Mechanisms of Inhibition of *Rhizobium etli* Pyruvate Carboxylase by L-Aspartate

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ABSTRACT: L-Aspartate is a regulatory feedback inhibitor of the biotin-dependent enzyme pyruvate carboxylase in response to increased levels of tricarboxylic acid cycle intermediates. Detailed studies of L-aspartate inhibition of pyruvate carboxylase have been mainly confined to eukaryotic microbial enzymes, and aspects of its mode of action remain unclear. Here we examine its inhibition of the bacterial enzyme *Rhizobium etli* pyruvate carboxylase. Kinetic studies demonstrated that L-aspartate binds to the enzyme cooperatively and inhibits the enzyme competitively with respect to acetyl-CoA. L-Aspartate also inhibits activation of the enzyme by MgTNP-ATP. The action of L-aspartate was not confined to inhibition of acetyl-CoA binding, because the acetyl-CoA-independent activity of the enzyme was also inhibited by increasing concentrations of L-aspartate. This inhibition of acetyl-CoA-independent activity was demonstrated to be focused in the biotin carboxylation domain of the enzyme, and it had no effect on the oxamate-induced oxaloacetate decarboxylation reaction that occurs in the carboxyl transferase domain. L-Aspartate was shown to competitively inhibit bicarbonate-dependent MgATP cleavage with respect to MgATP but also probably inhibits carboxybiotin formation and/or translocation of the carboxybiotin to the site of pyruvate carboxylation. Unlike acetyl-CoA, L-aspartate has no effect on the coupling between MgATP cleavage and oxaloacetate formation. The results suggest that the three allosteric effector sites (acetyl-CoA, MgTNP-ATP, and L-aspartate) are spatially distinct but connected by a network of allosteric interactions.
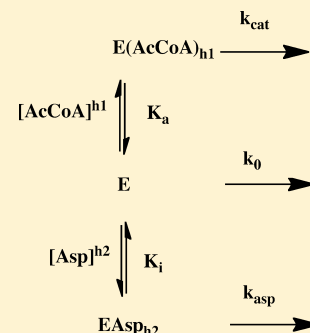
Pyruvate carboxylase (PC, EC 6.4.1.1) is a biotin-dependent carboxylase, which catalyzes carboxylation of pyruvate to oxaloacetate. This reaction is considered to be an important anaplerotic reaction because it replenishes tricarboxylic acid cycle intermediates that have been withdrawn for anabolic purposes.[1] PC is found in wide variety of organisms, including eubacteria, yeast, fungi, and animals (for reviews, see refs 1 and 2). In mammals, PC is also involved in gluconeogenesis in liver, *de novo* fatty acid synthesis in liver and adipose tissue, and neurotransmitter synthesis in astrocytes.[2,3] Furthermore, PC is also necessary for glucose-induced insulin secretion in pancreatic β-cells.[4] As PC has such diverse metabolic roles, dysregulation of this enzyme is involved in many diseases, including type 2 diabetes, obesity, and cancers.[3,5,6]

Pyruvate carboxylation catalyzed by PC proceeds through a series of reactions shown in Figure 1. Reactions 1 and 2, in which the biotin cofactor is carboxylated via a carboxyphosphate intermediate ($^-O_2COPO_3^{2-}$), occur in the biotin carboxylase (BC) domain. Reaction 3, in which the carboxyl group is transferred from the carboxybiotin to pyruvate to form oxaloacetate, occurs in the carboxyl transferase (CT) domain. PC is commonly an α_4 tetramer, and the overall pyruvate carboxylation reaction has been shown to proceed via intersubunit catalysis where the subunits act in pairs so that the biotin of one subunit is carboxylated in its own BC domain but transfers its carboxyl group to pyruvate in its partner subunit's CT domain.[7]

In the majority of organisms, the activity of PC is positively regulated by the allosteric activator acetyl-CoA as a result of an increased rate of fatty acid oxidation. This mechanism allows sufficient levels of oxaloacetate to oxidize β-oxidation-derived acetyl-CoA. In microbes, PC is negatively regulated by L-aspartate, which signals an abundance of tricarboxylic acid cycle intermediates. From structural studies of RePC[7] and *Staphylococcus aureus* PC,[8] the binding site for acetyl-CoA has been identified as an allosteric domain that is surrounded by the BC, CT, and biotin carboxyl carrier protein (BCCP) domains. The binding site for L-aspartate has yet to be identified.

While much has been learned about the action of acetyl-CoA in a wide variety of organisms (see ref 9 for a review), the action of L-aspartate has been most extensively studied in the eukaryotic microbial PCs from *Aspergillus nidulans*[10] and *Saccharomyces cerevisiae*.[11,12] In both cases, the inhibition was reported to be competitive with respect to acetyl-CoA; in *A. nidulans*, PC L-aspartate decreased the cooperativity of activation of the enzyme by acetyl-CoA, while in *S. cerevisiae* PC, it increased the cooperativity. Much less is known about

$$\text{enzyme-biotin + MgATP + HCO}_3^- \;\rightleftharpoons\; \text{enzyme-biotin}.\text{}^-\text{O}_2\text{COPO}_3^{2-} + \text{MgADP} \qquad - (1)$$

$$\text{enzyme-biotin}.\text{}^-\text{O}_2\text{COPO}_3^{2-} \;\rightleftharpoons\; \text{enzyme-biotin-CO}_2^- + \text{Pi} \qquad - (2)$$

$$\text{enzyme-biotin-CO}_2^- + \text{pyruvate} \;\rightleftharpoons\; \text{enzyme-biotin + oxaloacetate} \qquad - (3)$$

Figure 1. Partial reactions catalyzed by pyruvate carboxylase. Reactions 1 and 2 occur in the BC domain, and reaction 3 occurs in the CT domain.

the inhibitory effects of L-aspartate in bacterial PCs, and in general, the loci and mechanisms of action of L-aspartate are not well understood. In this study, we have performed a detailed steady-state kinetic analysis of the inhibitory effects of L-aspartate on RePC, which has been extremely thoroughly characterized in structural and mechanistic terms,[7,13−15] to investigate the loci of action and inhibitory mechanisms of L-aspartate.

■ EXPERIMENTAL PROCEDURES

Materials. Sodium pyruvate, sodium oxaloacetate, ATP, sodium phosphoenolpyruvate, acetyl-CoA, NADH, malate dehydrogenase, lactate dehydrogenase, and pyruvate kinase were purchased from Sigma. 2′,3′-O-(2,4,6-Trinitrophenyl)-adenosine 5′-triphosphate (TNP-ATP) was obtained from Jena Bioscience. L-Aspartic acid was purchased from Fluka.

RePC Expression and Purification. N-Terminally His-tagged RePC was expressed in *Escherichia coli* BL21(DE3) and purified as described previously.[16] The purified RePC was resuspended and stored at −80 °C in storage buffer containing 30% (v/v) glycerol, 100 mM Tris-HCl (pH 7.8), and 1 mM dithioerythreitol.[17]

Pyruvate Carboxylation Activity Assay. The pyruvate carboxylating activities in the absence or presence of acetyl-CoA were determined by a coupled spectrophotometric assay in which the oxaloacetate produced was converted to malate with concomitant oxidation of NADH in a reaction catalyzed by malate dehydrogenase.[13] The assays were performed at 30 °C in 1 mL reaction mixtures containing 0.1 M Tris-HCl (pH 7.8), 20 mM NaHCO$_3$, 6 mM MgCl$_2$, 1 mM MgATP, 0.2 mM NADH, 10 mM sodium pyruvate, and 5 units of malate dehydrogenase. The concentrations of acetyl-CoA and L-aspartate were varied from 0 to 150 μM and from 0 to 8 mM, respectively.

To determine the effect of L-aspartate inhibition on the MgTNP-ATP activation of pyruvate carboxylation, MgTNP-ATP concentrations were varied from 0 to 100 μM at different L-aspartate concentrations (140 μg of enzyme was used per assay).

The k_{app} values were calculated by dividing the initial velocity by the enzymic biotin concentration in each assay.

Oxamate-Induced Oxaloacetate Decarboxylation. The activity of oxamate-induced oxaloacetate decarboxylation in the absence of acetyl-CoA was determined by a coupled spectrophotometric assay in which the pyruvate produced was converted to lactate with concomitant oxidation of NADH in a reaction catalyzed by lactate dehydrogenase.[18] All assays were performed at 30 °C in 1 mL of a reaction mixture containing 0.1 M Tris-HCl (pH 7.8), 0.22 mM NADH, 1 mM oxamate, and 4 units of lactate dehydrogenase. The concentrations of oxaloacetate and L-aspartate were varied between 0 and 250 μM and between 0 and 8 mM, respectively. The k_{cat} value was calculated from the initial velocity divided by the enzymic biotin concentration in each assay.

Bicarbonate-Dependent ATP Cleavage Activity Assay. The activity of bicarbonate-dependent ATP cleavage reactions in the absence of acetyl-CoA was determined by a coupled spectrophotometric assay in which the ADP produced was converted to ATP with concomitant dephosphorylation of phosphoenolpyruvate to form pyruvate in a reaction catalyzed by pyruvate kinase. The pyruvate thus formed was converted to lactate with concomitant oxidation of NADH in a reaction catalyzed by lactate dehydrogenase.[19] The reactions were performed at 30 °C in 1 mL mixture containing 0.1 M Tris-HCl (pH 7.8), 20 mM NaHCO$_3$, 5 mM MgCl$_2$, 0.22 mM NADH, 10 mM phosphoenolpyruvate, 5 units of pyruvate kinase, and 4 units of lactate dehydrogenase. Activities were determined by varying concentrations of MgATP and L-aspartate from 0 to 1000 μM and from 0 to 8 mM, respectively. The assay was initiated by the addition of the purified enzyme (493 μg/each assay).

**Coupling between Oxaloacetate Formation and P$_i$ Release in the Pyruvate Carboxylation Reaction.** To determine the effect of L-aspartate on the coupling between oxaloacetate formation and P$_i$ release in the pyruvate carboxylation assay, the initial rates of oxaloacetate formation and P$_i$ release were determined in the absence and presence (8 mM) of L-aspartate. The rate of P$_i$ release was measured using the assay of Black and Jones.[20] Briefly, 2% (w/v) ammonium molybdate·4H$_2$O, 14% (w/v) ascorbic acid in 50% (v/v) trichloroacetic acid, and 2% (w/v) trisodium citrate·2H$_2$O with 2% (w/v) sodium arsenite in 2% (v/v) acetic acid were prepared as reagents A−C, respectively; 200 μL of reagent A, 300 μL of reagent B, and 800 μL of water were added to the cuvette followed by the addition of 200 μL samples of the pyruvate carboxylation reaction mixture 4, 8, 12, and 16 min after the start of the reaction. The solution was immediately mixed and stood for 1 min, after which 1 mL of reagent C was added. The blue color developed within 4 min, and the absorbance was measured at 700 nm. A standard curve of a range of P$_i$ concentrations was prepared using a standard solution of NaH$_2$PO$_4$ in 0.1 M Tris (pH 7.8) and the assay described above. The P$_i$ content of the pyruvate carboxylation reaction mixtures was calculated from the standard curve. From plots of P$_i$ released versus time, the initial rates of P$_i$ release were calculated by linear regression analysis for triplicate time courses. The initial rates of oxaloacetate formation were determined from spectrophotometric assays using the malate dehydrogenase coupling reaction, as described above. The reactions were initiated by addition of the purified RePC (35 μg/assay). The coupling between oxaloacetate formation and P$_i$ release was calculated as the ratios of initial rates of these two reactions, and three separate sets of measurements were taken so that ratios are reported as the mean and standard deviations of these measurements.

Data Analysis. The acetyl-CoA dependence or MgTNP-ATP dependence of the pyruvate carboxylation reaction at each

concentration of L-aspartate was analyzed by using nonlinear least-squares regression fits of the data to eq 1.

$$k_{app} = [k_0 + k_{cat}(K_a/[A])^{h_1}]/[1 + (K_a/[A])^{h_1}] \quad (1)$$

where [A] is either the acetyl-CoA concentration or the MgTNP-ATP concentration, K_a is the activation constant, h_1 is the Hill coefficient of cooperativity, k_{app} is the apparent catalytic rate constant for the reaction at each concentration of L-aspartate, k_0 is the catalytic rate constant of the acetyl-CoA-independent reaction (determined experimentally), and k_{cat} is the catalytic rate constant of the acetyl-CoA-dependent reaction when acetyl-CoA is saturating. A global analysis of the acetyl-CoA activation of pyruvate carboxylating activities at the different, fixed concentrations of L-aspartate was performed by a nonlinear least-squares regression fit of the complete set of data initially to eq 2 that was derived for the reaction scheme in
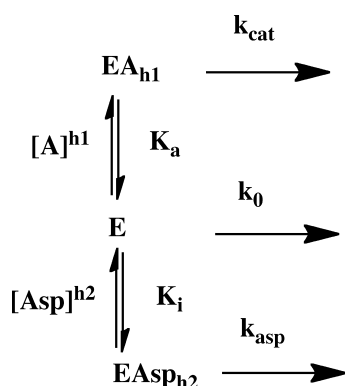
Figure 2. Reaction scheme showing the proposed inhibitory mechanism of pyruvate carboxylating activity by L-aspartate in the presence of acetyl-CoA, where k_0 and k_{cat} are catalytic rate constants for the acetyl-CoA-independent and -dependent reactions catalyzed by the enzyme (E) and enzyme·acetyl-CoA complex (EA$_{h1}$), respectively. The catalytic rate constant for the reaction catalyzed by the enzyme·L-aspartate complex (EAsp$_{h2}$) is k_{asp}. K_a is the apparent dissociation constant of the EA$_{h1}$ complex, and h_1 is the Hill coefficient for the activation process. K_i is the apparent dissociation constant of the enzyme·L-aspartate complex (EAsp$_{h2}$), and h_2 is the Hill coefficient for the inhibition by L-aspartate.

Figure 2, which describes competitive inhibition by L-aspartate with respect to acetyl-CoA but where k_{asp} is set to zero.

$$k_{app} = [k_0 + k_{cat}([A]/K_a)^{h_1}]/[1 + ([A]/K_a)^{h_1} + ([Asp]/K_i)^{h_2}] \quad (2)$$

where k_0, k_{cat}, K_a, and h_1 are as described for eq 1 except they are the values of these parameters in the absence of L-aspartate, [Asp] is the concentration of L-aspartate, K_i is the dissociation constant of the enzyme–aspartate complex, and h_2 is the Hill coefficient of cooperativity for the binding of L-aspartate to the RePC tetramer.
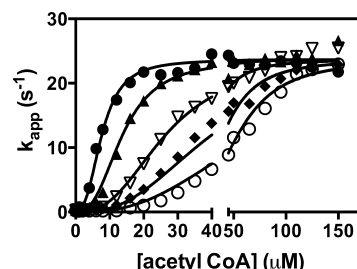
Inactivation of pyruvate carboxylating activity by L-aspartate in the absence of acetyl-CoA was analyzed by using nonlinear least-squares regression fits of the data to eq 3.

$$k_{app} = [k_0 + k_{asp}([Asp]/K_i)^{h_2}]/[1 + ([Asp]/K_i)^{h_2}] \quad (3)$$

where the parameters are as described for eq 2, except for k_{asp}, which is the catalytic rate constant for the pyruvate

carboxylation reaction conducted by the enzyme·L-aspartate complex. The value of k_0 was determined experimentally.

The data in Figure 3 were subsequently fit to eq 4, where the value of k_{asp} was set at the value determined from the analysis of

Figure 3. Effect of increasing concentrations of L-aspartate [(●) 0, (▲) 2, (▽) 4, (◆) 6, and (○) = 8 mM] on the activation of the pyruvate carboxylation reaction by acetyl-CoA. The lines represent a global, nonlinear least-squares regression fit of the data to eq 2. Reaction conditions were as described in Experimental Procedures.

the effect of L-aspartate on acetyl-CoA-independent pyruvate carboxylation described above.

$$k_{app} = [k_0 + k_{cat}([A]/K_a)^{h_1} + k_{asp}([Asp]/K_i)^{h_2}]$$
$$/[1 + ([A]/K_a)^{h_1} + ([Asp]/K_i)^{h_2}] \quad (4)$$

The kinetics of oxamate-induced oxaloacetate decarboxylation in the absence of acetyl-CoA at different L-aspartate concentrations were analyzed by nonlinear least-squares regression fits of the data to eq 5.

$$k_{app} = k_{cat}/(1 + K_{OAA}/[OAA] + [OAA]/K_I) \quad (5)$$

where K_{OAA} and [OAA] are the Michaelis–Menten constant for and the concentration of oxaloacetate, respectively. K_I is the substrate inhibition constant for oxaloacetate.

The data describing inhibition of the bicarbonate-dependent MgATP cleavage reaction by L-aspartate were analyzed by a nonlinear least-squares regression fit initially to eq 6 that describes mixed inhibition and then to eq 7 that describes competitive inhibition.

$$k_{app} = k_{cat}/[K_m(1 + [Asp]/K_i)/[MgATP] + 1 + [Asp]/K_{is}] \quad (6)$$

$$k_{app} = k_{cat}/[K_m(1 + [Asp]/K_i)/[MgATP] + 1] \quad (7)$$

where K_m is the Michaelis–Menten constant for MgATP, K_i is the dissociation constant of the enzyme·L-aspartate complex, and K_{is} is that for the enzyme·L-aspartate·MgATP complex.

■ RESULTS

Effect of Increasing L-Aspartate Concentrations on the Activation of Pyruvate Carboxylation by Acetyl-CoA. Table 1 shows the parameters estimated from the fits of eq 1 to each individual set of data at each concentration of L-aspartate shown in Figure 3 and k_0. This clearly shows that K_a increases with increasing L-aspartate concentrations, whereas there is no strong trend of change in either k_{cat} or h_1. However, k_0 does appear to decrease with increasing L-aspartate concentrations. As mentioned earlier, inhibition of fungal PCs by L-aspartate has been found to be competitive with respect to acetyl-CoA. To determine if this could be the case here, we

Table 1. Kinetic Parameters for Acetyl-CoA Activation of the Pyruvate Carboxylation Reaction Catalyzed by RePC in the Presence of Different Concentrations of L-Aspartate[a]

[L-aspartate] (mM)	K_a for acetyl-CoA (μM)	Hill coefficient (h_1)	k_{cat} (s^{-1})	k_0 (s^{-1})
0	7.3 ± 0.9	2.47 ± 0.21	22.9 ± 0.5	0.32
2	14.5 ± 0.5	2.87 ± 0.26	23.9 ± 0.6	0.27
4	23.7 ± 0.7	2.48 ± 0.16	24.9 ± 0.6	0.23
6	35.6 ± 1.1	2.74 ± 0.22	22.4 ± 0.6	0.21
8	60.5 ± 3.0	2.54 ± 0.21	25.3 ± 1.2	0.19

[a]Assay conditions: 0.1 M Tris-HCl (pH 7.8), 20 mM NaHCO$_3$, 6 mM MgCl$_2$, 1 mM MgATP, 0.2 mM NADH, 10 mM sodium pyruvate, and 5 units of malate dehydrogenase (MDH). The concentrations of acetyl-CoA and L-aspartate were varied from 0 to 150 μM and from 0 to 8 mM, respectively. The parameters (±standard errors) were estimated from a nonlinear regression fit of the data at each L-aspartate concentration to eq 1 except k_0, which was measured directly.

developed a model (Figure 2) that describes this type of inhibition and from which eq 2 was derived. The data in Figure 3 were globally fit to this equation, and the solid lines in Figure 3 represent the fit. As one can see, the fit was good with an R^2 of 0.992. The following parameters were estimated from the fit: k_{cat} = 23.7 ± 0.2 s^{-1}, K_a = 7.70 ± 0.27 μM, h_1 = 2.65 ± 0.09, K_i = 1.26 ± 0.08 mM, and h_2 = 2.78 ± 0.11. The value of h_2 suggests that the binding of l-aspartate to RePC is also cooperative with a Hill coefficient similar to that for acetyl-CoA. A fit of eq 2 in which h_2 was set to 1 (no allosteric binding of L-aspartate) failed to give a good fit to the data.
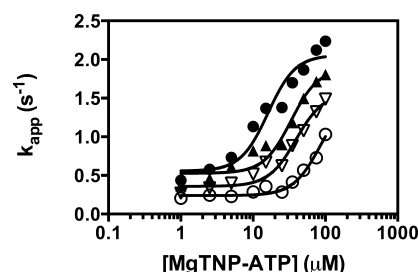
Effect of Increasing L-Aspartate Concentrations on the Pyruvate Carboxylation Reaction in the Absence of Acetyl-CoA. Figure 4 shows a plot of k_{app} at different
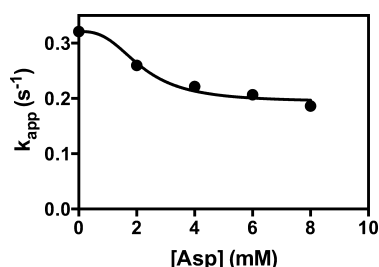
Figure 4. Effect of increasing concentrations of L-aspartate on the acetyl-CoA-independent pyruvate carboxylation reaction. Data points at zero acetyl-CoA concentration were replotted from Figure 3. The line represents a nonlinear least-squares regression fit of the data to eq 3.

concentrations of L-aspartate in the absence of acetyl-CoA. The data were fit to eq 3 that describes inhibition that depends on [L-aspartate]^{h_2}, using the value of h_2 estimated above. The fit to eq 3 gave values of K_i and k_{asp} of 2.2 ± 0.2 mM and 0.19 ± 0.01 s^{-1}, respectively. The value of K_i is somewhat higher than that estimated for the global fit of the data in Figure 3, but the data set in Figure 4 is much smaller. The positive value of k_{asp}, which is significantly different from zero (t test; $p < 0.01$), indicated that at a saturating concentration of L-aspartate, the enzyme retains some residual activity. Refitting the data in Figure 3 to eq 4, which accounts for this residual activity in the enzyme·L-aspartate complex, resulted in only very small changes to the estimated parameters: k_{cat} = 23.7 ± 0.2 s^{-1}, K_a = 7.70 ± 0.27 μM, h_1 = 2.69 ± 0.09, K_i = 1.25 ± 0.07 mM, and h_2 = 2.81 ± 0.11.

Effect of Increasing L-Aspartate Concentrations on the Activation of Pyruvate Carboxylation by MgTNP-ATP. Figure 5 shows the effects of L-aspartate on the activation

Figure 5. Effect of increasing concentrations of L-aspartate [(●) 0, (▲) 2, (▽) 4, and (○) 8 mM] on the activation of the pyruvate carboxylation reaction by MgTNP-ATP. The lines represent a nonlinear least-squares regression fit of each set data at a particular concentration of L-aspartate to eq 1, where h_1 was set to 2.3, the value determined previously.[13] Reaction conditions were as described in Experimental Procedures.

of pyruvate carboxylation by MgTNP-ATP, with each set of data at individual L-aspartate concentrations fit to eq 1. The estimated parameters from these fits are listed in Table 2. It is

Table 2. Kinetic Parameters for MgTNP-ATP Activation of the Pyruvate Carboxylation Reaction Catalyzed by RePC in the Presence of Different Concentrations of L-Aspartate[a]

[L-aspartate] (mM)	K_a for MgTNP-ATP (μM)	k_{cat} (s^{-1})	k_0 (s^{-1})
0	16.1 ± 2.9	2.1 ± 0.1	0.48
2	33.5 ± 5.6	1.9 ± 0.1	0.43
4	39.1 ± 6.2	1.6 ± 0.1	0.23
8	85.1 ± 17.6	1.5 ± 0.3	0.16

[a]Assay conditions: 0.1 M Tris-HCl (pH 7.8), 20 mM NaHCO$_3$, 6 mM MgCl$_2$, 1 mM MgATP, 0.2 mM NADH, 10 mM sodium pyruvate, and 5 units of malate dehydrogenase (MDH). The concentrations of MgTNP-ATP and L-aspartate were varied from 0 to 100 μM and from 0 to 8 mM, respectively. The parameters (±standard errors) except k_0, which was measured directly, were estimated from a nonlinear regression fit of the data at each L-aspartate concentration to eq 1, where h_1 was set to 2.3, the value determined previously.[13]
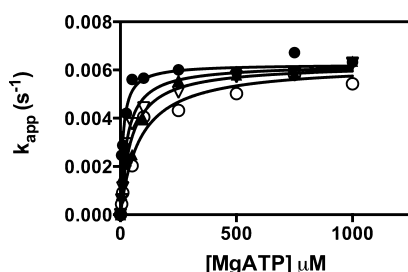
clear that with increasing concentrations of L-aspartate the value of K_a increases markedly and, as expected, that of k_0 decreases. The value of k_{cat} also appears to decrease with increasing concentrations of L-aspartate; however, the value of k_{cat} at 8 mM L-aspartate is not significantly different from that at 0 mM L-aspartate (t test; $p > 0.1$).

Effect of L-Aspartate on the Coupling between MgATP Cleavage and Pyruvate Carboxylation. The coupling between MgATP cleavage in the BC domain and pyruvate carboxylation in the CT domain was examined by measuring the rates of the formation of oxaloacetate and release of P$_i$ in the absence and presence (8 mM) of L-aspartate under identical reaction conditions. The ratios of oxaloacetate formation to P$_i$ release in the absence and presence of L-aspartate (8 mM) were 0.96 ± 0.06 and 0.89 ± 0.06, respectively. The means ± SD of coupling ratios in the absence and presence of L-aspartate were found to be not significantly different using a t test ($p > 0.2$). This suggests that binding of L-aspartate to the enzyme has no effect on the coupling between the reactions in the BC and CT domain reactions.

Effect of Increasing L-Aspartate Concentrations on the Partial Reactions of RePC in the Absence of Acetyl-CoA. To examine how L-aspartate has the observed inhibitory effect on pyruvate carboxylation in the absence of acetyl-CoA and to determine the locus of its action, the kinetics of oxamate-dependent oxaloacetate decarboxylation (CT domain)[18] and bicarbonate-dependent MgATP cleavage in the absence of pyruvate (BC domain)[19] were measured in the presence of increasing concentrations of L-aspartate.

Kinetic parameters for oxamate-dependent oxaloacetate decarboxylation (data not shown) in the absence of L-aspartate and in the presence of 8 mM L-aspartate were obtained by fitting the data to eq 5. Comparison of the estimates of these parameters and their accompanying standard errors using t tests showed that there were no significant differences among the values of k_{cat}, K_{OAA}, and K_I in the presence of 0 and 8 mM L-aspartate ($p > 0.2$ in all cases).

The effects of increasing concentrations of L-aspartate on the kinetics of bicarbonate-dependent MgATP cleavage in the absence of pyruvate are shown in Figure 6. The solid lines

Figure 6. Effect of increasing concentrations of L-aspartate [(●) 0, (▲) 2, (▽) 4, and (○) 8 mM] on the bicarbonate-dependent MgATP cleavage reaction in the absence of acetyl-CoA and pyruvate. The lines represent global fits of the data to eq 7. Reaction conditions were as described in Experimental Procedures.

represent global fits of the data to eq 7, which describes competitive inhibition and gave the following estimates for parameters: $k_{cat} = 0.0062 \pm 0.0001$ s^{-1}, $K_m = 11 \pm 2$ μM, and $K_I = 1.2 \pm 0.3$ mM. A fit to eq 6, which describes mixed inhibition, gave a fit of the data that was not significantly better than the fit to eq 7 by the variance ratio test ($p > 0.1$).

■ DISCUSSION

Previously, IC$_{50}$ values for the inhibition by L-aspartate for PC have been reported to be in the range of 0.6–4 mM,[10,11,21] while Dunn et al.[22] reported that 10 mM L-aspartate only marginally inhibited the enzyme from *Sinorhizobium meliloti*. Thus, the K_i value of 1.3 mM obtained for RePC in this study is at the lower end of the range.

As with PC from *S. cerevisiae*,[11,12] increasing concentrations of L-aspartate resulted in an increase in the apparent K_a for activation of the enzyme by acetyl-CoA. However, unlike in the *S. cerevisiae* enzyme where increasing L-aspartate concentrations markedly increased the Hill coefficient of cooperativity for the activation by acetyl-CoA,[11,12] L-aspartate had little effect on the cooperativity of acetyl-CoA activation in RePC. The competitive nature of the inhibition of RePC by L-aspartate with respect to acetyl-CoA activation was also noted in the fungal PCs,[10–12] and the antagonistic nature of the inhibition with respect to acetyl-CoA was noted but not analyzed for PC from the bacterial enzyme *Rhodobacter capsulatus*.[23] The cooperative

nature of the binding of L-aspartate to RePC has also been observed in the fungal PCs,[10,11] where Hill coefficients of 1.53 and 2.1 have been reported for the enzymes from *S. cerevisiae* and *A. nidulans*, respectively. In a bacterial PC from *Arthrobacter globiformus*,[24] a Hill coefficient of 2.0 was reported. Thus, the value of the Hill coefficient of 2.8 reported here for RePC inhibition by L-aspartate was somewhat higher than these values, indicating increased cooperativity of binding. In RePC, the value of the Hill coefficient for L-aspartate inhibition is similar to that of acetyl-CoA activation, but in the case of PC from *S. cerevisiae*,[11] the Hill coefficient for L-aspartate inhibition is greater than that of acetyl-CoA binding; in *A. globiformus*,[24] it is lower and acetyl-CoA does not activate *A. nidulans* PC in the absence of L-aspartate.[10]

The inhibition of RePC in the absence of acetyl-CoA in this work has also been reported to occur in the fungal PCs,[10,11,13] but there has been little investigation of the mechanism of this inhibition. We have shown that L-aspartate affects reactions in the BC domain but not the CT domain of RePC. It is clear that one mode of action is that of competitive inhibition with respect to MgATP binding in the bicarbonate-dependent MgATP cleavage reaction. However, this does not explain the inhibition of the pyruvate carboxylation reaction of RePC that was performed in the presence of close to saturating concentrations of MgATP.[13] Because the effect of L-aspartate does not lie in the CT domain, it is likely that this inhibition of pyruvate carboxylation occurs in the BC domain. It has been reported that acetyl-CoA enhances the coupling between the cleavage of MgATP (as measured by P$_i$ release) and the formation of carboxybiotin[25] and oxaloacetate in RePC.[15] However, L-aspartate does not appear to significantly affect the coupling between MgATP cleavage and oxaloacetate formation. This indicates that the inhibitory effect of L-aspartate at saturating concentrations of MgATP may be an effect on the rate of either carboxybiotin formation or the translocation of carboxybiotin to the CT domain.

As with activation of PCs by acetyl-CoA, there seems to be considerable variation in the parameters that describe the inhibition of PCs by L-aspartate, even between bacterial species. Generally, however, L-aspartate inhibition appears to be competitive with respect to acetyl-CoA and cooperative. The question of the mechanism of this competitive inhibition by L-aspartate then arises. The simplest model would be one in which L-aspartate and acetyl-CoA compete for the same binding site on PC. There is, however, some evidence to suggest that this is not the case. First, Libor et al.[21] showed that modification of the thermophilic *Bacillus* PC with trinitrobenzenesulfonate specifically inhibited acetyl-CoA-dependent enzyme activity but had no effect on the acetyl-CoA-independent activity or its inhibition by L-aspartate. Moreover, the presence of acetyl-CoA protected the enzyme against modification. Second, Adina-Zada et al.[26] showed that mutation of key residues in the acetyl-CoA binding site that increased the K_a between 76- and 252-fold had virtually no effect on the inhibition of RePC by L-aspartate in the absence of acetyl-CoA. Thus, it is probable that there is a distinct binding site for L-aspartate that exerts its effects on acetyl-CoA binding through allosteric effects. RePC has been crystallized in the presence of L-aspartate, but because of the high B factors the binding site for the inhibitor could not be located.[27] Adina-Zada et al.[13] showed that inhibition of RePC by MgTNP-ATP was also competitive with respect to acetyl-CoA but that its binding site was separate from that of the allosteric activator. Like L-

aspartate, MgTNP-ATP was also shown to have allosteric effects in the absence of acetyl-CoA, but MgTNP-ATP allosterically activated the enzyme. In this work, we have shown that L-aspartate also inhibits MgTNP-ATP activation of RePC. Thus, it is possible that the binding site for L-aspartate is separate from both that of acetyl-CoA and that of MgTNP-ATP but that there are allosteric interactions among all three binding sites.

In summary, we have shown that the inhibition of RePC by L-aspartate is multifaceted. As with other microbial PCs, the inhibition is competitive with respect to the allosteric activator, acetyl-CoA, but there is a component of the inhibition that is independent of acetyl-CoA. The locus of this part of the inhibition lies in the BC domain and is comprised in part of competitive inhibition of MgATP binding and biotin carboxylation and/or carboxybiotin translocation. Because it is unlikely that the L-aspartate binding site is in the MgATP binding site, the acetyl-CoA binding site, or the MgTNP-ATP binding site, the effects of L-aspartate on acetyl-CoA activation, MgTNP-ATP activation, and MgATP binding and the effects on biotin carboxylation and/or carboxybiotin translocation are allosteric, induced from a remote L-aspartate binding site. Further understanding of the mechanism of action of L-aspartate must await a structural resolution of its binding site.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

acetyl-CoA, acetyl-coenzyme A; PC, pyruvate carboxylase; RePC, *Rhizobium etli* pyruvate carboxylase; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; BC, biotin carboxylase; CT, carboxyl transferase.

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