Biochemistry

Volume 13, Number 4 February 12, 1974

Aspartate Transcarbamylase from *Streptococcus faecalis*. Purification, Properties, and Nature of an Allosteric Activator Site†

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ABSTRACT: Aspartate transcarbamylase has been purified to near homogeneity from *Streptococcus faecalis* extracts. The purity of the final enzyme preparations, as judged from analytical polyacrylamide gel electrophoresis, is between 90 and 95%. The molecular weight of the native enzyme is 128,000 ± 6000 as determined by sedimentation equilibrium and sucrose gradient centrifugation. In the presence of sodium dodecyl sulfate, the denatured enzyme gives a single band in polyacrylamide gel electrophoresis, the molecular weight of which is 32,500 ± 500. Consequently, this aspartate transcarbamylase appears to possess four subunits of identical or nearly identical size. The kinetic properties of this enzyme have been investigated. At low buffer concentration (5 mM Tris-HCl (pH 8.5)), enormous anion activation effects (20- to 50-fold activation with 10 mM anion concentrations) and nonlinear kinetics (carbamyl phosphate and aspartate saturation curves deviate from normal Michaelis–Menten type curves) are observed. At high salt concentrations, $1/V$ *vs.* 1/[carbamyl phosphate] and $1/V$ *vs.* 1/[aspartate] plots can be brought back to linear curves, and the apparent K_m of carbamyl phosphate is reduced dramatically from 13 to 0.2 mM. The data are consistent with the existence of an allosteric activator site, different from the substrate sites, which is sensitive to all anions that have been tested, including substrates and products of the reaction. Other possibilities to explain the data are also discussed.

Aspartate transcarbamylase derived from various bacterial species can be grouped into at least three distinct classes on the basis of both the kinetic and gel filtration characteristics of the particular enzyme (Neumann and Jones, 1964; Bethell and Jones, 1969). Of these three classes, the aspartate transcarbamylases which have the smallest molecular weights (approximately 100×10^3 to 140×10^3; class C) are unlike the other two enzyme types (classes A and B), for they are not affected specifically by pyrimidine nucleotides. Aspartate transcarbamylase from *Streptococcus faecalis* was selected as a representative of the class C enzyme in order to learn more about the nature of the differences between enzymes of these three groups. Aspartate transcarbamylase from *S. faecalis* was partially purified (about 300-fold) and some of its general properties were investigated (Prescott and Jones, 1970; Prescott, 1969a,b); from those studies it became apparent that this enzyme has very interesting and unique kinetic properties, particularly in its response to anions.

In this paper, we report further purification of this enzyme to a purity of 90–95%, and the study of its physical and molecular properties. This more detailed kinetic study with the pure enzyme firmly establishes the existence and importance of an allosteric activator site present in this enzyme.

Experimental Section

Chemicals and Reagents. Streptococcus faecalis (ATCC 8043) was grown by the procedure of Jones (1962), scaled up for use with a 500-l. fermenter. About 3 kg of bacterial paste was obtained in this way. The paste was stored frozen at −20°.

Enzymes and proteins used as standards were purchased from Sigma Chemical Co. Sodium dodecyl sulfate and ammonium sulfate (pure grades) were purchased from Mann Research Laboratories and used without recrystallization. [^{14}C]Carbamyl phosphate (dilithium salt) was obtained from New England Nuclear with a specific activity of 1.97 mCi/mol. It was recrystallized from cold ethanol according to the procedure of Adair and Jones (1972). Nonradioactive carbamyl phosphate came from Sigma Chemical Co. DEAE-cellulose

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was obtained from Bio-Rad Laboratories (exchange capacity of 0.7 mequiv/g, control No. 4471). It was washed with base and acid before use and was regenerated (Peterson and Sober, 1962) and used again. DEAE-Sephadex A-50 (exchange capacity 3.5 mequiv/g) was from Pharmacia. Hydroxylapatite was synthesized according to the method described by Prescott and Jones (1970); preparations of reproducible quality have been made in this way several times. All other chemicals and reagents were commercial preparations of analytical grade. All salt solutions were and must be neutralized to pH 8.5 before use.

Aspartate Transcarbamylase Assays. Aspartate transcarbamylase activity was determined in one of two ways. A colorimetric measurement of carbamyl aspartate production developed by Prescott and Jones (1969) was used most frequently in enzyme purification analysis and specific activity determination. This assay in our hands is reproducible and linear from 0.01 to 0.2 μmol of carbamyl aspartate with optical density (OD) values ranging from 0.04 to 0.65 at 466 nm.

The unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of 1 μmol of carbamyl aspartate or phosphate per min under standard assay conditions. The standard assay mixture contained 0.1 M Tris-HCl (pH 8.5), 10 mM carbamyl aspartate, 50 mM L-aspartate, and enzyme to yield a final volume of 1 ml or 0.5 ml. The reaction was started by addition of either carbamyl phosphate or enzyme after preincubation of the reaction mixture for 1 min at 25°. The incubation time for the assay was usually 10 min. The reaction was terminated by the addition of 1 N perchloric acid and part of the total reaction mixture was taken for the colorimetric carbamyl aspartate assay. Sometimes the reaction was terminated directly by addition of the antipyrine–acetyl monoxime solution (Prescott and Jones, 1969) used for the colorimetric carbamyl aspartate assay.

When more rigorous kinetic analysis was performed, the radioactive [^{14}C]carbamyl phosphate assay of Bethell *et al.* (1968), as modified by Adair and Jones, (1972), was used. The counting efficiency with a Beckman Model LS-100L scintillation counter was usually 78%. Non-enzyme controls are essential for both enzyme assays.

The protein concentration was determined using the method of Oyama and Eagle (1956). Crystalline bovine serum albumin (Calbiochem A grade) was used as standard. Catalase activity was assayed by the method of Beers and Sizer (1952). Lactate dehydrogenase was assayed by the method described in the Boehringer Mannheim catalog.

Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed at pH 9.5, 2 mA/tube, by the method described by Davis (1964) and at pH 7.6 by the method of Williams and Reisfeld (1964); Bromophenol Blue was used as a marker. After electrophoresis, the gels were fixed in 12.5% trichloroacetic acid and stained for protein with Coomassie Blue (Chrambach *et al.*, 1967). After staining and destaining, the protein patterns in the gels were scanned at 610 nm using a Gilford spectrophotometer equipped with an automatic gel scanner. An optical density of 3.00 was chosen for maximum absorption; the scanning rate was 1 cm/min. Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out by the method of Weber and Osborn (1969) with 5% acrylamide gel. Reductive denaturation with sodium dodecyl sulfate and renaturation were according to the method of Weber and Kuter (1971).

Sucrose Density Gradient Centrifugation. The method used was that of Martin and Ames (1961). The sample to be studied, in a volume of 0.25 ml (aspartate transcarbamylase concentration 0.1 mg/ml, 7 units), was layered on top of a linear 5–20% sucrose gradient. The sucrose solutions were made in 10 mM potassium phosphate buffer (pH 6.6). The 11-ml linear gradient was generated by an ISCO gradient former, Model 570. The centrifugation was performed in a Beckman SW 41 rotor with a Beckman-Spinco Model L-2 65 ultracentrifuge for 20 hr, at 4° and a speed of 39,000 rpm. The gradient was fractionated into 63 fractions of about 0.18 ml each with an ISCO density gradient fractionator, Model 183. Hemoglobin, lactate dehydrogenase, and catalase were used as standards.

Sedimentation Equilibrium Centrifugation. The equilibrium ultracentrifugation experiment was performed according to the method of Van Holde and Baldwin (1958), in a Beckman Model E ultracentrifuge equipped with Schlieren optics. The enzyme was dialyzed in 5 mM sodium phosphate buffer (pH 6.6) at 4°. The dialyzed enzyme had an OD of 1.5 at 280 nm as measured with a Zeiss spectrophotometer. The experiment was performed using a 12-mm thick, 4° single sector plastic cell containing 50 μl of sample and 0.4 ml of fluorocarbon. Centrifugation was carried out at 21° and 4059 rpm for 6.75 hr; then the speed was reduced to 2089 rpm for 16.5 hr and brought back to 4059 rpm for 4 additional hr. After reaching equilibrium, pictures were taken at three different phase angles (40, 50, and 60°). A synthetic boundary run using the same sample and solvent was performed to obtain the initial concentration. The precision of this method is believed to be within 3% (Yphantis, 1960).

Kinetic Experiments. The detailed conditions are given in figure legends. The velocity, V, is always expressed as micromoles of products formed per minute per milligram of protein unless it is specifically stated otherwise.

Results

Purification Procedure

Step I: Disruption of Cells and Extraction of Enzyme. Cells were disrupted with a Braun Model MSK Mechanical Cell homogenizer (Brownwill Scientific) using 0.17–0.18-mm glass beads, according to the method of Bleiweis *et al.* (1964). Frozen *S. faecalis* cells (0.52 kg) were thawed, suspended in 1.56 l. of ice-cold, 20 mM potassium phosphate buffer at pH 6.6, and held at 4°. Tri-*n*-butyl phosphate (14 ml) was added as antifoaming agent. For each single operation, 30 ml of diluted cell paste and 30 g of glass beads were mixed in a 75-ml glass flask. The stoppered flask was shaken for 3 min at 4000 oscillations/min in a stream of liquid CO_2 delivered at a rate sufficient to prevent heating of the chamber. After the disruption step was complete, the entire homogenate was centrifuged with a Sorvall GSA rotor at 10,000 rpm at 4° for 30 min; the supernatant was collected and dialyzed against three changes of 16 l. of 20 mM potassium phosphate buffer (pH 6.6) at 4°. The dialyzed homogenate had a total volume of 1950 ml.

Step II: Streptomycin Sulfate Precipitation. This step was carried out essentially as described by Prescott and Jones (1970) except that less streptomycin was added. Streptomycin sulfate solution (324 ml of a 5% solution in H_2O) was added dropwise to 1950 ml of homogenate which had a protein concentration of 25.8 mg/ml. After the addition of streptomycin, the entire solution was kept overnight at 4° without disturbance to allow for the complete precipitation of nucleic acids.

Step III: pH 4.8 Precipitation. The procedure of Prescott and Jones (1970) was used here without modification.

Step IV: Hydroxylapatite Chromatography. A column of 16 cm diameter made in a Buchner funnel with fritted disk (porosity C, 3000-ml capacity) was packed with hydroxylapatite gel and equilibrated with 1 mM potassium phosphate buffer (pH 6.6) at 4°. The column height can vary from 5 to 9.5 cm without affecting the resolution of the column. Flow rate of the column was kept at about 200 ml/hr. The protein sample obtained from step III was dialyzed against 1 mM potassium phosphate buffer (pH 6.6) at 4° before applying it to the column. The amount of protein applied was 4–5 mg of protein/ml of bed volume. The column was first eluted with about two column volumes of 40 mM potassium phosphate buffer (pH 6.6). During this stage, large amounts of proteins emerged without aspartate transcarbamylase activity. The column was then eluted with 50 mM potassium phosphate buffer (pH 6.6); aspartate transcarbamylase activity was eluted with this buffer. A typical hydroxylapatite column chromatogram is shown in Figure 1. Usually, 3–4 column volumes of 50 mM potassium phosphate buffer were sufficient to elute the enzyme, but sometimes a severe "tailing effect" was seen and as many as 6 column volumes of 50 mM phosphate buffer were required in order to elute all of the aspartate transcarbamylase activity from the column. The fractions with aspartate transcarbamylase activity were pooled and concentrated using an Amicon ultrafiltration cell fitted with a PM-10 membrane to yield a solution containing 6–8 mg of protein/ml. The concentrated protein solution had an appreciable amount of insoluble material which was centrifuged down and discarded since it did not contain any aspartate transcarbamylase activity. The clear supernatant solution was dialyzed against three changes of 50 mM potassium phosphate buffer (pH 6.6) at 4°, and was stored at 4°. The hydroxylapatite column could be regenerated by washing with 4–5 column volumes of 0.4 M potassium phosphate buffer (pH 6.6) and used again several times.

Step V: Ammonium Sulfate Fractionation. The 43 ml of enzyme solution (6 mg of protein/ml) from step IV was brought to 43% saturation, as described by Prescott and Jones (1970). The mixture was kept at 4° for 1 hr, after which it was centrifuged at 1700 rpm with a Sorvall SS-34 rotor for 10 min; the precipitate was discarded. The supernatant was then brought to 54% saturation by adding 18.0 ml of saturated $(NH_4)_2SO_4$ solution at a rate of 2 ml/5 min. This material was then kept at 4° undisturbed for 1 day, after which it was centrifuged again; the precipitate was collected and dissolved in 4 ml of cold 50 mM potassium phosphate buffer (pH 6.6). This solution, which contained most of the aspartate transcarbamylase activity, was dialyzed against two changes of 1 l. of 10 mM potassium phosphate buffer (pH 6.6) containing 0.28 M KCl and 2 mM mercaptoethanol.

Step VI: DEAE-Sephadex Column Chromatography. A 1.5 × 15 cm DEAE-Sephadex A-50 column was packed at 4° and was equilibrated with 10 mM potassium phosphate buffer (pH 6.6) containing 0.28 M KCl and 2 mM mercaptoethanol; the pressure drop over the bed was kept near 17–18 cm throughout the eluting process, and the flow rate was about 26 ml/hr. A 5-ml sample containing 13 mg of protein/ml (from step V) was carefully layered on top of the column, after which a 300-ml linear KCl elution gradient, from 0.28 to 0.47 M, was used. A typical DEAE-Sephadex column chromatogram is shown in Figure 2. Under the conditions described above, two peaks with aspartate transcarbamylase activity are invariably obtained regardless of the amount of protein sample applied on the column (10–67 mg). This phenomenon can also be demonstrated with a DEAE-cellulose

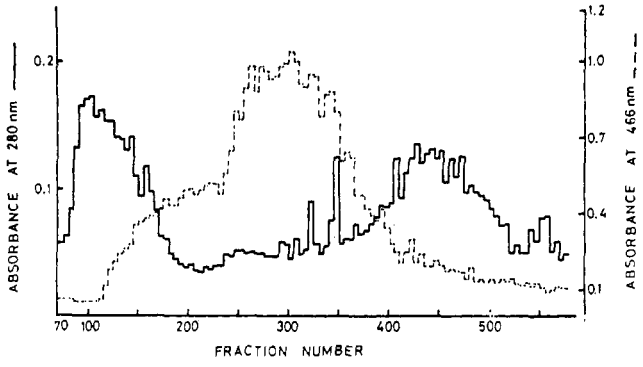

FIGURE 1: Hydroxylapatite column chromatography at 4°. The column size was 16 × 5 cm. Protein solution (100 ml) from step III (Table I), total activity 2770 units, was carefully layered on the column which had been equilibrated as described under Results. Fractions of 20 ml were collected. Potassium phosphate buffer (40 mM; 2 l.; pH 6.6) was used as the first eluent; then 50 mM potassium phosphate buffer (pH 6.6) was used. Aspartate transcarbamylase activity (absorbance at 466 nm) emerged right after the eluting buffer was changed. The flow rate was approximately 400 ml/hr. Protein peaks were measured as A_{280} with a Zeiss spectrophotometer. Fractions 150–500 were pooled and concentrated with PM-10 membrane filter (two fresh membranes were used). The specific activity of the concentrated enzyme was 39. The total units recovered numbered 2000.

column (Figure 3A). Rechromatography of peak I (the peak that is eluted earlier) and peak II (the second peak) (Figures 3B and 3C) established that peak I is an "artificial" peak (see Discussion). Therefore, only fractions from peak II were collected for further purification. Later it was found that the appearance of peak I could be totally prevented by running the DEAE-Sephadex or DEAE-cellulose column at a much faster flow rate, so the enzyme does not remain in the column too long ($\leq$2 hr for the DEAE-cellulose column, or $\leq$4 hr for the DEAE-Sephadex column). Inclusion of 2 mM mercaptoethanol in the eluting buffers seems to aid against production of peak I.

Step VII: DEAE-Cellulose Column Chromatography. A 22 × 0.9 cm DEAE-cellulose column was packed at room temperature and then placed in a 4° cold room. It was equilibrated with 10 mM potassium phosphate buffer (pH 6.6) containing 0.28 M KCl and 2 mM mercaptoethanol. The pressure over the bed should be high enough to give a flow rate of at least 50 ml/hr; the sample from step VI (5.5 ml, dialyzed once against 500 ml of equilibrating buffer at 4°) was carefully layered on top of the column. Equilibrating buffer (6 ml) was used to rinse the protein from the glass above the column bed and then the column was eluted with a 220-ml linear KCl elution gradient, from 0.28 to 0.47 M. Fractions containing aspartate transcarbamylase activity were collected, pooled, and concentrated in the usual manner.

Step VIII: DEAE-Sephadex Column Rechromatography. An 11 × 0.9 cm DEAE-Sephadex A-50 column was packed as described for step VI. It was equilibrated with 10 mM potassium phosphate buffer containing 0.34 M KCl and 2 mM mercaptoethanol. The flow rate was about 16 ml/hr. A sample from step VII (12 ml, dialyzed once in 1-l. equilibrating buffer at 4°) was layered on top of the column, and a 200-ml linear KCl gradient, from 0.34 to 0.47 M, was used as elutant. Of each 2-ml fraction, 1 μl was assayed. Those fractions which had a ratio of absorbance for carbamyl aspartate at 466 nm/absorbance for protein at 280 nm of 3.2–3.6 were pooled and concentrated. After being dialyzed against three changes of 1 l. of 5 mM sodium phosphate buffer (pH 6.6) this purest

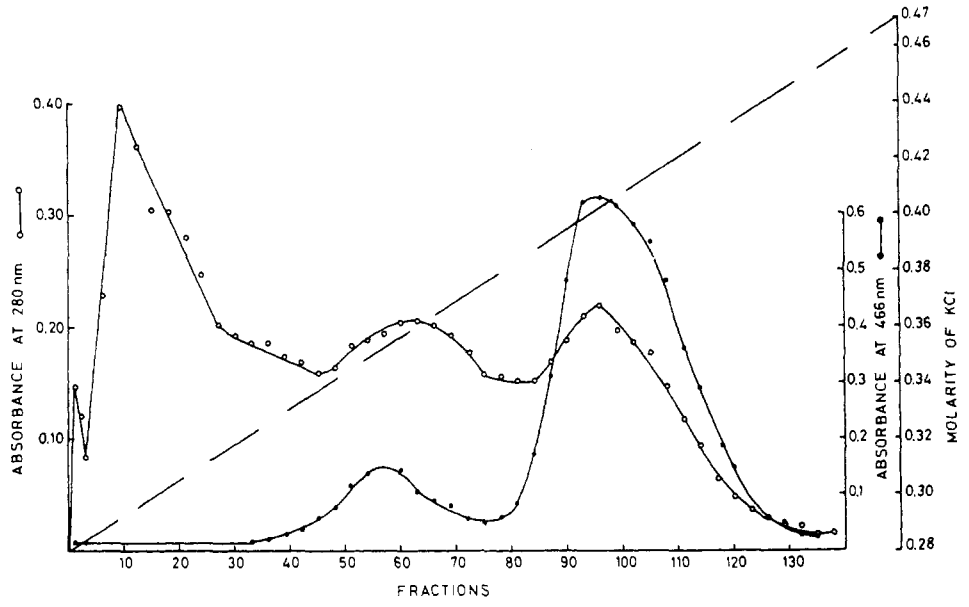

FIGURE 2: DEAE-Sephadex A-50 column chromatography. The detailed procedure is described in the text. The protein solution (5 ml) from step IV (Table I), protein concentration 13.4 mg/ml, total activity 6700 units, was used; 2-ml fractions were collected and a 1-μl aliquot was taken for the standard aspartate transcarbamylase assay. Fractions 80–132 were pooled and concentrated to 5.5 ml.

TABLE I: Purification of Aspartate Transcarbamylase from *Streptococcus faecalis*.

	Purification Stage	Vol (ml)	[Protein] (mg/ml)	Total Protein (mg)	Total Act. (Units)	Sp Act. (Units/mg)	Stage Recovery (%)
I	Homogenate	1950	25.8	50,200	14,600	0.29	(100)
II	Streptomycin supernatant	2250	16	36,000	13,700	0.38	94
III	pH 4.8 precipitate	358	45	16,100	12,200	0.76	90
IV	Hydroxylapatite column (eluate)	1840	0.2	368	9,570	26	70
	Amicon filtration, cell concn, and centrifugation	43	6.0	258			
V	Ammonium sulfate fraction (43–54% cut)	5.0	13.4[a]	67	6,720	100	70
VI	DEAE-Sephadex column eluate	5.5	3.5[a]	19.3	5,400[a]	280	80
VII	DEAE-cellulose column eluate	12	1.04[a]	12.5	4,000	320	74
VIII	DEAE-Sephadex column rechromatography eluate	7.0	1.28	9.0	3,140	350	78

[a] The protein concentrations were determined by measuring the absorbance at 280 nm and assuming that the ratio protein concentration (mg/ml)/OD_{280} = 1.2, since this ratio holds true for the purest enzyme fraction.

enzyme fraction is stored in glass tubes and kept at −20°. It was used for all of the physical and chemical studies in this and subsequent papers. The summary of a representative purification sequence is presented in Table I. This procedure has been successfully reproduced many times over a period of 2 years.

Criteria of Purity. The homogeneity of the purest enzyme fraction (specific activity = 350) was assessed by analytical gel electrophoresis at pH 9.5 according to the procedure of Davis (1964) (Figure 4B). The stained gel was scanned with a Gilford spectrophotometer equipped with an automatic gel scanner (Figure 4A). If the absorbance is a good estimate of protein, the enzyme was 90–95% pure. Polyacrylamide gel electrophoresis at pH 7.0, according to the procedure by Williams and Reisfeld (1964), gives a similar electrophoretic pattern. The electrophoretic pattern obtained after disc gel electrophoresis in the presence of sodium dodecyl sulfate according to the procedure of Weber and Osborn (1969) also confirmed that this enzyme was at least 90–95% pure. Moreover, it suggests that this enzyme has subunits of identical or nearly identical size.

Molecular Weight of the Native Enzyme and Subunit Structure. The molecular weight of the native enzyme is 128,000 ± 6000, determined by short column sedimentation equilibrium according to the procedure of Van Holde and Baldwin (1958).[1] Using sucrose gradient centrifugation according to the method of Martin and Ames (1961), the molecular weights of peak I and peak II enzyme (see Figure 3 and purification procedure step VI) are both 125,000 ± 1000.

The molecular weight of the subunits of aspartate transcarbamylase determined according to the method of Weber and Osborn (1969) is 32,500 ± 500 (see Figure 4C). Thus, this aspartate transcarbamylase contains four subunits of the same or nearly equal size.

[1] Assuming the partial specific volume of the aspartate transcarbamylase is 0.75.

General Properties. S. faecalis aspartate transcarbamylase is very stable in phosphate buffer at pH 6.6 (Prescott and Jones, 1970); this is also true of the pure enzyme. It is stable at 0.1–1 mg/ml concentration at −20° for at least 2 years, or at 4° for at least 2 months without changing its physical or kinetic properties. It is also stable in Tris-HCl buffer at pH 8.5 at 4° for at least 3 weeks. No effect of freezing and thawing has been detected. The ratio of the absorbance of aspartate transcarbamylase was $A_{280}/A_{260} = 1.80 \pm 0.05$. Enzyme activity is linear within a broad range of protein concentrations (0.022–33.6 μg/ml (see microfilm Figure 1[2])). The antigenic properties of this aspartate transcarbamylase are distinctly different from those of *Escherichia coli* aspartate transcarbamylase, for there is no cross-reaction between the enzyme from *S. faecalis* and *E. coli* aspartate transcarbamylase antibodies in an immunodiffusion analysis.[3]

Renaturation of Aspartate Transcarbamylase. Enzyme (14 units, 80% pure, 0.24 mg/ml, 0.2 ml) can be reversibly denatured by sodium dodecyl sulfate according to the procedure of Weber and Kuter (1971) (see Experimental Section); however, only 4% of the original activity was recovered after renaturation. If mercaptoethanol is omitted during the renaturation step, virtually no activity can be recovered.

General Steady-State Kinetic Properties and Demonstration of an Activator Site in the Enzyme Molecule. The aspartate saturation curve at low buffer concentration (5 mM Tris-HCl (pH 8.5); see Figure 5A) deviates from the typical Michaelis–Menten hyperbolic curve. The velocity *vs.* substrate plot is sigmoidal, followed by substrate inhibition. A double reciprocal plot clearly shows a curve that is concave upward, followed by a minimum at low 1/[aspartate] regions. The normal hyperbolic type saturation curve can be obtained when a high level of sodium acetate (400 mM) is present in the incubation mixture; again substrate inhibition prevails at high aspartate concentrations (Figure 5B). The carbamyl phosphate saturation curve at low buffer concentration is normally hyperbolic; however, substrate activation is clearly seen when carbamyl phosphate concentrations are increased to ≥ 2 mM (Figure 6, upper curve). This activation cannot be due to either carbamyl aspartate or orthophosphate (products of the aspartate transcarbamylase reaction), since the rate of the enzyme reaction is still linear with time until 20% of the aspartate is consumed, using 4 mM carbamyl phosphate and 1 mM aspartate as the initial substrates (see offset in Figure 6). Carbamyl phosphate activation no longer exists when a high level of sodium acetate (200 mM) is present (Figure 6, lower curve).

At low levels of carbamyl phosphate (0.1 mM), aspartate (1 mM), and buffer (5 mM Tris-HCl (pH 8.5)) many inorganic or organic anions greatly activate the reaction, but each anion activates to a different extent (20- to 100-fold activations at salt concentrations ranging from 10 to 50 mM; Figure 7 and Table II). In fact, under properly chosen conditions, every type of anion tested in our laboratory, including ATP and CTP, can stimulate the reaction to a certain extent. Urea and glycerol, two un-ionized compounds, have a minimal effect on the enzyme.

Nature of the Activator Site. Table III demonstrates that the activation effects of chloride ion vary with the nature of the countercation used in the experiment. Nitrogen salts

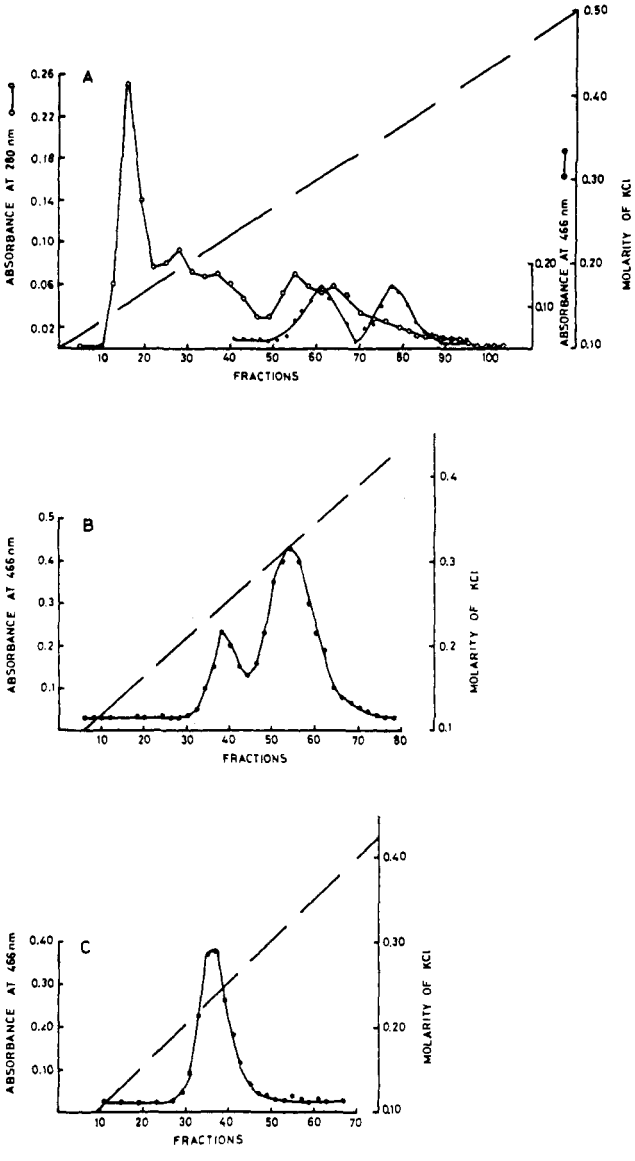

FIGURE 3: DEAE-cellulose column chromatography to investigate the nature of the two peaks of aspartate transcarbamylase activity. (A) DEAE-cellulose chromatography showing the appearance of two peaks of aspartate transcarbamylase activity. The column size was 1.5 × 9 cm; 48 ml of protein solution (580 aspartate transcarbamylase units) from step IV was applied to the column, which was then eluted at 4° with a linear gradient formed by 200 ml of 0.1 M KCl in 10 mM potassium phosphate (pH 6.6) and 200 ml of 0.5 M KCl in 10 mM potassium phosphate (pH 6.6). No mercaptoethanol was included in the eluting solvent. Fractions of 4 ml were collected. The total elution time was about 5 hr. The total activity recovered was about 400 units. (B) DEAE-cellulose rechromatography of peak II. The column size was 0.6 × 3.6 cm. Eluting buffers used were the same as described in A, except that only 50 ml of each solution was used; fractions of 1 ml were collected. The sample applied to the column was the combined fractions numbered 77–83 obtained from the DEAE column shown in A; the total aspartate transcarbamylase activity of this sample was 150 units. The elution time was about 2.5 hr. The total activity recovered was about 120 units. (C) DEAE-cellulose rechromatography of peak I. The column and gradient solutions used here were the same as used in B experiment. The pooled sample, however, was composed of fractions numbered 55–62, obtained from the DEAE column shown in A. The total activity of this sample was 42 units. The elution time was ~5 hr. Fractions of 1 ml were collected. The total activity was about 30 units.

[2] See paragraph at end of paper regarding suppplementary material.

[3] The *E. coli* aspartate transcarbamylase antibodies to holoenzyme and catalytic subunit were the generous gift of Professor Lawrence Levine, Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. 02154; the antibodies were prepared by the procedure of Bethell *et al.* (1968).

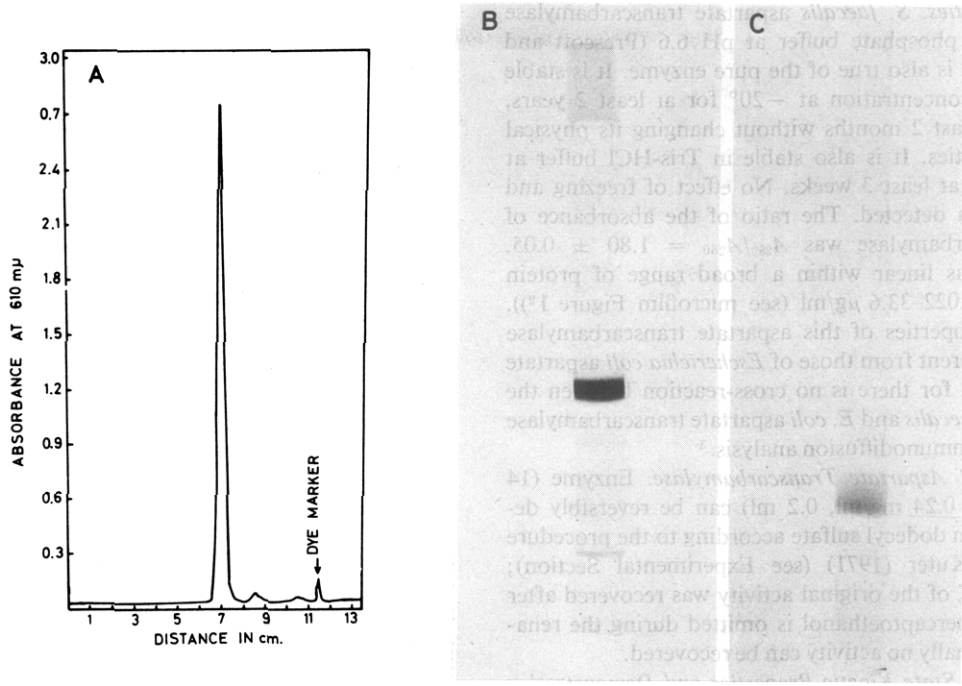

FIGURE 4: Analytical polyacrylamide gel electrophoresis of the purest aspartate transcarbamylase fraction. (A) Gel scanning with a Gilford spectrophotometer equipped with automatic scanner, at 610 nm. The sample gel shown in B was used for scanning. (B) Gel electrophoresis at pH 9.4, according to the method of Davis (1964). Enzyme (40 μg) was layered on each gel. The current applied was 5 mA/gel for about 2 hr at room temperature. Bromophenol Blue (the farthest moving band in the picture) was used as a marker. (C) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The current applied was 5 mA/gel for about 4 hr at room temperature. Enzyme (20 μg) was layered on each gel.

TABLE II: Per Cent Activation of Control by Various Organic Acids at 25 mM.[a]

Salt Used	% Activation
Formate	5600
Propionate	5000
Butyrate	5700
Isobutyrate	5800
L-α-Aminobutyrate	1400
γ-Aminobutyrate	400
D-Aspartate	5700
L-Alanine	800
β-Alanine	600
Carbamyl-β-alanine	5000

[a] Sodium was used as countercation. The assay conditions used here are as described in Figure 7. The control, without added salt, gives 50 cpm.

TABLE III: Per Cent Activation[a] of Various Nitrogen Salts (Using Chloride as Counteranion).

	Concentration		
	10 mM	20 mM	30 mM
NaCl	680	1250	1730
Triethanolamine-HCl (pK_a = 7.7)	770	1420	1950
Hydroxylamine-HCl (pK_a = 5.96)	850	1530	2120
Aminoethanol (pK_a = 9.45)	860	1660	2310
Tris-HCl (pK_a = 8.1)	960	1880	2620
Imidazole-HCl (pK_a = 7)	1580	2000	2390
NH_4Cl (pK_a = 9.25)	1090	2120	2710

[a] The control, in the absence of any added salt, is 230 cpm. The reaction mixture (1 ml) contained 3 mM Tris-HCl (pH 8.5), 1×10^{-4} M [^{14}C]carbamyl phosphate (0.573×10^6 cpm/μmol), 2 mM L-aspartate, 0.037 μg of aspartate transcarbamylase, and varying concentrations of salts. The reaction time at 25° was 20 min. The [^{14}C]carbamyl phosphate assay was used.

always give better activation than simple inorganic salts. Figure 8 shows that the effect of acetate, one of the better activator molecules tested, is competitive in nature against L-aspartate as the variable substrate. This suggests that at high aspartate levels addition of an activator would have no effect on the enzyme reaction; in other words, acetate and aspartate appear to compete for the same activator site. The two best aspartate analogs for aspartate transcarbamylases, maleate and succinate (Jones, 1962; Gerhart and Pardee, 1964), can activate the *S. faecalis* aspartate transcarbamylase reaction at very low levels of aspartate (2 mM); however, when the aspartate level is raised to 6 mM, maleate becomes only an inhibitor while succinate activates slightly before it inhibits the reaction (see Figure 2 of the microfilm version[2]).

Discussion

The purification procedure presented here results in about 1200-fold purification with satisfactory yields and a final purity of 90–95%. Each step is easily reproducible. For the

streptomycin precipitation step, care must be taken so that only an adequate amount of streptomycin is added gradually, for if too much streptomycin was added (more than one-fifth volume of the total protein solution which was adjusted to 25.8 mg/ml in protein concentration), the specific activity after step IV would only reach around 10 units/mg rather than 26 units/mg. Usually, the recovery from the hydroxylapatite column chromatography is satisfactory (60–80%), but the specific activity of the enzyme at this particular stage can vary and has ranged from 20 to 60. In general, regenerated hydroxylapatite columns (see Results) yield enzyme with higher specific activity than those obtained by using fresh hydroxylapatite preparations. The peak I enzyme emerging from DEAE-Sephadex or DEAE-cellulose columns has the same molecular weight as the "native enzyme," and is believed to be an artifact, presumably due to the interaction between the native enzyme and the DEAE group during the interval required for the column elution. It is known that DEAE columns are capable of denaturing or partially denaturing many enzymes or proteins (Peterson and Sober, 1962).

To the authors' knowledge, there are theoretically at least four reasonable mechanisms known to be capable of generating sigmoidal enzyme–substrate saturation curves (for a review, see Cleland, 1970).

(1) The positive cooperativity models, among which the Monod *et al.* (1965) model and the Koshland *et al.* (1966) model are most commonly considered. The cooperativity observed experimentally in binding or catalytic ability of the enzyme is thought to be due to conformational changes in enzyme subunits which affect subunit interactions. The native *E. coli* aspartate carbamylase, which exhibits sigmoidal saturation kinetics with both substrates (carbamyl phosphate and aspartate), has been shown to be best described by this type of model although the detailed mechanism for this enzyme is far from being solved (for a review, see Gerhart, 1970). The work by Gerhart and Pardee (1964) on *E. coli* aspartate carbamylase showed that maleate, a competitive inhibitor with aspartate as substrate, can activate the enzyme reaction at very low aspartate concentrations. The fact that a substrate analog can stimulate the reaction at low substrate concentration is taken to be excellent kinetic evidence that "cooperativity" is involved in the catalytic process (Atkinson *et al.*, 1965; Ferdinand, 1966; Kirtley and Koshland, 1967). In our case, we are unable to rule out this possibility at this stage. However, whether or not there are cooperative subunit interactions, a nonspecific allosteric activator site (demonstrated kinetically under Results), sensitive to all kinds of anions including substrates, has to exist. Moreover, the change of double reciprocal plots for carbamyl phosphate and aspartate from nonlinear to linear in the presence of a high level of acetate makes us believe that it is very unlikely that the "cooperativity model" *alone* can fit all of the experimental data.

(2) A rapid protein association–dissociation model such that a ligand can influence the state of association of that protein can also yield cooperative binding of that ligand (Frieden, 1967). The extent of cooperative binding will depend on the ratio of the binding constants for the ligand to different forms of the protein. If the intrinsic activities of the different molecular weight forms are sufficiently different, nonlinear kinetics may also occur. In our case, no multiple forms of aspartate transcarbamylase can be detected by molecular weight determinations (Table II) or disc gel electrophoresis. Also, the specific activity of aspartate transcarbamylase is constant over a 1500-fold range of enzyme concentrations (Figure 1 of the microfilm version[2]). Therefore, we do not think the data support this model.

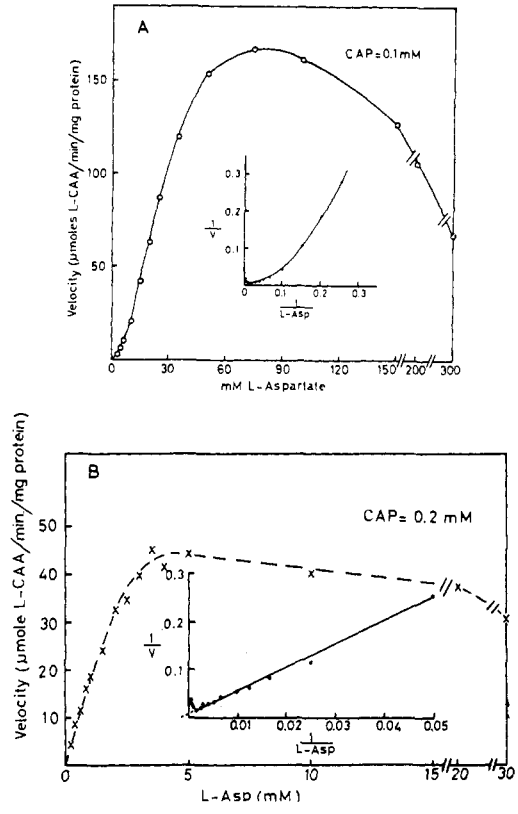

FIGURE 5: Aspartate saturation curves in the absence or presence of added acetate. (A) Aspartate saturation curve at low buffer concentration, without added salt. One milliliter of reaction mixture contained 5 mM Tris-HCl (pH 8.5), 0.1 mM [^{14}C]carbamyl phosphate (0.764×10^6 cpm/μmol), and 0.074 μg of aspartate transcarbamylase (for aspartate levels at 4 for 10 mM range), or 0.1 μg of aspartate transcarbamylase (for aspartate levels above 10 mM). The reaction time at 25° was 10 min. The [^{14}C]carbamyl phosphate assay was used. The inset shows the double reciprocal plot of the same datum. (B) Aspartate saturation curve in the presence of 400 mM sodium acetate. One milliliter of reaction mixture contained 5 mM Tris-HCl (pH 8.5), 400 mM sodium acetate, 0.2 mM [^{14}C]-carbamyl phosphate (0.573×10^6 cpm/μmol), and 0.074 μg of aspartate transcarbamylase. The reaction time at 25° was 10 min. The [^{14}C]carbamyl phosphate assay was used. The inset shows the double reciprocal plot of the same datum. Abbreviations used are: CAA, carbamyl aspartate; CAP, carbamyl phosphate; L-Asp, L-aspartate.

(3) The random addition of the bireactant substrates model is based on the theory that a random bi reaction (nomenclature according to Cleland, 1963a) would result in reciprocal plots that are 2:1 functions which can be either concave up or down (Cleland, 1963b; Frieden, 1964; Ferdinand, 1966; Sanwal and Cook, 1966; Sweeny and Fisher, 1968; Moffet and Bridger, 1970). However, as pointed out by Cleland (1970), for realistic values of rate constants, the expected curvature is very slight, and random mechanisms tend to give linear reciprocal plots which appear to be in rapid equilibrium (*i.e.*, the only rate-limiting step is the chemical transformation step (Alberty, 1952)). To date, no one has clearly identified a nonlinear reciprocal plot caused by random addition of substrates (Cleland, 1970). Moreover, in our case, it is very difficult to explain the enormous activation effects of many salts with ordinary random mechanisms. As will be shown in subsequent papers (Chang and Jones, 1974a,b), the kinetic mechanism of this aspartate transcarbamylase is probably

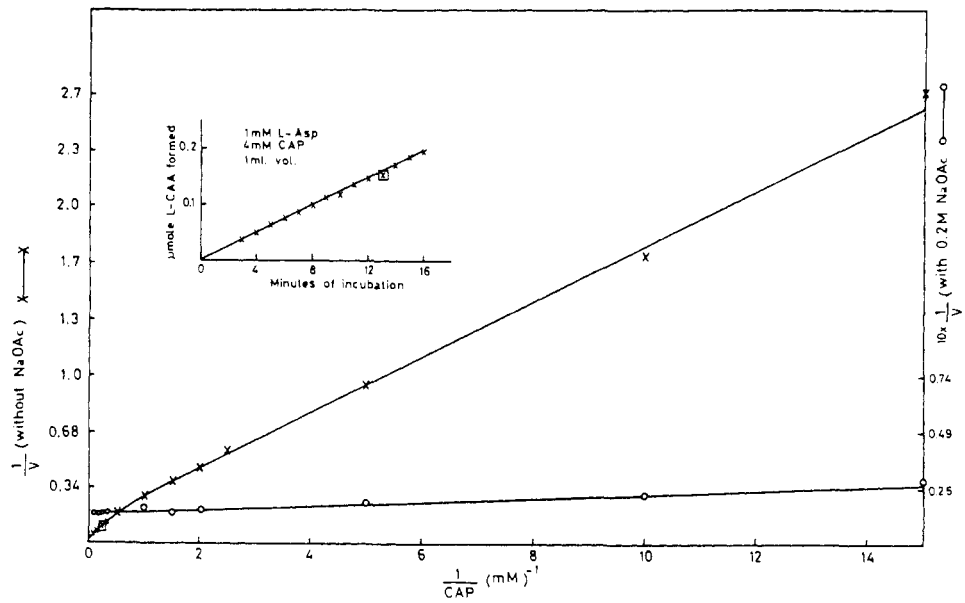

FIGURE 6: Double reciprocal plot of carbamyl phosphate saturation curve in the absence or presence of 20 mM sodium acetate. For the upper curve, 0.5 ml of reaction mixture contained 5 mM Tris-HCl (pH 8.5), 1 mM L-aspartate, 1.48 μg of aspartate transcarbamylase for carbamyl phosphate (1.146×10^5 cpm/μmol) levels from 0.667×10^{-4} to 2×10^{-3} M, or 0.74 μg of aspartate transcarbamylase for the rest. The reaction time at 25° was 10 min. The [^{14}C]carbamyl phosphate assay was used. For the lower curve, the 0.5-ml reaction mixture contained 5 mM Tris-HCl (pH 8.5), 1 mM L-aspartate, and 0.111 μg of aspartate transcarbamylase. The reaction time at 25° was 5 min. The [^{14}C]carbamyl phosphate assay was used. The inset shows the linearity of the initial velocity within about 20% aspartate consumption at 1 mM aspartate. The 20-ml reaction mixture contained 5 mM Tris-HCl (pH 8.5), 18.5 μg of aspartate transcarbamylase, 1 mM aspartate, and 4 mM carbamyl phosphate; the incubation was at 25°. At appropriate time intervals, 1-ml portions were withdrawn and transferred into test tubes which contained 1 ml of the colorimetric carbamyl aspartate assay mixture (antipyrine and acetyl monoxime). The point specified by a square in the inset is equivalent to the point specified by a square in the upper curve. Abbreviations used are: L-Asp, L-aspartate; CAP, carbamyl phosphate; CAA, carbamyl aspartate.

random, but this does not seem to be the main reason for the nonlinear kinetics of this enzyme.

(4) A "substrate binds twice to the enzyme" model has been considered by Cleland (1963c) and other workers where one molecule of a given reactant serves as a substrate, and a second molecule of the same reactant serves as a substrate or an effector per each active species of the enzyme molecule. When a substrate is added two times during the reaction sequence, the reciprocal plot will be parabolic (concave up), if the two points for addition of substrate are reversibly connected. Though it is rarely recognized by practicing enzymologists, this mechanism is theoretically and experimentally well documented in the literature (Worcel *et al.*, 1965; Frieden, 1964; Khoo and Russell, 1970). It is obvious that if one assumes that there is an allosteric activator site present in this aspartate transcarbamylase, which can bind aspartate as an activator, then this model would easily and satisfactorily explain the sigmoidal saturation curve for aspartate, and the activating and inhibitory effects of maleate and succinate at different levels of aspartate (see Figure 2 of the microfilm version[2]). The substrate activation effect of carbamyl phosphate in the absence of added salt (Figure 6) and the large activation effects by many anions (Figure 7 and Table II) can also be easily explained if one assumes that the enzyme activator site is rather nonspecific and sensitive to all kinds of anions. The data in Figures 6 and 8 suggest that the major function of the activator molecule is to influence (facilitate) the binding of substrate(s) rather than to facilitate the chemical transformation step(s) during the reaction. The dissociation constant for the activator molecule is probably in the millimolar range. The fact that nonlinearity is not seen in the upper curve of Figure 6 until the carbamyl phosphate level is raised to 2 mM or higher is probably due to the large differences in the dissociation constants between binding of carbamyl phosphate as substrate (K_d = 0.1 mM; Chang and Jones, 1974a,b) and binding of carbamyl phosphate as activator. Without acetate, the substrate inhibition by aspartate becomes obvious between 60 and 90 mM L-aspartate, but with 400 mM acetate, it can be observed at 3–5 mM aspartate. We think that this inhibition is probably due to the formation of an abortive enzyme–phosphate–aspartate complex (see Chang and Jones, 1974a,b).

The activating effects of various anions apparently also depend in part on the nature of the countercation of the salts used. From the data in Table III, it is difficult to draw any conclusion with regard to the relationship between activation efficiency and the structural and charge differences among various nitrogen salts. These data, however, make us believe that L-aspartate may be the "preferred" activator molecule, since it has two anionic carboxylic groups and an unhindered NH_3^+ in the same molecule. In fact, a preliminary estimation can be made (Chang and Jones, 1974b) that the dissociation constant for the activator site is probably below 0.5 mM, a value lower than the K_d for aspartate ($\geq$1.7 mM; Chang and Jones, 1974a). This activator site is apparently exposed and readily available to the solvent, so that any anion can activate, and the activation is nonspecific. From the present study, it is obvious that buffers used in the assay should be carefully controlled; buffers of unnecessarily high concentration generally have to be avoided, for they can mask the true events severely, unless one specifically wishes to prevent carbamyl phosphate or aspartate and its analogs from occupying the activator site in order to study only the true substrate binding sites.

The aspartate transcarbamylase from *S. faecalis*, by binding L-aspartate twice, should exhibit sensitive rate response

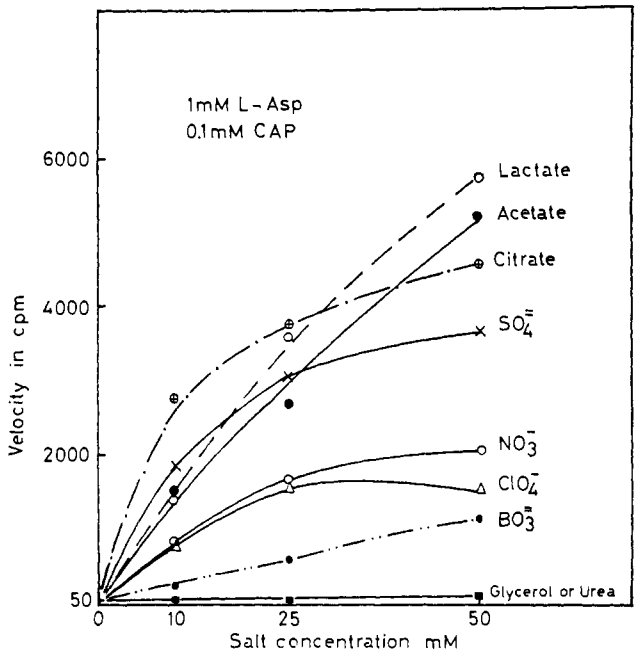

FIGURE 7: Activation effects of various inorganic or organic salts. All stock solutions of salt (0.1, 0.25, and 0.5 M) were prepared in 50 mM Tris-HCl and neutralized to pH 8.5 at room temperature. One milliliter of reaction mixture contained a total of 6 mM Tris-HCl, 1 mM L-aspartate (L-Asp), 0.1 mM [^{14}C]carbamyl phosphate (CAP) (1.146×10^6 cpm/μmol), and 1.85×10^{-2} aspartate transcarbamylase (pH 8.5). The reaction time at 25° was 15 min. The [^{14}C]carbamyl phosphate assay was used. The controls (without added salts) gave a net cpm of 50. The same curve is obtained with either D- or L-lactate. Sodium was the countercation in all cases.

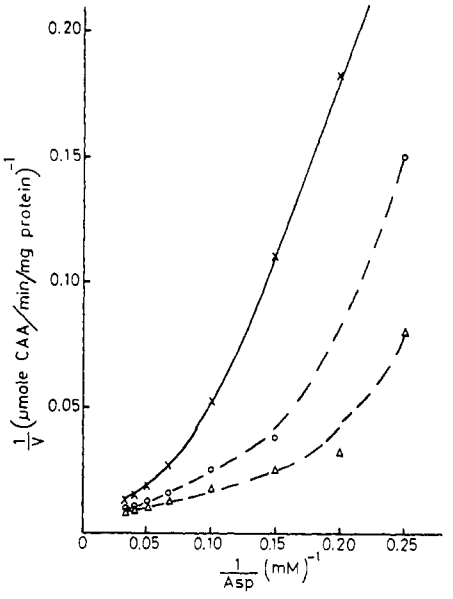

FIGURE 8: Double reciprocal plots using L-aspartate as a variable substrate at different concentrations of sodium acetate: (×) 0 mM; (○) 10 mM; (△) 20 mM acetate. One milliliter of reaction mixture contained 5 mM Tris-HCl (pH 8.5), 0.1 mM [^{14}C]carbamyl phosphate (0.764×10^6 cpm/μmol), 0.037 μg of, aspartate transcarbamylase and different concentrations of sodium acetate. The reaction time at 25° was 10 min (at 0 mM sodium acetate) or 5 min (at 10 and 20 mM sodium acetate). The [^{14}C]carbamyl phosphate assay was used. Abbreviation used is: CAA, carbamyl aspartate.

to changes of aspartate concentrations *in vivo*. We think that, as time goes on, more enzymes exhibiting similar binding behavior may be discovered.

Acknowledgment

We are most grateful to Dr. Samuel E. Allerton of this University for the sedimentation equilibrium analysis, to Dr. G. D. Novelli for growth of a large batch of *S. faecalis* cells, and to A. Shahinian for fine technical assistance. We are also grateful to Dr. L. Raijman for lively, critical discussions.

Supplementary Material Available

Supplementary material describing this experiment will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-74-629.

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Aspartate Transcarbamylase from *Streptococcus faecalis*. Steady-State Kinetic Analysis†

Ta-Yuan Chang‡ and Mary Ellen Jones*

ABSTRACT: The kinetic mechanism of *Streptococcus faecalis* aspartate transcarbamylase has been studied using steady-state kinetic analysis in the absence or presence of moderate levels of acetate to partially saturate an anion activator site in the enzyme molecule (Chang, T. Y., and Jones, M. E. (1974), *Biochemistry 13*, 629). Initial velocity studies indicate that the enzyme catalyzes the reaction by a sequential mechanism. Product inhibition analysis yields three competitive patterns and one noncompetitive pattern, a result which is more consistent with a random than an ordered mechanism, and which also supports the existence of a dead-end complex: enzyme–carbamyl phosphate–carbamyl aspartate. Pyrophosphate and maleate were chosen as substrate analogs of carbamyl phosphate and L-aspartate, respectively, for dead-end inhibition analysis. Two competitive inhibition patterns and two noncompetitive inhibition patterns are obtained in this analysis; this result also indicates the absence of a compulsory binding order between the points or addition of carbamyl phosphate and aspartate to the enzyme. The lower limits for the dissociation constants are 1×10^{-4} M for K_d(carbamyl phosphate) and 1.7×10^{-3} M for K_d(L-aspartate). Binding of carbamyl phosphate to the enzyme first hinders the binding of aspartate, while binding of aspartate first facilitates the binding of carbamyl phosphate. An asymmetrical random mechanism has been constructed as a tentative scheme for the kinetic mechanism for this enzyme since it can qualitatively explain all of the data.

In a previous paper (Chang and Jones, 1974a) we reported the purification of aspartate transcarbamylase from *Streptococcus faecalis* to near homogeneity and some kinetic evidence for an anion activator site of this enzyme. The activator site is sensitive to many anions including the anionic substrates and products of the reaction. With kinetic techniques, it is not possible to be certain whether the activator site is absolutely essential for the catalytic reaction of this enzyme or not. Fortunately, normal hyperbolic plots for the aspartate and carbamyl phosphate saturation curves can be obtained when acetate concentrations are high, because when acetate has partially saturated the activator site the substrates bind only once to the catalytic sites of this enzyme at low substrate concentrations. Under these conditions, the concentrations of aspartate, carbamyl phosphate, phosphate, and carbamyl aspartate can be varied to a reasonable extent without perturbing the activator site so that conventional steady-state kinetic analysis of the initial velocity, as well as product inhibition and dead-end inhibition studies, can be used to elucidate the kinetic mechanism of the reaction including the possible role of the activator molecule during enzyme catalysis.

Experimental Section

Chemicals and Reagents. The aspartate transcarbamylase used was 90–95% pure and is the purest enzyme available (Chang and Jones, 1974a). It can be stored for 2 months at 4°, at a concentration near 0.1 mg/ml, in 5 mM potassium phosphate buffer (pH 6.6). The stock enzyme solutions (about 1 mg/ml, in 5 mM potassium phosphate buffer (pH 6.6)) are stored at −20° and are stable for at least 2 years.

[^{14}C]Carbamyl phosphate (dilithium salt) used here is the same as mentioned in the previous paper (Chang and Jones, 1974a). It contains a minor radioactive contaminant, which is not converted to $^{14}CO_2$ afer acidification and boiling, that

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