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Plant aspartate transcarbamylase: kinetic properties of the enzyme from mung bean (*Phaseolus aureus*) seedlings

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Abstract. Aspartate transcarbamylase (EC 2.1.3.2) catalyzes the bi substrate reaction —carbamyl phosphate + L-aspartate ⇌ carbamyl aspartate + phosphate. The order of addition of substrates and release of products for the homogeneous aspartate transcarbamylase from *Phaseolus aureus* seedlings has been investigated by using the kinetic methods of analysis.

Initial velocity studies indicated that the mechanism might be a sequential one. Product inhibition studies showed that phosphate was a linear competitive inhibitor with respect to carbamyl phosphate and was an *S* (slope) and *I* (intercept) linear non-competitive inhibitor with respect to aspartate. Carbamyl aspartate was a non-competitive inhibitor with respect to both the substrates. These inhibition patterns agreed with an ordered mechanism of reaction with carbamyl phosphate as the leading substrate and phosphate as the last product to leave the enzyme surface. The presence of dead end complexes and the rapid equilibrium random mechanism were ruled out by the absence of inhibition by the substrate(s) and the linear replot slope *vs.* the inhibitor concentration.

Acetyl phosphate, an analog ue of carbamyl phosphate was a non-competitive inhibitor with respect to aspartate. This result could be explained both in terms of an ordered as well as a random mechanism. On the other hand, succinate, an analog ue of aspartate was an uncompetitive inhibitor with respect to carbamyl phosphate, indicating that the mechanism was ordered.

The transition state analog ue, N-(phosphonoacetyl)-L-aspartate, binds much more tightly than either of the two substrates. This analog ue was a linear competitive inhibitor with respect to carbamyl phosphate and a linear noncompetitive inhibitor with respect to aspartate. These results are compatible with an ordered mechanism rather than a random one.

Keywords. Aspartate transcarbamylase; mung bean; kinetic mechanism.

1. Introduction

Aspartate transcarbamylase (carbamyl phosphate: L-aspartate carbamyl transferase, EC 2.1.3.2) catalyzes the first specific step in the *de novo* pathway of pyrimidine biosynthesis. This enzyme was purified to homogeneity from *Phaseolus aureus* seedlings as reported earlier (Achar *et al* 1974). The availability of the homogeneous enzyme permitted a more detailed analysis of the kinetic properties of the enzyme.

The first detailed kinetic analysis of this reaction was made by Porter *et al* (1969) on the catalytic subunit of *Escherichia coli* enzyme. The steady state kinetic analysis indicated that the binding of substrates by the catalytic subunit of aspartate transcarbamylase was ordered with carbamyl phosphate as the leading substrate and three dead end complexes were formed. Heyde *et al* (1973 a, b) repeated the experiments of Porter *et al* (1969) along with a few more additional methods of analysis and proposed that the mechanism was of the rapid equilibrium random type with

three dead end complexes. Wedler and Gasser (1974) in an attempt to resolve the above discrepancy used isotope exchange technique and concluded that the mechanism was ordered. More recently Jacobson and Stark (1975) using ultra violet (UV) difference spectroscopy and steady state kinetics confirmed that the mechanism was ordered.

Unlike the *E. coli* enzyme, the enzyme from *Streptococcus faecalis* (Chang and Jones 1974 a, b, c) with a molecular weight similar to the plant enzyme (Achar *et al* 1974) functioned by a rapid equilibrium random mechanism although it was not subject to feedback inhibition by end products. The mung bean enzyme activity was regulated by UMP and was found to belong to the *K* system (Savithri 1977; Savithri *et al* 1978). It was therefore of interest to study the kinetic mechanism of the mung bean enzyme.

This paper describes initial velocity, product, substrate and transition state analogue inhibition studies. The data are compatible with an ordered mechanism with carbamyl phosphate as the first substrate to add and phosphate as the last product to leave the mung bean aspartate transcarbamylase.

2. Materials and methods

L-Aspartic acid, carbamyl aspartic acid, dilithium carbamyl phosphate, diacetyl monooxime, acetyl phosphate and DEAE cellulose (fine) were from Sigma Chemical Company, St. Louis, MO, USA. Diphenylamine-4-sulfonic acid (sodium salt) was from Fluka AG, Bucks, Switzerland. [U-^{14}C]-L-Aspartic acid was from Radioactive Chemical Center, Amersham, U.K. 1,4-Di-ε-2-(5-phenyloxazolyl)-benzene (POPOP) and 2,5-diphenyloxazole (PPO) were from Koch-Light Laboratories Limited, Colnbrook, Bucks, England. Sephadex G-25 and G-100 were products of Pharmacia, Uppsala, Sweden. N-(Phosponoacetyl)-L-aspartate was a gift from Dr G R Stark, Department of Biochemistry, Stanford University School of Medicine, Palo Alto, Ca, USA. All other chemicals were of analytical reagent grade. Mung bean seeds were purchased from the local market.

2.1. *Enzyme*

Aspartate transcarbamylase was isolated from 48 h germinated mung bean seedlings and purified as described earlier (Achar *et al* 1974) and used as the enzyme in these studies.

2.2. *Enzyme assay*

A standard reaction mixture consisting of 5 mM carbamyl phosphate (carbamyl phosphate was purified by the method of Gerhart and Pardee (1962) and was freshly prepared before use), 10 mM aspartate (titrated with 0·5M NaOH to pH 8·0), 0·1M Tris-HCl pH 8·0, enzyme and water to make up a total volume of 1 ml was incubated at 30°C. At the end of 20 min of incubation, the reaction was terminated by the addition of 0·3N perchloric acid (1 ml). The tubes were centrifuged at 1000 g for 5 min to remove the denatured proteins. The carbamyl aspartate formed was estimated by the colorimetric method of Gerhart and Pardee (1962). This colorimetric assay was not suitable for some of the kinetic analyses especially for the

inhibition studies with carbamyl aspartate. Therefore the assay described by Porter *et al* (1969) using [U-^{14}C]-aspartate was employed.

2.3. *Assay for aspartate transcarbamylase using [U^{14}-C]-aspartate*

The reaction mixture contained carbamyl phosphate (5 mM), [U-^{14}C]-aspartate (0·05 μCi), cold aspartate (10 mM), enzyme and 0·2M Tris-HCl buffer pH 8·0. The reaction was started by the addition of carbamyl phosphate and at the end of 20 min of incubation at 30°C, it was stopped by adding 1·5 ml of 0·02M CH_3COOH. A 1·5 ml aliquot of this mixture was loaded on to a Dowex $50\times8H^+$ form (50μ–100μ) column ($0{\cdot}8\times10$ cm). (The H^+ form of the resin was prepared by washing the resin with 0·5N NaOH, 0·5N HCl and water until the washing was free of chloride). The carbamyl aspartate was eluted from the column by washing with 4 ml water. The effluent was collected directly into the counting vials and evaporated to dryness at 80°C. The residue in the vial was dissolved in 0·6 ml water and scintillation fluid (5 ml) was added. The scintillation fluid contained Triton X-100 (10% v/v) and toluene containing PPO (7 g/lit) and 0·3 g (POPOP)/lit in the ratio of 1 : 2. The radioactivity in the samples was determined using Beckman LS-100 scintillation spectrometer. The reaction was linear up to 20 min and the protein concentration used was within the linear range. The enzyme functioned optimally at pH 8·0 and 10·2 (Achar *et al* 1974). Although the regulation of the activity of the enzyme was studied at both the pH values, the kinetic mechanism was examined only at pH 8·0.

Protein was estimated by the method of Lowry *et al* (1951) using crystalline bovine serum albumin as the standard protein.

One unit of enzyme activity was defined as μmol of product formed/min. Specific activity was expressed as units/g protein.

3. Results

3.1. *Initial velocity studies*

The enzyme (100 μg) was preincubated with carbamyl phosphate at concentrations indicated in figure 1 for 10 min at 30°C. The reaction was started by the addition of 1 mM, 4 mM and 10 mM aspartate respectively to the three sets of assay tubes and the amount of carbamyl aspartate formed was estimated (Gerhart and Pardee 1962). The reciprocal of velocity was plotted against the reciprocal of carbamyl phosphate concentration (figure 1). The parabolic nature of the curves indicated that the enzyme exhibited homotropic interactions with carbamyl phosphate. The cooperative effect was also evident from the Hill coefficients of greater than 1 (not shown in the figure). The existence of homotropic interactions would make further analysis of kinetic data difficult. One method of abolishing such an interaction would be the use of activators which convert sigmoid saturation curve to a classical hyperbolic saturation pattern (Monod *et al* 1964).

The enzyme was preincubated with either aspartate (10 mM) or carbamyl phosphate (5mM) for 10 min. The reaction was started by the addition of carbamyl phosphate (0·25 to 5 mM) in the case of enzyme preincubated with as partate and with aspartate (10 mM) in the case of the enzyme preincubated with carbamyl phosphate.

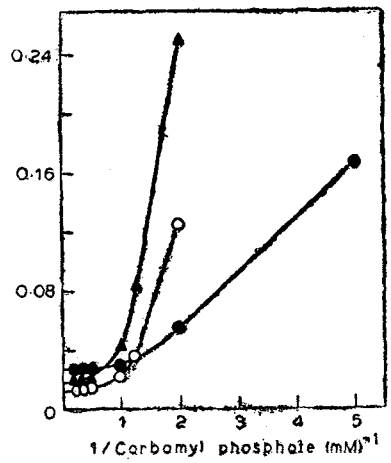

Figure 1. Reciprocal plot of aspartate transcarbamylase activity for the variation of carbamyl phosphate, at fixed levels of aspartate.
The reaction mixtures contained carbamyl phosphate (0·25 to 5 mM), aspartate (1, 4 and 10 mM), Tris-HCl buffer pH 8·0 (100 mM), enzyme (100 μg) and water to make up a total volume of 1 ml. The enzyme was preincubated with carbamyl phosphate for 10 min at 30°C prior to starting the reaction with aspartate. Carbamyl aspartate was estimated as described in materials and methods. The reciprocal of velocity (μmol of carbamyl aspartate formed/min/g of protein) was plotted against reciprocal of carbamyl phosphate concentration. ● ——— ● 1 mM aspartate; ▲ ——— ▲ 4 mM aspartate; O ——— O 10 mM aspartate.

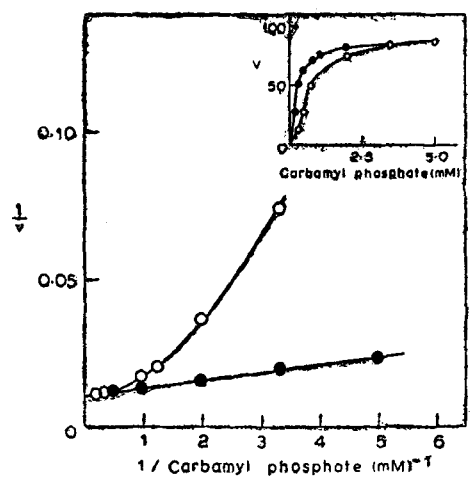

Figure 2. Effect of preincubation with substrates on the activity of the enzyme when the carbamyl phosphate concentration is varied.
The enzyme (100 μg) was preincubated with either aspartate (10 mM) or carbamyl phosphate (0·25 to 5 mM) prior to starting the reaction with the other substrates. The reciprocal velocity was plotted against the reciprocal of concentration of carbamyl phosphate. The inset shows the increase in velocity as a function of carbamyl phosphate concentration. O ——— O Enzyme preincubated with carbamyl phosphate. ● ——— ● Enzyme preincubated with aspartate.

The reciprocal plot of the velocity against carbamyl phosphate concentration is shown in figure 2. The inset shows the saturation curves. There was no time dependence for the activation by aspartate or for the effect of carbamyl phosphate.

Sigmoid saturation curve and hence a parabolic curve was obtained in the double reciprocal plot when the enzyme was preincubated with carbamyl phosphate. On the other hand, preincubation with aspartate resulted in a hyperbolic saturation curve and a linear reciprocal plot for the variation of carbamyl phosphate (figure 2). It is clear that preincubation of the enzyme with aspartate abolished the sigmoidicity of the carbamyl phosphate saturation curve. In all the kinetic analyses, described in this paper, the enzyme was preincubated with aspartate prior to the addition of carbamyl phosphate.

3.2. *Effect of aspartate on the reaction rate at fixed levels of carbamyl phosphate*

The initial velocity of the reaction was determined at 0·25, 0·5 and 5 mM concentrations of carbamyl phosphate and the concentration of aspartate was varied from 0·25 to 10 mM. The reciprocal plot of the saturation curves indicated that these were converging straight lines (figure 3). The slope and the intercept of the least square lines fitting the data were computed using the program (FIT) written by the authors in collaboration with M R Narasimha Murthy (Department of Organic Chemistry, Indian Institute of Science, Bangalore). The program outputs the parameters of a weighted least square line also, the weights were taken to be proportional to the fourth power of velocities so that it gave equal variances for all the measurements (Cleland 1963). From a replot of the slope *vs* the reciprocal of the concentra-

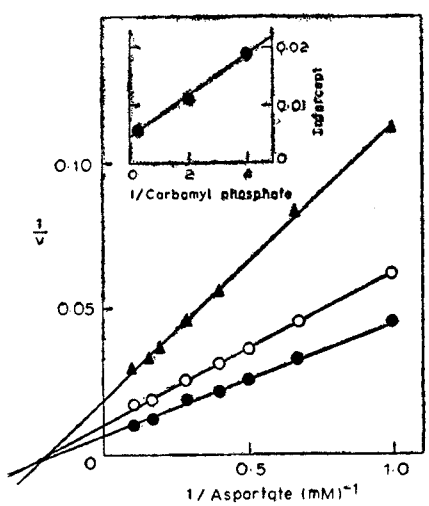

Figure 3. Reciprocal plot for aspartate transcarbamylase activity: aspartate varied at fixed levels of carbamyl phosphate. The enzyme (95 μg) was preincubated with concentrations of aspartate indicated in the figure and assayed at fixed levels of carbamyl phosphate (0·25, 0·5 and 5·0 mM). The reciprocal of velocity was plotted against reciprocal of concentration of aspartate. The inset shows the replot of the intercept at different fixed concentrations of carbamyl phosphate *vs* the reciprocal of carbamyl phosphate concentration. The V, K_B (aspartate) and K_A (carbamyl phosphate) are calculated from the figure to be 166 μmol/min/g, 5·0 mM and 0·6 mM, respectively. ▲——▲ 0·25 mM carbamyl phosphate; ○——○ 0·5 mM carbamyl phosphate; ●——● 5·0 mM carbamyl phosphate.

tion of carbamyl phosphate, the V, K_A for carbamyl phosphate and K_B for aspartate were evaluated to be 166 μmole/min/g, 0·6 mM and 5·0 mM, respectively.

These results are compatible with a sequential mechanism. In order to differentiate between ordered and random mechanisms, product inhibition studies were performed.

3.3. *Product inhibition studies*

(a) *Phosphate*

Effect of phosphate on carbamyl phosphate saturation: At a saturating concentration of aspartate, the concentration of carbamyl phosphate was varied at different fixed levels of phosphate. From the plot of reciprocal of velocity *vs* the reciprocal of carbamyl phosphate concentration (figure 4), the nature of inhibition was found to be competitive. The replot of slope *vs* the concentration of phosphate (inset) indicated that the inhibition was of the linear competitive type. Competitive nature of inhibition was observed both at saturating and at nonsaturating (not shown in figure) levels of aspartate.

Effect of phosphate on aspartate saturation: At a saturating concentration of carbamyl phosphate, phosphate had no effect on the aspartate saturation curve. On the other hand, variation of aspartate at fixed levels of phosphate (0, 50 and 100 mM) and at nonsaturating concentration of carbamyl phosphate gave lines with a change in both the slope and intercept on a Lineweaver-Burk plot indicating that the inhibition was noncompetitive (figure 5). The replot of slope and intercept *vs* concentration of phosphate was linear (inset). The nature of inhibition could be described as S (slope) and I (intercept) linear noncompetitive.

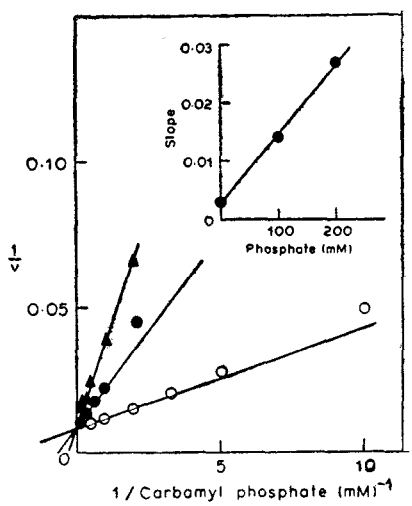

Figure 4. Reciprocal plot: Carbamyl phosphate varied, phosphate fixed. Different fixed concentrations of phosphate (0, 100 and 200 mM) were added to the three sets of reaction mixtures and assayed at varying concentrations of substrate (0·25–5 mM). The reciprocal of the velocity was plotted against the reciprocal of phosphate concentration. Inset shows the replot of the slope obtained from the reciprocal plot against phosphate concentration. O ——— O In the absence of phosphate; ● ——— ● 100 mM phosphate; ▲ ——— ▲ 200 mM phosphate.

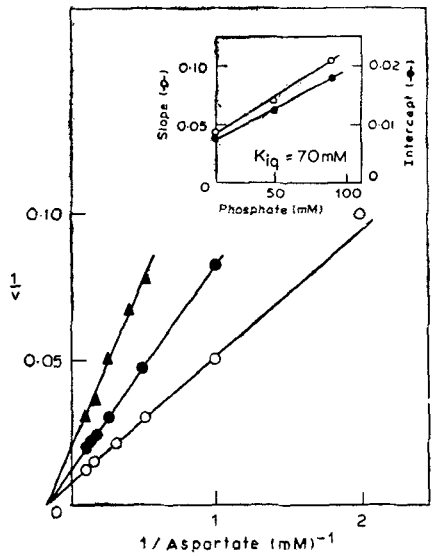

Figure 5. Reciprocal plot: Aspartate varied, phosphate fixed. The enzyme was preincubated with aspartate (0·25 to 10 mM) and different fixed concentrations of phosphate (0, 50 and 100 mM) were added to each set. The reaction was started by the addition of carbamyl phosphate (1 mM) and the reciprocal of the velocity was plotted against the reciprocal of aspartate concentration. The slope (o) and intercept (o) was replotted against the concentration of carbamyl phosphate. K_{iq} for phosphate was calculated to be 70 mM from the replot (inset).
O ——— O In the absence of added phosphate; ● ——— ● 50 mM phosphate; ▲ ——— ▲ 100 mM phosphate.

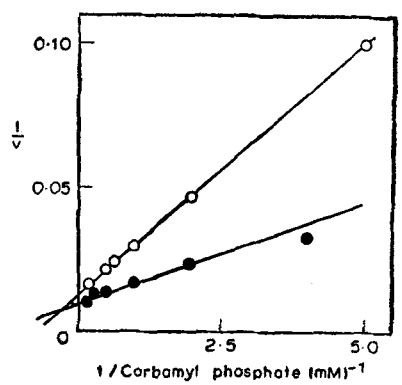

Figure 6. Reciprocal plot: Carbamyl phosphate varied, carbamyl aspartate fixed. The enzyme (100 μg) was preincubated with aspartate (10 mM). 100 mM carbamyl aspartate was added to one set of assay mixtures while a duplicate set without the added carbamyl aspartate served as the control. The reaction was started by the addition of carbamyl phosphate and the amount of carbamyl aspartate was determined using [U−^{14}C]-labelled aspartate. The reciprocal of the velocity was plotted against the reciprocal of carbamyl phosphate concentration.
● ——— ● In the absence of added carbamyl aspartate.
O ——— O 100 mM carbamyl aspartate.

(b) *Carbamyl aspartate*

Effect of carbamyl aspartate on carbamyl phosphate saturation: The activity of the enzyme at different concentrations of carbamyl phosphate in the presence of 100 mM carbamyl aspartate was measured using [U-^{14}C]-labelled aspartate as described under materials and methods. A duplicate set without carbamyl aspartate served as the control. The Lineweaver-Burk plot indicated that the nature of inhibition was noncompetitive with respect to carbamyl phosphate (figure 6). These experiments were repeated by varying the concentration of aspartate at 100 mM carbamyl aspartate. Similarly even with respect to aspartate, the inhibition by carbamyl aspartate was noncompetitive (not shown in the figure). The data from these inhibition studies suggest that the reaction proceeds by an ordered mechanism.

However, it is not possible to distinguish between an ordered mechanism and a rapid equilibrium random mechanism by product inhibition studies alone (Frieden 1976). The inhibition patterns for both these mechanisms would be similar, if in addition to rapid equilibrium, there is the formation of nonproductive *E.* second substrate complexes and *E.* first product complexes. These could be distinguished on the basis of dead end inhibition and isotope exchange studies.

3.4. *Dead end inhibition studies*

The inhibition pattern with the substrate analogues which may function as dead end inhibitors provide information about the mechanism of the reaction. Acetyl phosphate was chosen as the substrate analogue for carbamyl phosphate and succinate for aspartate in these studies. Acetyl phosphate and succinate were competitive inhibitors for carbamyl phosphate and aspartate, respectively (not shown in the figure).

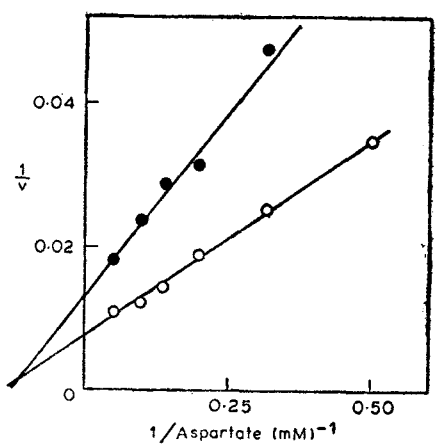

Figure 7. Reciprocal plot for the variation of aspartate at a fixed concentration of acetyl phosphate. The enzyme (100 μg) was preincubated with concentrations of aspartate indicated in the figure. To one set of assay tubes, 50 mM acetyl phosphate was added and the other set to which acetyl phosphate was not added served as the control. The reaction was started by the addition of carbamyl phosphate. The reciprocal of the velocity was plotted against the reciprocal of carbamyl phosphate concentration. O ——— O In the absence of acetyl phosphate. ● ——— ● 50 mM acetyl phosphate.

(a) *Effect of acetyl phosphate on aspartate saturation*

Acetyl phosphate was both an inhibitor and a pseudosubstrate for the *E. coli* enzyme (Porter *et al* 1969). For the mung bean enzyme acetyl phosphate was found to function only as an inhibitor and not as a pseudosubstrate as N-acetyl-L-aspartate was not formed (estimated by the method of Perchere and Capony 1968). Aspartate concentration was varied from 0·25–10 mM at 0 and 50 mM acetyl phosphate and at saturating levels of carbamyl phosphate. The lines on the reciprocal plot (figure 7) of velocity of the reaction *vs* the reciprocal of the concentration of aspartate, indicated the inhibition to be noncompetitive. The noncompetitive inhibition pattern could be explained both in terms of an ordered as well as a random mechanism.

(b) *Effect of succinate on carbamyl phosphate saturation*

Variation of carbamyl phosphate at different fixed levels of succinate (0, 2, 4 and 6 mM) resulted in uncompetitive inhibition (figure 8). This result could be explained only in terms of the ordered mechanism and not by a random mechanism.

3.5. *Experiments with transition state analogue N-(phosphonoacetyl)-L-aspartate*

Compounds structurally similar to the transition state can be expected to bind more tightly than either substrates or products. N-(Phosphonoacetyl)-L-aspartate is one such example. It combines in one molecule the structural features of aspartate and carbamyl phosphate. Collins and Stark (1971) have synthesised this compound and have used it to differentiate between the various kinetic mechanisms. We have, in a similar manner used N-(phosphonoacetyl)-L-aspartate in our system to elucidate the kinetic mechanism.

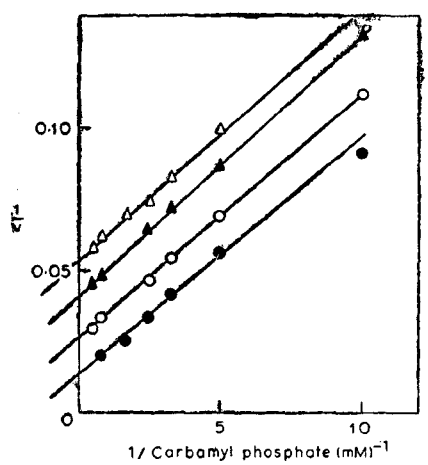

Figure 8. Reciprocal plot: Carbamyl phosphate varied, succinate fixed. The enzyme (100 μg) was preincubated, with aspartate (5 mM) and different fixed concentrations of succinate (0, 2, 4 and 6 mM), and the reaction was started by the addition of carbamyl phosphate at concentrations indicated in the figure. The reciprocal of the velocity was plotted against the reciprocal of carbamyl phosphate concentration at different fixed concentrations of succinate. ● ——— ● in the absence of succinate; O ——— O 2 mM succinate; ▲ ——— ▲ 4 mM succinate; △ ——— △ 6 mM succinate.

(a) *Effect of N-(phosphonoacetyl)-L-aspartate on carbamyl phosphate saturation*

The concentration of N-(phosphonoacetyl)-L-aspartate was varied from 0 to 100 μM at saturating concentrations of carbamyl phosphate and aspartate. From the

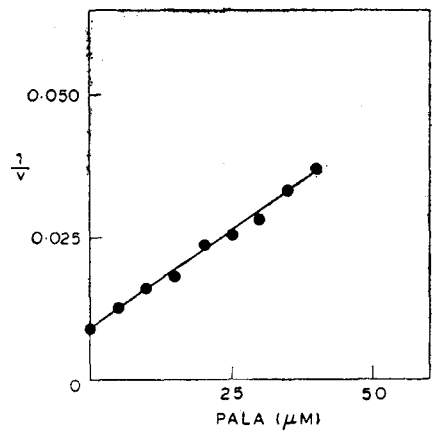

Figure 9. Dixon plot for the inhibition of enzyme activity by N-(phosphonoacetyl)-L-aspartate. The enzyme (100 μg) was preincubated with aspartate (10 mM). The reaction was started by the addition of carbamyl phosphate. The reciprocal of the velocity was plotted against the concentration of N-(phosphonoacetyl)-L-aspartate. The K_i value for N-(phosphonoacetyl)-L-aspartate was calculated to be 13 μM.

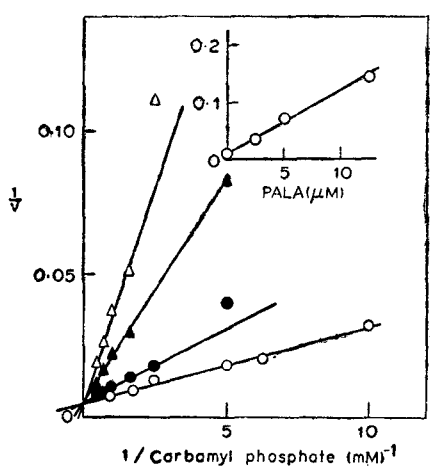

Figure 10. Reciprocal plot for the variation of carbamyl phosphate at different fixed levels of N-(phosphonoacetyl)-L-aspartate. The enzyme (100 μg) was preincubated with aspartate (10 mM). Different fixed concentrations of N-(phosphonoacetyl)-L-aspartate was added and the reaction was started by the addition of carbamyl phosphate at concentrations indicated in the figure. The reciprocal of carbamyl phosphate concentration at different fixed concentrations of N-(phosphonoacetyl)-L-aspartate. The inset shows the replot of slope *vs* the concentration of N-(phosphonoacetyl)-L-aspartate.

○ ——— ○ In the absence of N-(phosphonoacetyl)-L-aspartate;
● ——— ● 2·5 μM N-(phosphonoacetyl)-L-aspartate;
▲ ——— ▲ 5 μM N-(phosphonoacetyl)-L-aspartate;
△ ——— △ 10 μM N-(phosphonoacetyl)-aspartate.

Dixon plot (figure 9) the K_i value was calculated to be 13 μM. It is obvious that N-(phosphonoacetyl)-L-aspartate can bind much more tightly than either carbamyl phosphate or aspartate.

The concentration of N-(phosphonoacetyl)-L-aspartate was fixed at 0, 2·5, 5 and 10 μM and the concentration of carbamyl phosphate was varied from 0 to 10 mM and the reaction velocity was measured. The reciprocal plots for the saturation curves are shown in figure 10. The lines had the same intercept but different slopes. The inhibition was competitive.

(b) *Effect of N-(phosphonoacetyl)-L-aspartate on aspartate saturation*

At fixed levels of N-(phosphonoacetyl)-L-aspartate (0, 2·5, and 7·5 μM), the concentration of aspartate was varied at saturating concentration of carbamyl phosphate. A least square fit of the data on a reciprocal plot indicated that the inhibition was noncompetitive (figure 11). This result could be explained only in terms of the ordered mechanism.

4. Discussion

The results presented above are compatible with a compulsory ordered mechanism as given below.

Table 1 gives a comparison of the predicted patterns for the ordered mechanism (figure 12) along with the observed patterns. It can be seen that the observed patterns are in good agreement with the expected patterns.

A sigmoid saturation pattern was obtained with carbamyl phosphate but a hyperbolic pattern was obtained with aspartate. The abolition of sigmoidicity of car-

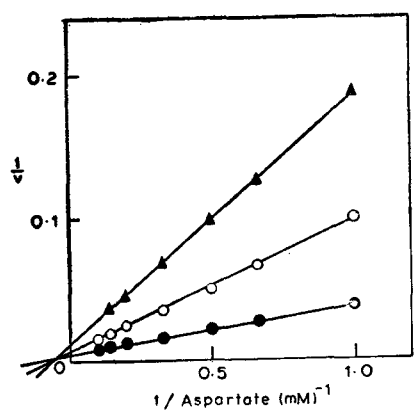

Figure 11. Reciprocal plot: Aspartate varied, N-(phosphonoacetyl)-L-aspartate fixed. The enzyme (95 μg) was preincubated with aspartate at concentrations indicated in the figure. Different fixed concentrations of N-(phosphonoacetyl)-L-aspartate was added to the three sets of reaction mixtures and the reaction was started by the addition of carbamyl phosphate (5 mM). The reciprocal of the velocity was plotted against the reciprocal of aspartate concentration. ● ——— ● In the absence of N-(phosphonoacetyl)-L-aspartate. O ——— O 2·5 μM N-(phosphonoacetyl)-L-aspartate. ▲ ——— ▲ 7·5 μM N-(phosphonoacetyl)-L-aspartate.

bamyl phosphate saturation curve on preincubation with aspartate (figure 2) indicated that aspartate was an activator in addition to being a substrate. This type of change in the substrate saturation pattern is a common feature of allosteric enzymes (Monod *et al* 1964). In addition to aspartate, phosphate, succinate and several other dianions brought about this change even at low concentrations (Savithri *et al* 1978). Chang and Jones (1974a, b and c) have shown that for the *S. faecalis* enzyme also enormous activation effects are observed on addition of dianions. However, in this case there is a large increase in V value on addition of dianions, whereas, in our system there is no change in V but there is a change in $S_{0.5}$ values (1·2 mM to 0·6 mM). The conversion of sigmoid saturation patterns to hyperbolic curves per-

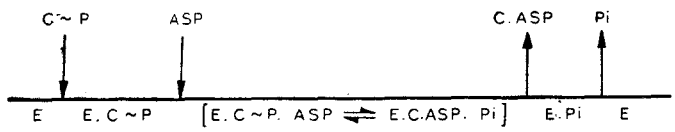

Figure 12. Kinetic mechanism for the reaction catalyzed by mung bean aspartate transcarbamylase; $C \sim P$=carbamyl phosphate; asp=aspartate; *C*.asp=carbamyl aspartate; P_i=phosphate.

Table 1. Comparison of the observed patterns of mung bean aspartate transcarbamylase (ATCase) with the predicted patterns for an ordered mechanism.

Property	Predicted pattern for an ordered mechanism	Observed pattern
1. Initial velocity: carbamyl phosphate varied. Aspartate (Asp) fixed. Lineweaver-Burk plot.	Lines coincidental	Parabolic, becomes coincidental on preincubation with aspartate.
Asp varied carbamyl phosphate fixed	Lines coincidental	Lines coincidental
2. Product inhibition: Carbamyl phosphate varied. P_i fixed.		
Asp saturated	Competitive	Competitive
Asp unsaturated	Competitive	Competitive
Asp varied, P_i fixed:		
Carbamyl phosphate unsaturated	Noncompetitive	Noncompetitive
Carbamyl phosphate saturated	No inhibition	No inhibition
Carbamyl aspartate fixed, carbamyl phosphate varied:		
Asp unsaturated	Noncompetitive	Noncompetitive
Asp saturated	Uncompetitive	Not determined
Asp varied:		
Carbamyl phosphate unsaturated	Noncompetitive	Noncompetitive
Carbamyl phosphate saturated	Noncompetitive	Noncompetitive
3. Dead end inhibition: Succinate fixed,		
Asp varied	Competitive	Competitive
Carbamyl phosphate varied	Uncompetitive	Uncompetitive
Acetyl phosphate fixed:		
Asp varied	Noncompetitive	Noncompetitive
carbamyl phosphate varied	Competitive	Competitive
4. Transition state analog: PALA fixed		
carbamyl phosphate varied	Competitive	Competitive
Asp varied	Noncompetitive	Noncompetitive

mits classical kinetic approaches to be used for the study of the reaction mechanism.

The coincidental lines (figure 3) obtained when the concentration of aspartate was varied at fixed levels of carbamyl phosphate indicated that a sequential mechanism was operative and ruled out any chemically meaningful ping pong mechanism.

The linear competitive nature of the inhibition obtained when the concentration of carbamyl phosphate was varied at different fixed levels of phosphate (figure 4) indicated that both carbamyl phosphate and phosphate might be binding to the same form of the enzyme i.e., the free enzyme. On the other hand, the inhibition pattern with respect to aspartate was noncompetitive (figure 5) indicating that aspartate was binding to a form different from the form to which phosphate was binding (*E* form). However, at saturating levels of carbamyl phosphate all the free enzyme would be present as *E.* carbamyl phosphate complex and hence phosphate would not be able to bind to the free enzyme. In this situation, reversible connections between the various enzyme forms are broken and therefore, no inhibition is expected. The absence of inhibition is in support of this argument. The linear replots of both slope and intercept and the absence of inhibition by both the substrates at high concentration ruled out dead end complexes of the type $E.(P_i)_2$, E(carbamyl phosphate)$_2$, E(aspartate P_i) or $E.(P_i$. carbamyl phosphate).

When the concentration of carbamyl phosphate was varied at different fixed levels of succinate, uncompetitive inhibition (figure 8) was obtained. This result could be explained only in terms of the ordered mechanism and not by a random mechanism. Succinate binds to the *E.* carbamyl phosphate form while phosphate binds to the *E.* form of the enzyme. These two forms are not reversibly connected. Therefore, the inhibitor in this case has only an *I* effect (Plowmann 1972). In other words the pattern expected is uncompetitive. If the mechanism was random then noncompetitive inhibition pattern would be obtained.

Since N-(phosphonoacetyl)-L-aspartate has structural features of both carbamyl phosphate and aspartate, it can bind only to the free enzyme and not to *E.* carbamyl phosphate or *E.* aspartate form. The competitive inhibition pattern observed with carbamyl phosphate showed that both of them must be binding to the same form of the enzyme i.e., the free enzyme. On the other hand, the inhibition was noncompetitive with respect to aspartate indicating that N-(phosphonoacetyl)-L-aspartate and aspartate are binding to two different forms of the enzyme. If the mechanism was random, competitive inhibition pattern would be expected even with respect to aspartate.

The *P. aureus* enzyme resembles the catalytic subunit of the *E. coli* enzyme in its molecular weight and steady state kinetics of the reaction. However, it differs from the catalytic unit in that it still possesses the ability to regulate the activity by the end products of the pathways. Ong and Jackson (1972) have made a less detailed analysis on a partially purified enzyme from *P. aureus* seedlings. Their product inhibition patterns agree well with our results. They have also proposed an ordered mechanism with carbamyl phosphate as the leading substrate and phosphate as the last product to leave the enzyme surface. However, this conclusion is not in agreement with their initial velocity pattern which point towards a ping pong mechanism.

It can be reasonably concluded from the above results that the enzyme follows an ordered mechanism with carbamyl phosphate as the first substrate to add and phosphate as the last product to leave the enzyme surface. This mechanism however, needs to be confirmed by isotope exchange and binding studies.

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