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

Chaiyos Sirithanakorn,‡ Abdussalam Adina-Zada,‡ John C. Wallace,§ Sarawut Jitrapakdee,*† and Paul V. Attwood*‡

‡Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
§School of Chemistry and Biochemistry, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

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 enzyme, 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to be an important anaplerotic reaction because it replenishes tricarboxylic acid cycle intermediates that have been withdrawn for anabolic purposes.‡ PC is found in wide variety of organisms, including eubacteria, yeast, fungi, and animals (for reviews, see refs 1 and 2).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.‡,§ Furthermore, PC is also necessary for glucose-induced insulin secretion in pancreatic β-cells.⁵ As PC has such diverse metabolic roles, dysregulation of this enzyme is involved in many diseases, including type 2 diabetes, obesity, and cancers.³,⁴,⁶

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 (–O₂CP(O)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 α₄ 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 RePC⁷ and Staphylococcus aureus PC,⁶ 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 nidulans⁵ 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
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 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
dithioerythritol.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.13 The assays were performed at 30 °C in 1 mL reaction mixtures containing 0.1 M Tris-HCl (pH 7.8), 20 mM NaHCO₃, 5 mM MgCl₂, 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
100 μM and from 0 to 8 mM, respectively. The assay was
initiated by the addition of the purified enzyme (493 μg/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 NaHCO₃, 5 mM MgCl₂, 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
100 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 Pi
Release in the Pyruvate Carboxylation Reaction.** To
determine the effect of l-aspartate on the coupling between
oxaloacetate formation and Pi release in the pyruvate
carboxylation assay, the initial rates of oxaloacetate formation
and Pi release were determined in the absence and presence (8
mM) of l-aspartate. The rate of Pi release was measured using
the assay of Black and Jones.20 Briefly, 2% (w/v) ammonium
molybdate-4H₂O, 14% (w/v) ascorbic acid in 50% (v/v)
trichloroacetic acid, and 2% (w/v) trisodium citrate-2H₂O 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 Pi concentrations was prepared using a standard
solution of NaH₂PO₄ in 0.1 M Tris (pH 7.8) and the assay
described above. The Pi content of the pyruvate carboxylation
reaction mixtures was calculated from the standard curve. From
plots of Pi released versus time, the initial rates of Pi 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 Pi
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

\[
\text{enzyme-biotin + MgATP + HCO}_3^- \rightleftharpoons \text{enzyme-biotin} \cdot \text{O}_2 \text{COPO}_4^{2-} + \text{MgADP} \quad (1)
\]

\[
\text{enzyme-biotin} \cdot \text{O}_2 \text{COPO}_4^{2-} \rightleftharpoons \text{enzyme-biotin} \cdot \text{CO}_2 + \text{Pi} \quad (2)
\]

\[
\text{enzyme-biotin} \cdot \text{CO}_2 + \text{pyruvate} \rightleftharpoons \text{enzyme-biotin} + \text{oxaloacetate} \quad (3)
\]

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

\[
k_{\text{app}} = \left[ k_0 + k_{\text{cat}}(K_a/[A])^{h_1} \right] / \left[ 1 + (K_a/[A])^{h_1} \right]
\]

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, \(k_{\text{app}}\) 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 \(k_{\text{cat}}\) 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, which describes competitive inhibition by L-aspartate with respect to acetyl-CoA but where \(k_{\text{app}}\) is set to zero.

\[
k_{\text{app}} = \left[ k_0 + k_{\text{cat}}([A]/K_a)^{h_1} \right] / \left[ 1 + ([A]/K_a)^{h_1} \right] + ([\text{Asp}]/K_{\text{asp}})^{h_2}
\]

where \(k_{\text{app}}\), \(k_{\text{cat}}\), \(K_a\), and \(h_1\) are as described for eq 1 except they are the values of these parameters in the absence of L-aspartate, \([\text{Asp}]\) is the concentration of L-aspartate, \(K_{\text{asp}}\) is the dissociation constant of the enzyme-L-aspartate complex, and \(h_2\) is the Hill coefficient for the activation process. \(K_i\) is the apparent dissociation constant of the enzyme-L-aspartate complex and \(h_1\) is the Hill coefficient for the inhibition by L-aspartate.

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 \(k_{\text{cat}}\) are catalytic rate constants for the acetyl-CoA-independent and -dependent reactions catalyzed by the enzyme (E) and enzyme-acetyl-CoA complex (EAh1), respectively. The catalytic rate constant for the reaction catalyzed by the enzyme-L-aspartate complex (EAsph2) is \(k_{\text{app}}\). \(K_i\) is the apparent dissociation constant of the EAh1 complex, and \(h_1\) is the Hill coefficient for the activation process. \(K_{\text{asp}}\) is the dissociation constant of the enzyme-L-aspartate complex, and \(h_2\) is the Hill coefficient for the inhibition by L-aspartate.

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

\[
k_{\text{app}} = \left[ k_0 + k_{\text{cat}}([A]/K_a)^{h_1} + k_{\text{app}}([\text{Asp}]/K_{\text{asp}})^{h_2} \right] / \left[ 1 + ([A]/K_a)^{h_1} + ([\text{Asp}]/K_{\text{asp}})^{h_2} \right]
\]

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.

\[
k_{\text{app}} = k_{\text{cat}}/[1 + K_{\text{OAA}}/(\text{OAA}) + [\text{OAA}]/K_i]
\]

where \(K_{\text{OAA}}\) and \([\text{OAA}]\) 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.

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

\[
+ 1 + [\text{Asp}]/K_m]
\]

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

where \(K_m\) is the Michaelis–Menten constant for MgATP, \(K_i\) is the dissociation constant of the enzyme-L-aspartate complex, and \(K_m\) 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 \(k_{\text{cat}}\) 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

| [l-aspartate] (mM) | \(K_v\) for acetyl-CoA (\(\mu\)M) | Hill coefficient \(h_i\) | \(k_{ap}\) (s\(^{-1}\)) | \(k_{0i}\) (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              |

*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 \(\mu\)M 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 \(k_{ap}\) which was measured directly.

Effect of Increasing L-Aspartate Concentrations on the Pyruvate Carboxylation Reaction in the Absence of Acetyl-CoA. Figure 4 shows a plot of \(k_{ap}\) at different 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]\(^{-1}\), using the value of \(h_i\) estimated above. The fit to eq 3 gave values of \(K_v\) and \(k_{ap}\) of 2.2 ± 0.2 \(\mu\)M and 0.19 ± 0.01 s\(^{-1}\), respectively. The value of \(K_v\) 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 \(k_{ap}\) 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: \(k_{ap} = 23.7 ± 0.2\) s\(^{-1}\), \(K_v = 7.70 ± 0.27\) \(\mu\)M, \(h_1 = 2.69 ± 0.09\), \(K_i = 1.25 ± 0.07\) mM, and \(h_2 = 2.81 ± 0.11\).

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

| [l-aspartate] (mM) | \(K_v\) for MgTNP-ATP (\(\mu\)M) | \(k_{ap}\) (s\(^{-1}\)) | \(k_{0i}\) (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              |

*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 \(\mu\)M and from 0 to 8 mM, respectively. The parameters (±standard errors) except \(k_{ap}\) which was measured directly, were estimated from a nonlinear regression fit of the data at each l-aspartate concentration to eq 1, where \(h_i\) was set to 2.3, the value determined previously.13 Reaction conditions were as described in Experimental Procedures.

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 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 clear that with increasing concentrations of l-aspartate the value of \(K_v\) increases markedly and, as expected, that of \(k_{ap}\) 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 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 \(k_{\text{cat}}, K_{\text{ox}}\), 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 represent global fits of the data to eq 7, which describes competitive inhibition and give the following estimates for parameters: \(k_{\text{cat}} = 0.0062 \pm 0.0001 \text{s}^{-1}, K_m = 11 \pm 2 \mu\text{M}, \text{and } K_i = 1.2 \pm 0.3 \text{ mM} \). 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 \(K_i\) 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*. \(^{25}\) 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 Pi 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 \(K_i\) 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...
Further understanding of the mechanism of action of L-MgTNP-ATP activation, and MgATP binding and the onset biotin carboxylation and/or carboxybiotin translocation are binding sites. 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.

**AUTHOR INFORMATION**

**Corresponding Authors**
*Department of Biochemistry, Mahidol University, Bangkok, Thailand. Phone: +662-201-5600. Fax: +61-8-354-7174. E-mail: sarawut.jit@mahidol.ac.th.*

*School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia, Australia. Telephone: +61-8-6488-3329. Fax: +61-8-6488-1148. E-mail: paul.attwood@uwa.edu.au.

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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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