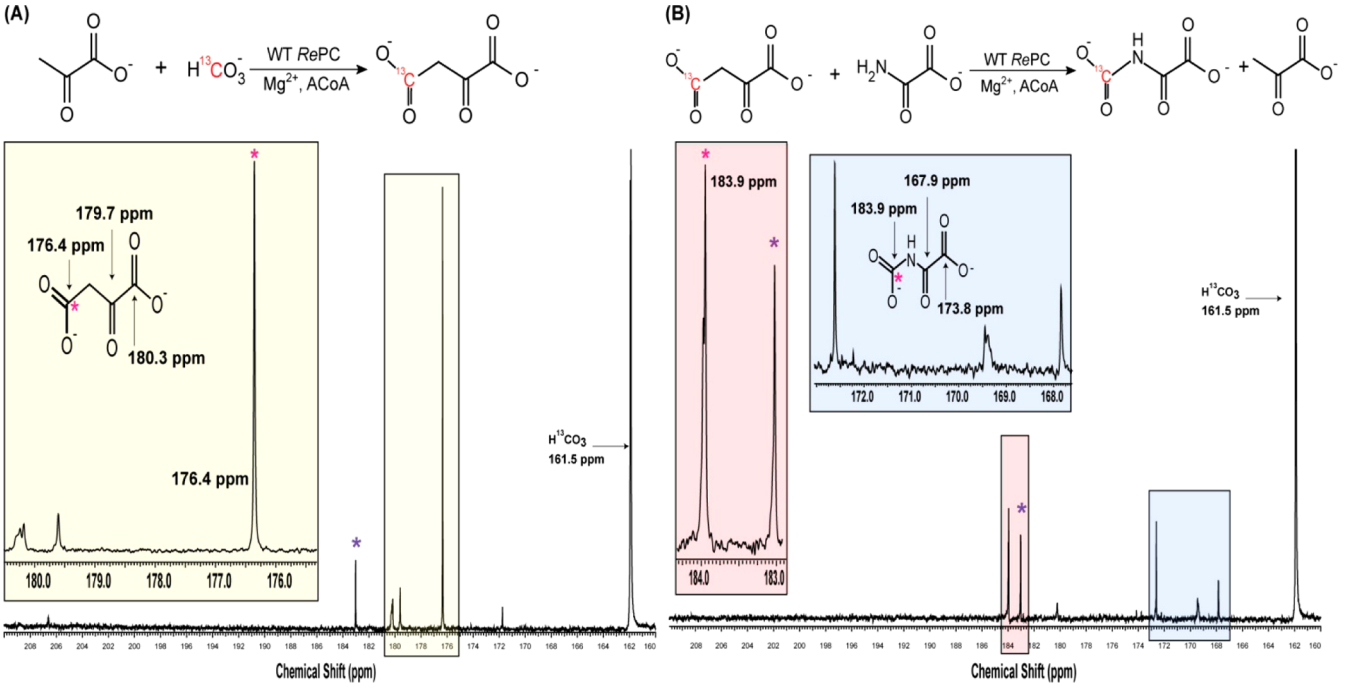

Figure 3. ^{13}C NMR spectra of the *in situ* PC-catalyzed formation of [4-^{13}C]oxaloacetate from pyruvate and $H^{13}CO_3^-$ (A) and subsequent carboxylation of oxamate to form the carbamylated oxamate product (B). The inset in panel A shows the ^{13}C NMR peaks assigned to [4-^{13}C]oxaloacetate {δ 180.3 [C-1, $C(O)CO_2$], 179.6 [C-2, $CH_2C(O)$], 176.4 (^{13}C-4, CH_2CO_2)}. The inset in panel B shows the acid-labile ^{13}C NMR peaks corresponding to the newly formed carbamylated oxamate species {δ 183.9 (^{13}C-4, -$NHCO_2$), 173.8 [C-1, $C(O)CO_2$], 167.9 [C-2, $NHC(O)$]}. Chemical shifts of ^{13}C peaks were referenced to DMSO-d_6. Purple asterisks in both spectra denote acetate, formed during the regeneration of MgATP from acetyl phosphate and acetate kinase. Red asterisks denote the location of the ^{13}C-enriched atom in each of the structures.

determine if C-4 of oxaloacetate was transferred to oxamate to form the carbamylated product, $H^{13}CO_3^-$ was used to selectively generate ^{13}C-labeled [4-^{13}C]oxaloacetate *in situ* using wild-type *Re*PC in the presence of pyruvate and MgATP (Figure 3A). ^{13}C NMR spectra indicated the nearly complete conversion of pyruvate to [4-^{13}C]oxaloacetate. The ^{13}C-enriched peak at δ 176.4 (Figure 3A, inset), as well as peaks at δ 180.3 and 179.6, is entirely consistent with published chemical shift values for oxaloacetate.[14] These peaks were unique to samples that contained PC and were not observed in the control reactions (see Figures S2−S7 of the Supporting Information for full ^{13}C NMR spectra of the enzymatic and control reactions). Once the *in situ* formation of [4-^{13}C]-oxaloacetate was confirmed, 20 mM oxamate was added to both the enzymatic and control reaction mixtures. After incubation, several new peaks were observed in ^{13}C NMR spectra of the enzymatic reaction (Figure 3B). The diagnostic peak for the formation of the oxamate carbamate product was observed at δ 183.9 (Figure 3B, red inset). Slight changes, when compared to the oxamate control, in peak intensity and chemical shift of the other carbons in the oxamate carbamate are attributed to weak coupling with the ^{13}C-enriched carboxyl group.[14] Under acidic conditions (pH ~3), the diagnostic peak corresponding to the carboxyl group of the carbamate ($-N^{13}CO_2$) had disappeared, consistent with the predicted acid lability of the carbamate product.[15]

DISCUSSION

The origin of the sigmoidal initial velocity plots and oxaloacetate substrate inhibition in the presence of oxamate are difficult to explain without invoking a complicated kinetic scheme that cannot be evaluated with the current kinetic data. Even though both oxaloacetate and oxamate exhibit substrate inhibition (Figure S1A of the Supporting Information), this alone does not explain the sigmoidicity of the initial velocity plots at low levels of oxaloacetate. The nonlinearity of the initial velocity plots may hint at the existence of cooperative interactions between the multiple CT domains in the *Re*PC tetramer,[16] but we cannot currently say with any certainty if such a mechanism is being exploited in these reactions. Even so, there are several definitive conclusions that can be made from this study about the mechanism by which oxamate stimulates the PC-catalyzed decarboxylation of oxaloacetate.

Inspection of the reciprocal plots (Figure S1B of the Supporting Information) clearly shows an intersecting pattern, indicating (V/K) varies with oxamate concentration, which is contrary to the expected parallel patterns for the putative ping-pong-type mechanism. For the first time, the ^{13}C NMR spectra have plainly shown that the stimulatory effects of oxamate can be, in part, attributed to the transfer of CO_2 from oxaloacetate to oxamate, thus forming the acid-labile carbamate. On the basis of our steady-state kinetic analysis, isotope effects, and ^{13}C NMR experiments, we propose a catalytic mechanism for the oxamate-induced decarboxylation of oxaloacetate in the CT domain of PC (Scheme 2). Oxaloacetate is initially decarboxylated in the active site to form carboxybiotin.[5−7] After carboxybiotin formation, the active site opens and allows for the coordination of oxamate to the Lewis acid metal center in the CT domain active site, thus favoring the placement of carboxybiotin in the CT domain.[3,4,6,9] Carboxybiotin transfers the CO_2 to oxamate, resulting in the formation of the carbamylated oxamate. Interestingly, the intersecting patterns

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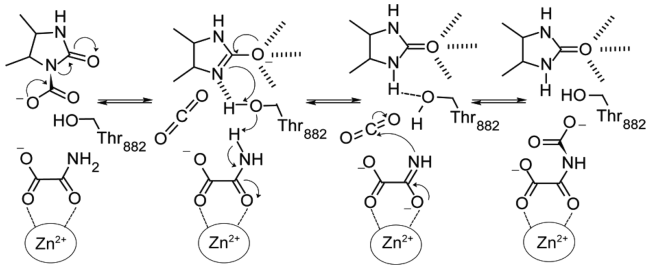
Scheme 2. Proposed Mechanism of Transfer of a Carboxyl from Carboxybiotin to Oxamate To Form the Oxamate Carbamate



in the initial velocity plots suggest that at low concentrations of oxamate, the carboxybiotin intermediate is relatively unstable and will decarboxylate, either in the CT or in the biotin carboxylase domain, rather than react with oxamate. This alternative kinetic pathway, in which oxaloacetate decarboxylation and oxamate carboxylation are uncoupled, occurs more regularly at low concentrations because binding of oxamate to the active site does not occur as often as it would at saturating concentrations. Increasing concentrations of oxamate overcome the "nonproductive" decarboxylation of carboxybiotin, thereby increasing the (V/K) for the oxaloacetate decarboxylation reaction. Therefore, the intersecting patterns of the initial velocity plots indicate there are two kinetic mechanisms once the relatively unstable carboxybiotin intermediate is formed, a nonproductive decarboxylation of carboxybiotin at low concentrations of oxamate and a classical ping-pong-type mechanism at higher concentrations of oxamate.

Further, the substrate inhibition at concentrations of oxaloacetate greater than 20 μM observed in the presence of oxamate (Figure S1 of the Supporting Information) is consistent with a normal ping-pong-type mechanism in which occupation of the CT domain by oxaloacetate blocks the access of oxamate to the active site and effectively decreases the rate of decarboxylation. The transfer of a carboxyl from carboxybiotin to oxamate proceeds in a manner similar to that proposed for the carboxylation of pyruvate in the CT domain.[4] Once formed, the oxamate−enolate intermediate reacts with the liberated CO_2, thus forming the oxamate−carbamate intermediate observed in the ^{13}C NMR spectra. The artificial suppression of the apparent K_m for oxaloacetate by oxamate and the magnitude of the normal observed $^{13}(V/K)$ isotope effect of 1.2% on C-4 of oxaloacetate are both consistent with the transfer of CO_2 from carboxybiotin to oxamate being partially rate-limiting relative to the initial decarboxylation of oxaloacetate and formation of the carboxybiotin intermediate.[17] In conclusion, we have presented the mechanism by which oxamate stimulates the enzymatic decarboxylation of oxaloacetate in the reverse reaction of PC based on steady-state kinetic analysis, isotope effect experiments, and NMR studies. These results indicate that the stimulatory effect of oxamate can be attributed to the ability of oxamate to drive the overall reaction through a more kinetically favored pathway by acting as a suitable carboxyl acceptor in the enzymatic reaction.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full details of wild-type *Re*PC purification, isotope effect data, steady-state kinetic data, and full ^{13}C NMR spectra of enzymatic and control reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written with contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

acetyl-CoA, acetyl-coenzyme A; BC, biotin carboxylase; CT, carboxyl transferase; IPTG, isopropyl β-D-thiogalactopyranoside; *Re*PC, *R. etli* pyruvate carboxylase; PC, pyruvate carboxylase.

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