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Mechanism of the Reaction Catalyzed by the Catalytic Subunit of Aspartate Transcarbamylase. Kinetic Studies with Carbamyl Phosphate as Substrate†

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ABSTRACT: The catalytic subunit prepared by treatment of native aspartate transcarbamylase with *p*-mercuribenzoate has been used to investigate the mechanism of the reaction. The results of initial velocity, product inhibition, dead-end inhibition, isotope transfer, and binding studies are consistent with the reaction having a random mechanism for which the rate of interconversion of the central complexes appears to be slow compared with all other steps of the reaction sequence. Further, they indicate that the mechanism involves the formation of three dead-end complexes: enzyme–aspartate–P_i, enzyme–aspartate–carbamyl aspartate, and enzyme–carbamyl phosphate–carbamyl aspartate. In addition, initial velocity and product inhibition studies have been made using the catalytic subunit obtained by subjecting the native aspartate transcarbamylase to limited proteolytic digestion by trypsin. The resulting data are qualitatively similar to those obtained with the mercurial catalytic subunit and in accord with the reaction approximating to a rapid equilibrium, random mechanism with three dead-end complexes. This postulate contrasts with previous conclusions that the reaction has an ordered mechanism. Dissociation constants have been determined for the reaction of substrates with various enzyme forms. However, some of these are not precise because of the difficulties associated with obtaining accurate, steady-state kinetic data under conditions where carbamyl phosphate binds strongly to the enzyme.

Both the native form of aspartate transcarbamylase, isolated from *Escherichia coli*, and the catalytic subunit derived from it by treatment with *p*-mercuribenzoate catalyze the reaction: carbamyl phosphate + aspartate → carbamyl aspartate + P_i. From investigations of the binding of succinate, an inhibitory analog of aspartate, to the catalytic subunit, it was reported that the binding was dependent on the presence of carbamyl phosphate (Changeux *et al.*, 1968). Thus, it was inferred that the reaction involving aspartate would occur *via* an ordered mechanism with carbamyl phosphate as the first substrate to add to the enzyme. This conclusion was supported by the results of steady-state kinetic studies from which it was concluded that carbamyl aspartate was released from the enzyme before P_i (Porter *et al.*, 1969).

By contrast with the above findings, the results of Collins and Stark (1969) showed that succinate did combine with the catalytic subunit although its reaction was considerably enhanced in the presence of carbamyl phosphate. In addition, these authors showed that the catalytic subunit is capable of binding aspartate. Moreover, aspartate influences the rate of digestion of the native enzyme by proteolytic enzymes (McClintock and Markus, 1968, 1969). These results raise the possibility that substrates bind in a random manner to both the native enzyme and catalytic subunit.

At the time of publication of the paper by Porter *et al.* (1969), steady-state kinetic investigations were being carried out with the catalytic subunit which can be obtained by limited digestion of the native aspartate transcarbamylase with trypsin (Heyde *et al.*, 1973) and whose physicochemical properties are similar to those of the subunit obtained using *p*-mercuribenzoate (Gerhart and Holoubek, 1967). Since it was considered that the mechanism of the reaction catalyzed by the latter catalytic subunit had not been definitely established, the kinetic investigations were extended to include work with this enzyme form. From the results of these studies, it has been concluded that the reactions catalyzed by both catalytic subunits conform to a random mechanism which appears to be of the rapid equilibrium type and which involves the formation of three dead-end complexes. The conclusion is supported by the data obtained from isotope transfer and binding studies as well as by the results of kinetic investigations with acetyl phosphate as a substrate (Heyde and Morrison, 1973).

Experimental Section

Materials. L-Aspartic acid (A grade), [2,3-^{14}C]succinic acid (10.2 mCi/mmol), carbamyl aspartate (DL-ureidosuccinic acid, A grade), dilithium carbamyl phosphate (B grade), and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid[1] (TES, A grade) were obtained from Calbiochem. The L isomer of carbamyl aspartate was prepared by the method of Korn (1957) and converted to the sodium salt by passage of a solution through a column of Zeo-Karb 225 (Na^+ form). Carbamyl phosphate was purified by repeated precipitations from solu-

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[1] Abbreviation used is: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

tion using ethanol (Gerhart and Pardee, 1962) until the purity was >90% as determined enzymically using aspartate transcarbamylase. ATP was purchased from P-L Biochemicals and recrystallized by the method of Berger (1956). Bovine serum albumin was a Sigma product. Potassium phosphate and succinic acid (analytical reagent grade) were from British Drug Houses and monomethyl phosphate was from K & K Laboratories. Acetyl phosphate was prepared by the method of Porter *et al.* (1969), but as a gel formed on addition of lithium acetate in methanol, an equal volume of water was added before precipitation of the dilithium salt of acetyl phosphate with 2 vol of ethanol. The product appeared to be only 70% pure by enzymic assay with excess aspartate transcarbamylase, although analysis indicated that any impurity did not contain carbon or phosphorus. A pure preparation of dilithium acetyl phosphate was obtained by passing a solution of the impure material through a column of Zeo-Karb 225 (Li^+ form) and treating the effluent with 7 vol of ethanol.

[8-^{3}H]ATP (21.7 Ci/mmol) was purchased from Schwarz BioResearch, [U-^{14}C]aspartic acid (106 or 229 mCi/mmol) and potassium [^{14}C]cyanate (24 mCi/mmol) from the Radiochemical Centre, Amersham, and [^{32}P]P_i (1500 Ci/mmol) from Lucas Heights, Sydney, Australia. [2,3-^{3}H]Aspartic acid (24 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). [1,2,3,4-^{14}C]Carbamyl aspartate was prepared enzymically from [U-^{14}C]aspartic acid as described by Porter *et al.* (1969). [^{14}C]Carbamyl phosphate was prepared by a slight modification of the method of Davies *et al.* (1970) while carbamyl [^{32}P]phosphate was prepared by the same procedure except that the proportions of inorganic phosphate and cyanate were reversed.

Visking tubing (size 20/32) came from the Visking Co. (Chicago, Ill.) and was washed before use (Englund *et al.*, 1969). Diaflo membranes were supplied by Amicon and the strain of *E. coli* (K12) was a gift from Dr. J. C. Gerhart.

Methods. Preparation of enzymes. Native aspartate transcarbamylase was isolated from extracts of *E. coli* (K12) and converted to the catalytic subunit by treatment with *p*-mercuribenzoate as described by Gerhart and Holoubek (1967). This preparation of the catalytic subunit is a trimer (Weber, 1968) and has been called the mercurial catalytic subunit. A second preparation of catalytic subunit was obtained by separating on Sephadex G-100 the products of limited digestion of the native enzyme in the presence of aspartate (Heyde *et al.*, 1973). This preparation has a similar sedimentation coefficient to that of the mercurial catalytic subunit and will be referred to as the tryptic catalytic subunit. Both types of catalytic subunit were stable during assay at pH 8.0 in TES buffer using concentrations of 0.1 μg/ml and above. No change in specific activity was observed when the enzyme was assayed at concentrations between 0.1 and 1.0 μg/ml (*cf.* Porter *et al.*, 1969).

Measurement of enzyme activity. Enzymic activity was determined at 28° in the presence of 0.05 M TES buffer (pH 8.0). The amount of enzyme added corresponded to 0.1 or 0.2 μg/ml for the mercurial catalytic subunit and 0.3 μg/ml for the tryptic catalytic subunit, while the total volume of reaction mixtures was either 0.5 or 1.0 ml depending on the sensitivity required for the assays. To ensure that initial velocities were being measured, reactions were run for at least two time periods. The method of stopping the reaction varied with the procedure used to determine the initial velocity of the reaction. Whenever possible, this was done by measuring the rate of carbamyl aspartate formation. The latter compound was assayed colorimetrically (Gerhart and Pardee, 1962) and the volume of reagent added to stop the reaction was adjusted so that the final assay volume was 3.0 ml. Irrespective of the volume added, the amounts of color-producing reagents were the same. When [^{14}C]aspartate was used as the substrate (stock solution 106 mCi/mmol), the formation of carbamyl [^{14}C]aspartate was determined by the procedure of Porter *et al.* (1969) in which the aspartate remaining in a 100-μl sample of the reaction mixture was removed by passage through a small column of Dowex-50. When carbamyl aspartate was present, reaction velocities were determined by measuring the rate of P_i formation (Herries, 1967).

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

Binding studies. These were performed as described by Paulus (1970) using Visking membranes (Heyde, 1973) and the mercurial catalytic subunit at a concentration of 500 μg/ml.

Determination of isotope transfer rates. Reaction mixtures contained, in a total volume of 0.2 ml: 0.05 M TES buffer; 0.01 μCi of [^{14}C]aspartate, 0.002 μCi of [^{14}C]carbamyl phosphate, or 0.03 μCi of carbamyl [^{32}P]phosphate; aspartate and carbamyl phosphate at final concentrations of 10.0 and 1.0 mM, respectively; 0.02 μg of mercurial catalytic subunit; and carbamyl aspartate at the concentrations indicated in Table V. The reactions were initiated by the addition of carbamyl phosphate and stopped at 1-min intervals up to 5 min as described below. The aspartate–carbamyl aspartate transfer reaction was stopped and labeled carbamyl aspartate separated from the remaining labeled aspartate by applying 10-μl samples of the reaction mixture to columns of Dowex-50 (Porter *et al.*, 1969). The carbamyl phosphate–carbamyl aspartate transfer reaction was stopped by adding an equal volume of 10% perchloric acid to the reaction mixture. This treatment brought about the conversion of the remaining [^{14}C]carbamyl phosphate to $^{14}CO_2$ which was removed by passing CO_2 through the solution for 30 min (Bethel *et al.*, 1968). The carbamyl phosphate–P_i transfer reaction was stopped by adding to the reaction mixture 1.0 ml of precipitating reagent consisting of 0.2 N perchloric acid, 0.08 M ammonium molybdate, and 0.01 M triethylamine hydrochloride (ice cold), as described by Sugino and Miyoshi (1964). After the addition of 1.0 μmol of cold P_i, the mixture was allowed to stand in an ice bath for 2 min. The precipitate was then collected on a Millipore filter and washed with the above precipitating reagent which had previously been saturated with the triethylamine–phosphomolybdate complex (Sugino and Miyoshi, 1964). The latter complex was prepared by adding phosphate to the precipitating reagent. The filter was dried at room temperature. [^{14}C]Carbamyl aspartate was counted in a Packard Tri-Carb liquid scintillation counter while [^{32}P]P_i was counted in a Nuclear-Chicago gas flow counter.

Analysis of data. The initial velocity data were plotted graphically in double reciprocal form to check the linearity of the curves and to determine the patterns of the plots. Analyses were then made by the method of least squares using the appropriate computer programs of Cleland (1963a) in which the velocities are assumed to have equal variance. Binding data which gave linear double reciprocal plots were fitted to eq 1 of Cleland (1963a). Kinetic data conforming to a sequential initial velocity pattern, linear competitive inhibition, and linear noncompetitive inhibition were fitted, respectively, to eq 7, 8, and 10 of Cleland (1963a). Initial velocity data that gave parabolic double reciprocal plots were fitted to eq 2 of

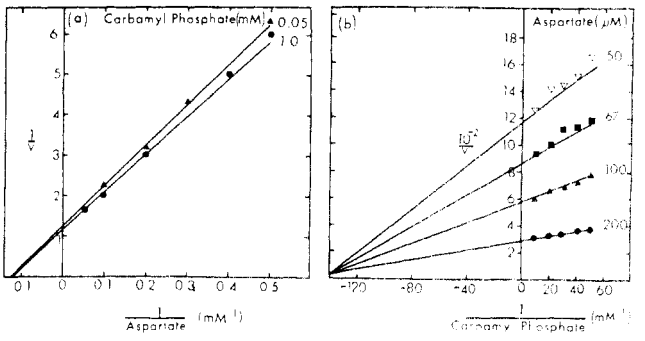


FIGURE 1: Effect of the concentration of carbamyl phosphate and aspartate on the initial velocity of the reaction: (a) effect of asparate concentration at two fixed concentrations of carbamyl phosphate, using the colorimetric assay; (b) effect of carbamyl phosphate concentration at four fixed concentrations of aspartate, using the radioactive assay. Velocities are expressed as micromoles of carbamyl aspartate formed per microgram of mercurial catalytic subunit per minute.

Cleland (1963a). The fitting of the complete data sets to these equations gave the best estimates of the values for the kinetic constants, together with their standard errors. The values so obtained were used to draw the families of lines in the figures and, where appropriate, to calculate true values for the kinetic constants. Weighted mean values and the standard errors of the means, as well as the standard errors of products and quotients, were calculated as described previously (Morrison and Uhr, 1966).

Results

Initial Velocity Studies. When the initial velocity of the reactions catalyzed by both the mercurial and tryptic catalytic subunits was followed by measuring the rate of carbamyl aspartate formation, there were severe limitations with respect to the range of fixed concentrations of carbamyl phosphate that could be used with aspartate as the variable substrate. This was due to the low sensitivity of the chemical assay for carbamyl aspartate relative to the Michaelis constant for carbamyl phosphate. The data obtained with the mercurial catalytic subunit (Figure 1a) show that a 20-fold increase in the concentration of carbamyl phosphate has only a small effect in decreasing the slope of the line and it was not feasible to reduce the concentration below 0.05 mM. The data of Figure 1a were fitted to eq 7 of Cleland (1963a), in which A and B represent carbamyl phosphate and aspartate, respectively, and reasonable values were obtained for the Michaelis constant for aspartate (K_b), as well as for the maximum velocity (V) of the reaction (Table I). However, the estimate for $K_{ia}K_b/K_a$, which is equivalent to K_{ib} for a rapid equilibrium random mechanism, was poor.

In an endeavor to obtain a value for the dissociation constant for the reaction of carbamyl phosphate with free enzyme (K_{ia}), assuming that such a reaction does occur, this substrate was varied over a range of lower concentrations while aspartate was held at fixed concentrations well below the value of its Michaelis constant. This procedure and the method of assay, which involved measurement of the rate of conversion of [^{14}C]aspartate to [^{14}C]carbamyl aspartate, were similar to those utilized by Porter *et al.* (1969). As expected, the fitting of the data of Figure 1b to eq 2 yielded a good value for K_{ia} (Table I). But the value determined for the Michaelis constant for carbamyl phosphate (K_a) was poor and this is a consequence of the use of fixed aspartate concentrations that are

TABLE I: Kinetic Constants of Aspartate Transcarbamylase Reaction as Determined from Initial Velocity Studies.[a]

Kinetic Constant	Mercurial Catalytic Subunit	Tryptic Catalytic Subunit
K_{ia} (μM)	7.2 ± 0.9	5.9 ± 1.1
K_a (μM)	<6	<3
K_b (mM)	8.1 ± 0.5	10.4 ± 0.5
V_m (μmol/min per μg of enzyme)	0.87 ± 0.02	0.20 ± 0.01

[a] The values for the kinetic parameters were obtained by fitting initial velocity data to eq 7 of Cleland (1963a). For the mercurial catalytic subunit, the data of Figure 1a were used to determine values for K_b and V_m while the value for K_{ia} was calculated from the data of Figure 1b. K_a and K_b represent Michaelis constants for carbamyl phosphate and aspartate, respectively. K_{ia} represents the dissociation constant for the reaction of carbamyl phosphate with free enzyme.

low in relation to the K_m value for this substrate. Further attempts were made to determine precisely the initial velocity pattern and to obtain accurate values for the kinetic constants by using more highly labeled aspartate and varying both substrates simultaneously in the region of their K_m values. However, [^{14}C]aspartate (229 mCi/mmol, 92% isotope abundance in all carbon atoms) was not of sufficiently high specific radioactivity and while the [2,3-^{3}H]aspartate (24 Ci/mmol) was potentially suitable, it was unexpectedly degraded during the separation of aspartate and carbamyl aspartate on a column of Dowex-50. Thus, it was impossible to determine the increase in the number of counts per minute due to the formation of relatively small amounts of [^{3}H]carbamyl aspartate. The use of the assay involving [^{14}C]carbamyl phosphate (Bethel *et al.*, 1968) was also precluded because the [^{14}C]-cyanate available for its synthesis was not of sufficiently high specific radioactivity.

The results illustrated in Figure 1b might be taken to indicate that the value for K_a is less than the apparent value of 6 μM which is obtained with aspartate at a fixed concentration of 0.2 mM. However, the data are not sufficiently precise to determine accurately the position of the crossover point in relation to the abscissa. If the lines were to intersect below the abscissa, the value for K_a would be considerably higher than the K_{ia} value and this would be more in keeping with the results of some other experiments (Heyde and Morrison, 1973).

The results obtained using the tryptic catalytic subunit were qualitatively similar to those reported above, but there were quantitative differences in the values for K_{ia}, K_b, and the maximum velocity (Table I). Although both sets of data were unsatisfactory with respect to obtaining precise values for all the kinetic constants, they nevertheless indicate that the reactions conform to a sequential mechanism.

Product Inhibition Studies. To obtain further information about the nature of the sequential reactions catalyzed by the catalytic subunits of aspartate transcarbamylase, product inhibition studies were undertaken. The patterns obtained with the mercurial catalytic subunit, as illustrated in Figure 2, indicate that P_i functions as a linear competitive inhibitor with respect to carbamyl phosphate and as a linear noncompetitive inhibitor in relation to aspartate, while carbamyl aspartate

TABLE II: Apparent Kinetic Constants Associated with Product Inhibition of the Reaction.[a]

Product Inhibitor	Varied Substrate	Apparent K_i (mM)		App K_m:K_i (Slope)
		Slope	Intercept	
Phosphate (Q)	Carbamyl phosphate (A)	3 ± 2		0.029 ± 0.004
		(0.1 ± 0.1)		(0.022 ± 0.001)
	Aspartate (B)	67 ± 20	8.5 ± 1.3	
		(45 ± 4)	(22 ± 1)	
Carbamyl aspartate (P)	Carbamyl phosphate (A)	0.4 ± 0.5	45 ± 3	0.066 ± 0.0004
		(2 ± 3)	(109 ± 21)	(0.0039 ± 0.0005)
	Aspartate (B)	25 ± 2	35 ± 5	
		(58 ± 10)	(164 ± 76)	

[a] The data were analyzed as described under Methods. The values recorded for apparent K_i are the weighted means of values from two experiments. All values represent constants for the mercurial catalytic subunit except for the numbers in parentheses which represent constants for the tryptic catalytic subunit.

acts as a linear noncompetitive inhibitor with respect to both carbamyl phosphate and aspartate. Similar inhibition patterns were obtained from studies with the tryptic catalytic subunit. The values determined for the apparent inhibition constants from analysis of the data of Figure 2 as well as the data obtained using the tryptic catalytic subunit are recorded in Table II. Because the slopes of the lines in the absence of carbamyl aspartate or P_i were close to zero, the values for K_i (slope) with carbamyl phosphate as the variable substrate could not be accurately determined. Nevertheless, they have been listed so as to give an indication of their magnitude. By contrast, it is possible to calculate with good precision the various ratios for apparent K_m:K_i (slope) and these values have been included in Table II.

Investigations were also made of the product inhibition by carbamyl aspartate when the concentrations of carbamyl phosphate and aspartate were varied in constant ratio. Since carbamyl phosphate was varied over a range of concentrations which was high compared with its K_m value, the data were fitted to eq 8 of Cleland (1963a). The results (Figure 3) show that the maximum velocity of the reaction is not reduced in the presence of carbamyl aspartate.

Dead-End Inhibition Studies. Succinate acted as an inhibitory analog of aspartate, giving rise to inhibitions that were linearly competitive with respect to aspartate (Figure 4a) and linearly noncompetitive in relation to carbamyl phosphate (Figure 4b). Monomethyl phosphate and ATP can be considered as inhibitory analogs of carbamyl phosphate since each compound causes linear competitive inhibition with respect to this substrate. Although acetyl phosphate acts as a poor alternative substrate for the enzyme (Heyde and Morrison, 1973), it can also be studied as an inhibitor under conditions where the formation of *N*-acetylaspartate would be small and not detected by the procedure used to assay carbamyl aspartate. The results obtained were similar to those for monomethyl phosphate and ATP. The apparent values for the kinetic constants obtained from analysis of the dead-end inhibition data are given in Table III which also includes ratios of apparent K_m:K_i (slope) as determined with carbamyl phosphate as the variable substrate.

Substrate Inhibition of Reaction by Carbamyl Phosphate and Aspartate. During the determination of the initial velocity patterns using the mercurial catalytic subunit, it was noted that higher concentrations of each substrate cause substrate inhibition of the reaction. More detailed investigations of these effects showed that aspartate causes noncompetitive substrate inhibition while it appears that the substrate inhibition by carbamyl phosphate is of the competitive type.

Binding of Reactants to Mercurial Catalytic Subunit. Investigations of the binding of substrates and products to the enzyme have been undertaken with the ultrafiltration method of Paulus (1970). But since Diaflo UM-10 membranes gave markedly variable results, they were replaced by Visking membranes (Heyde, 1973). None of the results was completely satisfactory although they indicated that the free enzyme interacts with aspartate and carbamyl aspartate. The dissociation constant for the enzyme–aspartate complex was determined to

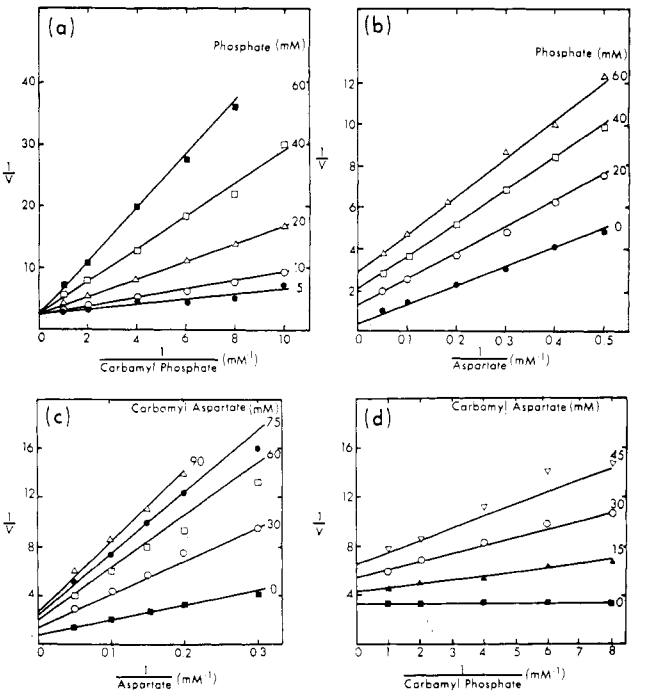


FIGURE 2: Product inhibition of the reaction by phosphate (a,b) and carbamyl aspartate (c,d): (a) carbamyl phosphate varied and the aspartate concentration fixed at 5.0 mM; (b) asparate varied and the carbamyl phosphate concentration fixed at 1.0 mM; velocities are expressed as micromoles of carbamyl aspartate formed per microgram of mercurial catalytic subunit per minute; (c) aspartate varied and the carbamyl phosphate concentration fixed at 1.0 mM; (d) carbamyl phosphate varied and the asparate concentration fixed at 5.0 mM. Velocities are expressed as micromoles of phosphate formed per microgram of mercurial catalytic subunit per minute.

TABLE III: Kinetic Constants for Inhibition of the Reaction by Substrate Analogs.

Inhibitor	Varied Substrate	Fixed Substrate (mM)	Apparent K_i (mM) Slope	Intercept	App K_m:K_i (slope)	True K_i (mM)
Succinate	Aspartate	1.0	1.4 ± 0.1 (2.9 ± 0.1)			0.98 ± 0.11[a]
	Carbamyl phosphate	0.2	0.26 ± 0.06		0.026 ± 0.003	0.26 ± 0.06 (K_i)
				1.02 ± 0.12		1.00 ± 0.15 (K_I)
Monomethyl phosphate	Aspartate	1.0	27 ± 2			0.19 ± 0.03 (K_i)
				67 ± 10		<0.4 (K_I)
	Carbamyl phosphate	5.0	0.63 ± 0.08		0.034 ± 0.001	<0.21[a]
ATP	Aspartate	0.5	2.3 ± 0.4 (7.0 ± 0.6)			0.04 ± 0.01 (K_i) (0.04 ± 0.01)
				3.2 ± 0.7 (20.5 ± 4.4)		<0.04 (K_I) (<0.06)
	Carbamyl phosphate	5.0	0.044 ± 0.021 (0.051 ± 0.016)		0.14 ± 0.01 (0.20 ± 0.01)	<0.04[a] (<0.06)
Acetyl phosphate	Aspartate	1.0	38 ± 4			0.28 ± 0.04 (K_i)
				67 ± 16		<0.18 (K_I)
	Carbamyl phosphate	5.0	0.41 ± 0.27		0.015 ± 0.001	<0.25[a]

[a] Value obtained by calculation, using the appropriate K_i and K_I values, for comparison with the directly determined value for the apparent K_i. The data were analyzed as described under Methods. The apparent K_i values are the weighted means of values from two experiments. The true values for the inhibition constants were calculated by assuming that the rate equation for the reaction in the presence of succinate is represented by the equation: $v = V[A][B]/(K_{ia}K_b + K_a[B] + K_b[A] + [A][B])$ with the $K_{ia}K_b$ and $K_b[A]$ terms multiplied by the factors $(1 + [I]/K_i)$ and $(1 + [I]/K_I)$, respectively, while that for the reaction in the presence of monomethyl phosphate, ATP, or acetyl phosphate is given by the above equation with the $K_{ia}K_b$ and $K_a[B]$ terms multiplied by the factors $(1 + [I]/K_i)$ and $(1 + [I]/K_I)$, respectively. The values of kinetic constants given in Table I were used in the calculations together with the values for the fixed concentrations of substrate. Values in parentheses are for the tryptic catalytic subunit, while all other values are for the mercurial catalytic subunit.

be 15 mM, but this is only an approximate value as the range within which it was practical to vary the aspartate concentration (0.66–4.0 mM) was unsuitable. A dissociation constant of 1.5 ± 0.2 mM for the enzyme–carbamyl aspartate complex was calculated on the basis that the variance associated with each determination was constant. However, the maximum number of moles of carbamyl aspartate bound per mole of enzyme (0.47 ± 0.04) was low (Heyde, 1973). Problems were encountered with attempts to determine the binding of succinate over the concentration range from 0.33 to 2.0 mM. These were due to the fact that the blank values obtained in the absence of enzyme were higher than the test values, even though the latter increased with increasing succinate concentrations.

Evidence for the binding of aspartate to the mercurial catalytic subunit also comes from the finding that aspartate, at a concentration of 10 mM, causes marked protection of the enzyme against heat inactivation at 45° (Table IV).

Studies of Isotope Transfer. The initial rates of the isotope transfer between carbamyl phosphate–P_i, carbamyl phosphate–carbamyl aspartate, and aspartate–carbamyl aspartate have been measured during the early stages of the reaction catalyzed by the mercurial catalytic subunit in the presence

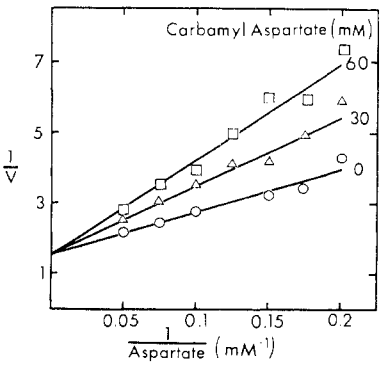


FIGURE 3: Product inhibition of the reaction by carbamyl asparate with the substrates varied in a constant ratio. The ratio of carbamyl phosphate to aspartate was 1:10. Velocities are expressed as micromoles of phosphate formed per microgram of mercurial catalytic subunit per minute.

TABLE IV: Effect of Aspartate in Protecting against Heat Inactivation of Aspartate Transcarbamylase.[a]

Time at 45° (min)	% of Original Act. Aspartate Present	Aspartate Absent
0	100	100
20	88	42
40	69	35
60	58	20

[a] Mixtures with and without 10 mM aspartate, containing 0.05 M TES buffer (pH 8.0) and 0.1 μg of mercurial catalytic subunit/ml, were incubated at 45°. Enzyme assays were then initiated at 28° by adding carbamyl phosphate, and aspartate when necessary, to give final concentrations of 1.0 and 10 mM, respectively.

TABLE V: Initial Velocity of Isotope Transfer Reactions in the Absence and Presence of Carbamyl Aspartate.[a]

Transfer Measured	Rate[b] at a [Carbamyl Aspartate] of (mM)		
	0	50	100
Aspartate–carbamyl aspartate (B–P)	0.24	0.17	0.13
Carbamyl phosphate–phosphate (A–Q)	0.24	0.19	0.16
Carbamyl phosphate–carbamyl aspartate (A–P)	0.25	0.22	0.19

[a] For the chemical reaction in the absence of carbamyl aspartate, 0.25 μmol of carbamyl aspartate was produced per min per μg of mercurial catalytic subunit, as measured by the colorimetric assay. [b] Micromoles of isotope incorporated into product per minute per microgram of mercurial catalytic subunit.

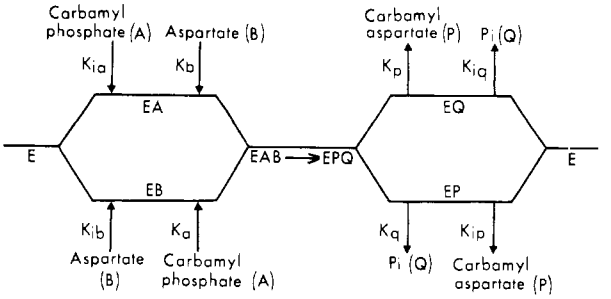
and absence of carbamyl aspartate (Table V). The results in the absence of carbamyl aspartate indicate that the initial velocity, as determined by measurement of each of the three transfer rates, is virtually the same as that obtained by chemical assay of the amount of carbamyl aspartate formed. This finding attests to the validity and accuracy of the procedures used to measure the transfer rates. The effect of adding carbamyl aspartate to reaction mixtures is to reduce the rate of each transfer, but it is apparent that there is little change in the ratio of any two of the three transfer rates.

Discussion

The kinetic investigations of the reaction catalyzed by the catalytic subunit of aspartate transcarbamylase have been hampered by the relatively low K_m value for carbamyl phosphate and the lack of a sensitive assay procedure for measuring initial velocities when this substrate is varied in the region of its K_m value. Consequently, it has not been possible to obtain a well-defined initial velocity pattern and accurate values for the kinetic constants associated with carbamyl phosphate. Nevertheless, the application of a wide range of kinetic techniques has led to results which can be most simply explained on the basis that the reaction conforms to a random mechanism rather than to an ordered mechanism as postulated by Porter *et al.* (1969). The reasons for reaching this conclusion are elaborated below.

The sequential initial velocity pattern (Figure 1) and the product inhibition patterns (Figure 2) are qualitatively in accord with the postulates that the reaction proceeds *via* either (a) an ordered mechanism in which carbamyl phosphate adds to the enzyme before aspartate and carbamyl aspartate dissociates before inorganic phosphate, or (b) a rapid equilibrium random mechanism involving the formation of three dead-end complexes, *viz.* enzyme–aspartate–P_i, enzyme–carbamyl phosphate–carbamyl aspartate, and enzyme–aspartate–carbamyl aspartate (Schemes I and II). The inhibitions of the reaction by monomethyl phosphate, acetyl phosphate, and ATP, that are competitive with respect to carbamyl phosphate and noncompetitive in relation to aspartate, do not permit a distinction to be made between the two mechanisms. But as succinate acts as a competitive inhibitor with respect to aspartate and as a noncompetitive inhibitor with respect to carbamyl phosphate (Figure 4), it follows that the substrates must add to the enzyme in a random manner. If the ordered mechanism were to apply, succinate would have acted as an uncompetitive inhibitor in relation to carbamyl phosphate. The aforementioned conclusion is also consistent with (a) the demonstrations that, in the absence of carbamyl phosphate, aspartate is bound to the enzyme (with a dissociation constant consistent with the kinetically determined dissociation constant), affects the absorption of a tyrosyl residue (Collins and Stark, 1969), protects against heat inactivation (Table IV), and influences the rate of proteolytic digestion (McClintock and Markus, 1968); (b) the competitive inhibition of the reaction by carbamyl aspartate when the substrates are varied in constant ratio (Figure 3); (c) the constancy of the transfer ratios as the concentration of carbamyl aspartate is increased (Table V) and the inability to observe back incorporation of [^{14}C]carbamyl aspartate into aspartate during the course of the reaction (it should, however, be noted that these results could also be a consequence of the essentially irreversible nature of the reaction); (d) the conclusion of Silverstein (1970) from isotope exchange studies at equilibrium that there is a random addition of substrates to the native enzyme.

SCHEME I



The possibility that the reaction occurs by means of a simple ordered mechanism, as proposed by Porter *et al.* (1969), can also be eliminated on quantitative grounds. The product inhibition data of Figure 2 have been analyzed using Cleland's (1963b) complete rate equation for an ordered mechanism, in which A, B, P, and Q represent carbamyl phosphate, aspartate, carbamyl aspartate, and P_i, respectively, and the appropriate relationships between true and apparent inhibition constants. The results (Table VI) indicate that the three values which can be calculated for K_pK_{iq}/K_q are not similar

SCHEME II

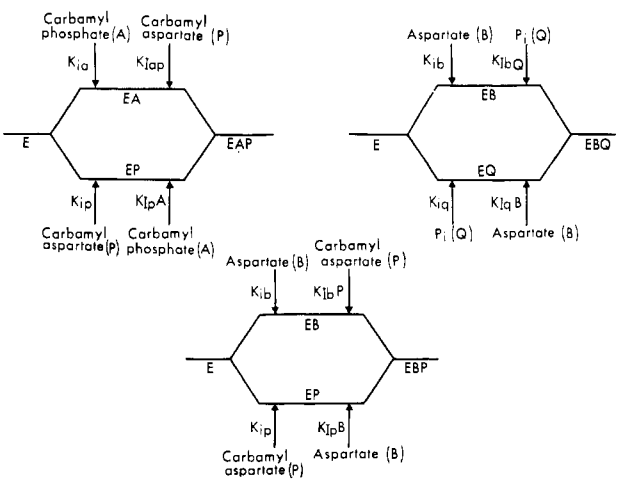


TABLE VI: Calculated Values of the Product Inhibition Constants for the Reaction.[a]

Product Inhibitor	Variable Substrate	Ordered Mechanism: Relationship between True and App Constants	Ordered Mechanism: Value (mM)	Random Mechanism: Relationship between True and Apparent Constants	Random Mechanism: Value (mM)
Q (phosphate)	A (carbamyl phosphate)	$K_{iq} = K_{is}$	3.2 ± 1.7 (0.12 ± 0.12)	$K_{iq} = K_{is}\left[\frac{1 + [(B]/K_{IqB})}{1 + ([B]/K_{ib})}\right]$	
	B (aspartate)	$K_{iq} = K_{is}\left[\frac{K_{ia}}{K_{ia} + [A]}\right]$	0.58 ± 0.20 (0.26 ± 0.05)	$K_{iq} = K_{is}\left[\frac{K_{ia}}{K_{ia} + [A]}\right]$	0.58 ± 0.20 (0.26 ± 0.05)
		$K_{iq} = K_{iI}\left[\frac{K_a}{K_a + [A]}\right]$	<0.05 (<0.07)	$K_{IbQ} = K_{iI}\left[\frac{K_a}{K_a + [A]}\right]$	<0.05 (<0.07)
P (carbamyl aspartate)	A (carbamyl phosphate)	$\frac{K_pK_{iq}}{K_q} = \left[\frac{K_{is}}{1 + (K_a[B]/K_{ia}K_b)}\right]$	<0.43 (<2.1)	$K_{ip} = K_{is}\left[\frac{1 + ([B]/K_{IpB})}{1 + ([B]/K_{ib})}\right]$	
		$\frac{K_pK_{iq}}{K_q} = \frac{K_{iI}K_b}{K_b + [B]} = \frac{K_{iI}[B]}{K_{ip}}$	55 ± 11 (102 ± 24)	$K_{IaP} = K_{iI}\left[\frac{K_b}{K_b + [B]}\right]$	28 ± 2 (91 ± 8)
	B (aspartate)	$K_pK_{iq}/K_q = K_{is}$	25 ± 2 (58 ± 10)	$K_{ip} = K_{is}\left[\frac{1 + ([A]/K_{IpA})}{1 + ([A]/K_{ia})}\right]$	
		$K_{ip} = K_{iI}\left[\frac{[A]}{K_a + [A]}\right]$	35 ± 5 (164 ± 76)	$K_{IbP} = K_{iI}\left[\frac{K_a}{K_a + [A]}\right]$	<0.21 (<0.49)

[a] The reaction was assumed to have either an ordered mechanism or a rapid equilibrium random mechanism with three dead-end complexes. Calculations were made by substituting into the given relationships between true and apparent constants the values for the apparent constants (Table II), the values for K_a, K_{ia}, and K_b (Table I), and the fixed substrate concentrations given in the legends to the appropriate figures. All values represent constants for the mercurial catalytic subunit, except for numbers in parentheses which represent constants for the tryptic catalytic subunit.

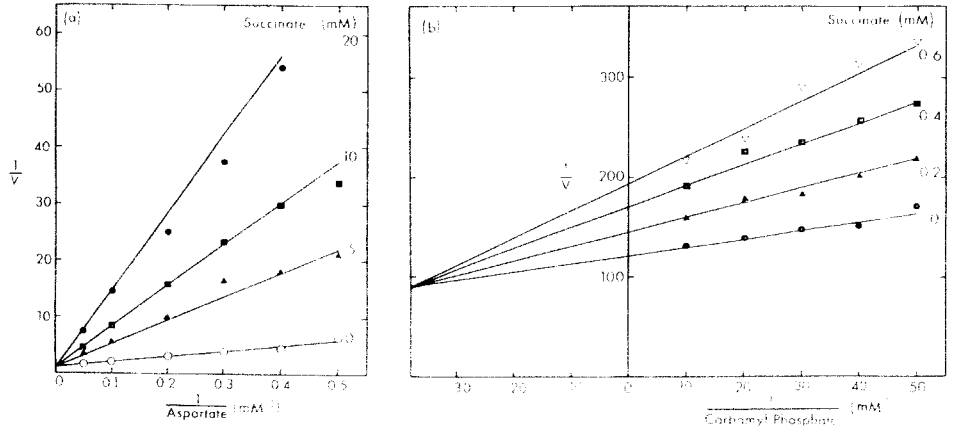


FIGURE 4: Dead-end inhibition of the reaction by succinate: (a) asparate varied and the carbamyl phosphate concentration fixed at 1.0 mM; (b) carbamyl phosphate varied and the asparate concentration fixed at 0.2 mM. Velocities are expressed as micromoles of carbamyl aspartate formed per microgram of mercurial catalytic subunit per minute.

TABLE VII: Summary of Values for Dissociation Constants[a] for Interaction of Reactants with Various Enzyme Forms, as Determined from Kinetic and Binding Studies on the Mercurial Catalytic Subunit.

Reactant	Kinetic Constant	Reaction	Value (mM)	Method
Carbamyl phosphate (A)	K_{ia}	E + A	0.007 ± 0.0009	Kinetic
	K_a	EB + A	<0.006	Kinetic
	K_{IpA}	EP + A	0.14 ± 0.03[b]	Kinetic + binding
Aspartate (B)	K_b	EA + B	8.1 ± 0.5	Kinetic
	$K_{ib} = K_{ia}K_b/K_a$	E + B	22 ± 6	Kinetic[e]
			~15	Binding
	K_{IqB}	EQ + B	3.5 ± 1.3[c]	Kinetic
	K_{IpB}	EP + B	2.7 ± 0.8[d]	Kinetic + binding
Carbamyl aspartate (P)	K_{ip}	E + P	1.5 ± 0.2	Binding
	K_{IaP}	EA + P	28 ± 2	Kinetic
	K_{IbP}	EB + P	<0.21	Kinetic
Phosphate (Q)	K_{iq}	E + Q	0.6 ± 0.2	Kinetic
	K_{IbQ}	EB + Q	<0.05	Kinetic
Succinate		E + succinate	0.26 ± 0.06	Kinetic
		EA + succinate	1.0 ± 0.2	Kinetic
ATP		E + ATP	0.039 ± 0.010	Kinetic
		EB + ATP	<0.038	Kinetic

[a] Dissociation constants for A, B, P, and Q were calculated on the basis of the relationships given in Table VI for the random rapid equilibrium mechanism involving three dead-end complexes. Those for succinate and ATP are from Table III. [b] From relationship in Table VI, using the value for K_{ip} as determined from binding experiments. [c] From the crossover point of Figure 2b. [d] From the crossover point of Figure 2c using K_{IpA} and the fixed concentration of A. [e] From the studies on the reaction with acetyl phosphate as substrate (Heyde and Morrison, 1973).

as required for a simple ordered mechanism.[2] Although there is considerable uncertainty associated with the values derived from the variation of the slopes of the lines for the noncompetitive inhibition by carbamyl aspartate with respect to carbamyl phosphate, it is nevertheless clear that they are one to two orders of magnitude less than those calculated from the intercept variations. Similar discrepancies are found with regard to the values of K_{iq}. Consequently, the reaction cannot conform to a simple ordered mechanism. The discrepancies in the values for K_pK_{iq}/K_q cannot be explained on the basis of carbamyl aspartate competing with carbamyl phosphate as well as acting as a product inhibitor because the slopes of the lines of Figure 2c and 2d do not vary as a parabolic function of the carbamyl aspartate concentration. It might be argued that the linear slope variations are due to the fact that carbamyl aspartate was varied over a range of concentrations which was low compared with the value of K_pK_{iq}/K_q. When the K_pK_{iq}/K_q term is considered to be negligible and allowance is made for the dead-end combination of carbamyl aspartate with free enzyme (K_{Ip}), the reciprocal form of the rate equation for an ordered bi–bi mechanism with carbamyl phosphate (A) as the variable substrate would be modified as shown in eq 1. From the variation of the vertical intercepts as a function of the carbamyl aspartate concentration (Figure 3), a value of 17.2 ± 1.3 mM was calculated for K_{ip} which represents the product inhibition constant for carbamyl aspartate. If this value were correct, then in the presence of 30 and 60 mM carbamyl aspartate a three- to fivefold variation of the intercepts of Figure 3 should have been observed.

$$\frac{1}{v} = \frac{K_a}{V}\left[\left(\frac{K_{ia}K_b}{K_a[B]} + 1\right)\left(1 + \frac{[P]}{K_{Ip}}\right)\right]\frac{1}{[A]} + \frac{1}{V}\left[\left(\frac{K_b}{[B]} + 1\right)\left(1 + \frac{[P]}{K_{ip}\left(1 + \frac{K_b}{[B]}\right)}\right)\right] \quad (1)$$

The above analysis demonstrates that, if the mechanism were ordered, the product inhibition constants, K_pK_{iq}/K_q and K_{ip}, must be sufficiently large in relation to the concentrations of carbamyl aspartate used so that no regular product inhibition occurs. Consideration has been given to the possibility that the reaction conforms to an ordered mechanism with carbamyl aspartate combining as a dead-end inhibitor with both free enzyme and the enzyme–carbamyl phosphate complex. Such a postulate is qualitatively in accord with the results of Figures 2c, 2d, and 3 and analysis of the data of Figures 2c and 2d has shown that there are no quantitative discrepancies. But as mentioned previously the dead-end inhibition patterns given by succinate (Figure 4) eliminate any mechanism involving the ordered addition of substrates. It should be noted that Porter *et al.* (1969) reached the conclusion that the mechanism was ordered on the basis of data obtained at relatively high (and even inhibitory) fixed concentrations of carbamyl phosphate and low fixed concentrations of aspartate. Under such conditions it would be difficult to detect by steady-state kinetic techniques the alternative pathway associated with a random mechanism which involves the formation of an enzyme–aspartate complex.

Collins and Stark (1971) have studied the inhibition of the reaction at pH 7.0 by *N*-phosphonoacetyl-L-aspartate which was considered to be an analog of the ternary transition state complex that is formed in the reaction. They reported that the inhibition by this compound is competitive with respect to carbamyl phosphate and simple noncompetitive in relation to

[2] In a personal communication, R. W. Porter has indicated that similar discrepancies are found on analysis of the data of Porter *et al.* (1969).

aspartate. From such results it follows that, under the chosen experimental conditions and irrespective of whether the mechanism is ordered or random, the inhibitor does not combine with an enzyme–carbamyl phosphate complex. If the latter combination were also to occur, the inhibition would be noncompetitive with respect to both substrates. Collins and Stark (1971) have endeavored to use their inhibition data in a quantitative manner to distinguish between the two possible reaction mechanisms. In an attempt to exclude the random mechanism, they assumed that the inhibitor does not react with an enzyme–aspartate complex. But if the dissociation constants for the binding of *N*-phosphonoacetyl-L-aspartate to free enzyme and the enzyme–aspartate complex were of comparable magnitude, then the results illustrated in Figure 4 of their paper would be compatible with a random mechanism. Binding studies by difference spectroscopy have demonstrated the similarity between the binding of *N*-phosphonoacetyl-L-aspartate to the enzyme–aspartate complex and to free enzyme, as well as the converse similarity between the binding of aspartate to the enzyme–*N*-phosphonoacetyl-L-aspartate complex and to free enzyme (E. Heyde, G. R. Jacobson, and G. R. Stark, unpublished observations). In connection with the aforementioned results it is interesting to note that carbamyl aspartate appears to combine more strongly with an enzyme–aspartate complex than with free enzyme (Table VII).

On the assumption that the reaction catalyzed by the mercurial catalytic subunit of aspartate transcarbamylase conforms to a rapid equilibrium random mechanism (Scheme I) with three dead-end complexes (Scheme II), eq 2 would

$$v = \frac{V_f[A][B] - \dfrac{V_r K_{ia} K_b [P][Q]}{K_{ip} K_q}}{\left\{ K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \dfrac{K_{ia}K_b[P]}{K_{ip}} + \dfrac{K_{ia}K_b[Q]}{K_{iq}} + \dfrac{K_{ia}K_b[P][Q]}{K_{ip}K_q} + \dfrac{K_b[A][P]}{K_{IaP}} + \dfrac{K_a[B][Q]}{K_{IbQ}} + \dfrac{K_{ia}K_b[B][P]}{K_{IpB}K_{ip}} \right\}} \quad (2)$$

apply. Using the relationships derived from this equation (Table VI) and the thermodynamically determined value for the reaction of carbamyl aspartate with free enzyme (K_{ip}, Table VII), values have been calculated for the dissociation constants of various enzyme–reactant complexes (Table VII). Not all of the values could be determined precisely, but within this limitation it would appear that some conclusions can be reached about the interactions of substrates and products with different enzyme forms. The data indicate that the binding of carbamyl phosphate is enhanced by the presence of aspartate and hindered by the presence of carbamyl aspartate on the enzyme. From the former result, it should follow that aspartate is bound better to the enzyme–carbamyl phosphate complex than to free enzyme. The most reliable estimate of K_{ib} [22 ± 6 mM, from initial velocity studies on the acetyl phosphate reaction (Heyde and Morrison, 1973) on the assumption that this reaction also is rapid equilibrium random] is higher than K_b and hence fulfills the prediction. The binding of aspartate to the enzyme–carbamyl aspartate and enzyme–phosphate complexes is also stronger than to free enzyme. Conversely, the presence of aspartate on the enzyme enhances the combination of carbamyl aspartate and phosphate. Further, carbamyl aspartate reacts less well with the enzyme–carbamyl phosphate complex than with free enzyme.

Finally, it is of interest to note that, qualitatively, similar properties are exhibited by the catalytic subunits prepared by either treatment with *p*-mercuribenzoate or partial proteolytic digestion of the native enzyme. However, it appears that there are quantitative differences with respect to the ability of these two enzyme forms to combine with reactants.

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