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BEEF BRAIN CYTOPLASMIC ASPARTATE AMINOTRANSFERASE

PURIFICATION, KINETICS, AND PHYSICAL PROPERTIES

MARY L. KRISTA AND MARGARET L. FONDA

Department of Biochemistry, University of Louisville School of Medicine, Louisville, Ky. 40201 (U.S.A.)

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SUMMARY

Beef brain cytoplasmic aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) was purified 380-fold. The existence of multiple forms (α, β, γ) of the enzyme was shown by chromatography on Sephadex CM-50 at pH 5.4 and by electrophoresis at pH 9.0 on polyacrylamide gel.

Analytical ultracentrifugation yielded a sedimentation coefficient of 5.62 S and a mol. wt of 90 000 $\pm$ 6000 for the brain aspartate aminotransferase. A Stokes radius of 39.5 Å and a mol. wt of 103 000 were determined for the enzyme by gel filtration on Sephadex G-200.

Apparent K_m values of 2 mM for L-aspartate and 0.17 mM for α-ketoglutarate were obtained with the brain aspartate aminotransferase. The enzyme was inhibited significantly by 1–3 mM α-ketoglutarate at pH 6.0. No inhibition of brain aspartate aminotransferase activity was noted with γ-aminobutyric acid. Most of these properties of the beef brain enzyme are the same as those obtained with beef heart cytoplasmic aspartate aminotransferase.

INTRODUCTION

Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) functions at a branch point between the citric acid cycle and the "γ-aminobutyric acid shunt pathway", a secondary metabolic route found only in nervous tissue. L-Glutamic acid decarboxylase, located in the central nervous system, catalyzes the formation of γ-aminobutyrate from L-glutamate. Evidence has been presented that L-glutamate, when discharged into the brain, promotes excitatory actions[1–3], whereas γ-aminobutyrate inhibits neurotransmittance[3–5]. The relative amounts of L-glutamate to γ-aminobutyrate in the neuronal system apparently need to be controlled carefully. Since glutamic acid in brain tissue has been shown to be utilized mainly by transamination[6,7], and since aspartate aminotransferase is at a branch point between the citric acid cycle and the γ-aminobutyrate shunt, it might

be predicted that in the brain aspartate aminotransferase plays a prominent role in metabolic control of both glutamate and γ-aminobutyrate concentrations.

Aspartate aminotransferase purified from several mammalian tissues[8–10] has been separated into mitochondrial and cytoplasmic isozymes. On starch gel electrophoresis, cytoplasmic aspartate aminotransferase has been fractionated further into α, β, and γ subforms[11]. The α subform was the most enzymatically active, whereas the γ subform was essentially inactive.

The kinetic and physical properties of cytoplasmic aspartate aminotransferase purified from mammalian heart and liver, have been investigated extensively. However, purification and characterization of cytoplasmic aspartate aminotransferase from mammalian brain has been reported only for the rat[10]. Because of the dual metabolic role of aspartate aminotransferase unique to the brain, it was thought that the enzyme may have a control function in the brain that it does not have in other tissues. It was of interest, therefore, to purify and characterize beef brain aspartate aminotransferase and compare it to the beef heart enzyme purified in a similar manner.

The behavior of beef brain asparatate aminotransferase on ion exchange and molecular sieve chromatography and polyacrylamide disc gel electrophoresis was investigated. The sedimentation coefficient, Stokes radius, and molecular weight were determined for the enzyme. Also, some of the kinetic properties of beef brain aspartate aminotransferase were investigated and compared to those reported for aspartate aminotransferase purified from beef heart and liver.

MATERIALS AND METHODS

Materials

The following reagent grade chemicals and proteins were purchased from Sigma Chemical Co.: β-NADH, pyridoxal phosphate, Trizma base, polyvinylpyrrolidone, 6-benzamido-4-methoxy-*m*-toluidinediazonium chloride (fast violet B salt), EDTA, *cis*-oxaloacetic acid, α-ketoglutaric acid, L-aspartic acid, γ-aminobutyric acid, bovine serum albumin, egg ovalbumin, and yeast alcohol dehydrogenase. Special enzyme grade $(NH_4)_2SO_4$ and coomassie brilliant blue were purchased from Schwarz/Mann Chemical Co.; malate dehydrogenase was secured from Boehringer Mannheim Corp.; and human serum albumin was obtained from Nutritional Biochemicals Corp. The Sephadex gels were purchased from Pharmacia Fine Chemicals Inc. Hydroxylapatite gel was prepared according to the method of Tiselius *et al.*[12].

Enzyme assays

Velocity measurements were carried out spectrophotometrically at 25 °C in a temperature-controlled cell compartment of a Zeiss PMQ II spectrophotometer.

Aspartate aminotransferase activity was initially assayed by measuring the formation of oxaloacetate according to the procedure of Cammarata and Cohen[13]; however, it was difficult to obtain linear rates with this assay. Therefore, the transaminase activity was measured by coupling the reaction with malate dehydrogenase and β-NADH according to the procedure of Karmen[14] and Amador and Wacker[15]. Unless otherwise specified, the assay solutions contained 6.7 mM α-ketoglutarate, 6.7 mM L-aspartate, 67 mM Tris–HCl (pH 7.4), 0.17 mg of β-NADH [10 mg/ml of

100 mM potassium phosphate (pH 8.0)], and 8.3 μg of malate dehydrogenase [1.25 mg suspended in 1 ml of 2.8 M $(NH_4)_2SO_4$] in a total volume of 1 ml. After starting the reaction by the addition of aspartate aminotransferase, the decrease in absorbance at 340 nm was recorded every 15 s for 3 min.

The velocity of the malate dehydrogenase-catalyzed reduction of oxaloacetic acid to L-malate and the simultaneous oxidation of NADH to NAD^+ was assayed according to the method of Thorne[16]. The reaction mixture contained 67 mM Tris–HCl (pH 7.4), 0.99 mM *cis*-oxaloacetic acid, and 0.17 mg of NADH in a total volume of 1 ml. An aliquot of malate dehydrogenase was added and the decrease in absorbance at 340 nm was recorded every 15 s for 3 min.

Protein determination

Protein was determined routinely according to the method of Lowry *et al.*[17] using human serum albumin as the standard.

Analytical ultracentrifugation

All ultracentrifugation studies were performed in a Beckman/Spinco Model E analytical ultracentrifuge at 5 °C using schlieren optics and a Kodak Wratten filter (No. 77A). The observed sedimentation velocity coefficients were corrected to $s_{20,w}$ values using the equation of Svedberg and Pederson[18].

Sedimentation equilibrium ultracentrifugation to determine the molecular weight of aspartate aminotransferase was carried out according to the method of Archibald[19]. The values of c_m, c_b, $(dc/dx)_m$, and $(dc/dx)_b$ were calculated from sedimentation equilibrium runs. Silicone fluid (0.1 ml) was introduced into a conventional 4 ° single sector ultracentrifuge cell. A 0.5-ml sample of beef brain aspartate aminotransferase (3 mg/ml in 50 mM sodium acetate (pH 5.4) with enzyme stabilizers) was added. The rotor speed was 8766 rev./min. The value of c_0 was obtained from a synthetic boundary run. A 0.3-ml sample of the same enzyme solution as previously centrifuged was added to a 4 ° sector valve-type synthetic boundary cell. The rotor speed was 11 272 rev./min.

Gel electrophoresis

Disc gel electrophoresis was performed at pH 9.0 according to the method of Ornstein[20] using 7% polyacrylamide gels. Samples of aspartate aminotransferase in a 20% sucrose solution were applied to the gels. Each protein sample was run in duplicate. One gel was stained with coomassie brilliant blue (0.05% in 10% trichloroacetic acid), a non-specific protein stain. The other gel of the same protein concentration was stained with fast violet B salt, as in the procedure of Decker and Rau[21] for the specific staining of aspartate aminotransferase activity on starch gels.

RESULTS

Purification of beef brain cytoplasmic aspartate aminotransferase

The purification procedure was similar to that used previously for pig heart aspartate aminotransferase[11]. All steps were performed at 4 °C unless specified differently, and the preparation was protected from light. After each step of purification, samples were taken for determination of enzyme activity and protein concentration.

Four beef brains (1633 g of tissue), acquired at the time of slaughter from Fischer Packing Co. (Louisville, Ky.), were cleaned, cut into small pieces, and homogenized in 250 mM sucrose and 0.1 mM EDTA. The 28% (w/v) homogenate was centrifuged at 12 800 rev./min for 60 min.

The supernatant was made 50 mM succinate and pH 6.0 by the addition of 500 mM succinate (pH 6). α-Ketoglutarate (2.5 mM), EDTA (0.1 mM), and pyridoxal phosphate (0.1 mM) were also added and were present in all subsequent steps except for the Sephadex column where the pyridoxal phosphate was omitted. The enzyme was precipitated by $(NH_4)_2SO_4$ in the fraction between 50 and 75% saturation. The precipitate was dissolved in 100 ml of 50 mM succinate (pH 6.0) and dialyzed against 4 changes of 2 l of the same buffer. The entire solution was placed in an 80 °C water bath and brought up to 71 °C within 16 min. Upon reaching 71 °C, the protein solution was removed immediately and cooled to 5 °C. Heat-denatured proteins were removed by centrifugation at 6000 rev./min for 30 min.

The enzyme was reprecipitated with $(NH_4)_2SO_4$ between 50 and 60% saturation. The precipitate was dissolved in 8 ml of 20 mM potassium phosphate (pH 6.8) and was dialyzed against 4 changes of 1 l of the same buffer. The protein was treated batch-wise with hydroxylapatite gel (1 g of gel/2.3 g of protein). After centrifugation at 8000 rev./min for 15 min, the supernatant was retained.

A Sephadex CM-50 column (2.5 cm × 40 cm) was equilibrated with 20 mM sodium acetate buffer (pH 5.4). The enzyme, 232 mg of protein in 12 ml, was dialyzed against the same buffer and applied to the column. The eluting acetate buffers at pH 5.4 were as follows: 44 ml of 40 mM acetate, 88 ml of 60 mM acetate, and a linear

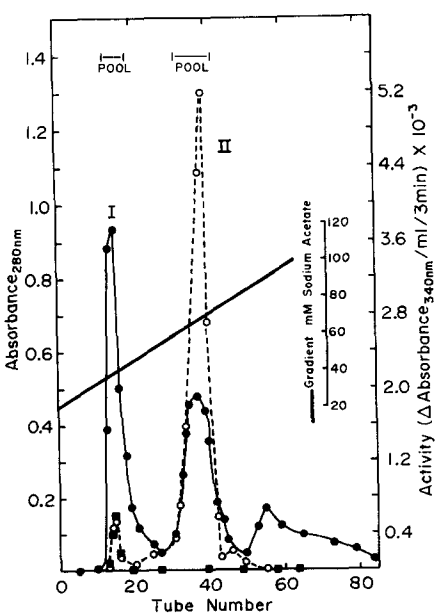

Fig. 1. Chromatography of beef brain cytoplasmic aspartate aminotransferase on Sephadex CM-50. Protein was eluted with sodium acetate (pH 5.4) in 6-ml fractions at a flow rate of 20 ml/h. Absorbance at 280 nm, ●—●; aspartate aminotransferase activity, ○---○; malate dehydrogenase activity, ■.

gradient of 440 ml of 60 mM acetate in the mixing chamber and 440 ml of 110 mM acetate in the reservoir. Two peaks of aspartate aminotransferase activity were eluted from the column as shown in Fig. 1. Peak I contained small amounts of both transaminase and malate dehydrogenase, whereas Peak II contained most of the aspartate aminotransferase activity.

The protein in Peaks I and II was precipitated separately with 90% $(NH_4)_2SO_4$. Each precipitate was dissolved in 2–4 ml of 50 mM sodium acetate (pH 5.4) and dialyzed against 2 changes of 1 l of the buffer. The enzyme was stored in 50 mM sodium acetate (pH 5.4) at −15 °C. An outline of the purification procedure is shown in Table I. The fluctuations in yields obtained in Steps 1–5 may be due to inaccuracy

TABLE I

PURIFICATION OF BEEF BRAIN CYTOPLASMIC ASPARTATE AMINOTRANSFERASE

Fraction	*Activity (units/ml)**	*Volume (ml)*	*Total activity (units)*	*Total protein (g)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification (-fold)*
1. Homogenate	4.3	4100	17 800	50.4	0.353	100	1.0
2. 1st $(NH_4)_2SO_4$ (50–75%)	98.9	180	18 100	2.7	6.71	102	19.0
3. Heat treatment	89.2	153	13 700	0.74	18.5	77	52.4
4. 2nd $(NH_4)_2SO_4$ (50–60%)	1060	14	14 900	0.28	53.2	84	151
5. Hydroxylapatite	1160	12	13 900	0.23	60.4	78	171
6. Sephadex CM-50							
Peak I	46.2	7.4	342	0.036	9.5	1.9	26.9
Peak II	948	4	3 820	0.028	136	21.5	385

* 1 unit equals oxidation of 1 μmole NADH per min at 25 °C.

in measuring the larger volumes or to removal of some enzyme inhibitors.

For comparison with the beef brain cytoplasmic enzyme, beef heart cytoplasmic aspartate aminotransferase was purified in a nearly identical manner.

Kinetic properties of beef brain aspartate aminotransferase

The effect of pH on aspartate aminotransferase activity was determined in 67 mM Tris–HCl at pH 7.7, 8.0, 8.3, 8.4, and 8.9. α-Ketoglutarate and L-aspartate both were added at a concentration of 6.7 mM. Maximum activity for beef brain cytoplasmic aspartate aminotransferase was obtained between pH 8.0 and 8.9. As was reported for pig heart cytoplasmic aspartate aminotransferase[22], activity of brain aspartate aminotransferase decreased below pH 8.0. This is probably due to inhibition by α-ketoglutarate at lower pH values.

Apparent K_m values of beef brain aspartate aminotransferase for the substrates α-ketoglutarate and L-aspartate were determined in the presence of 0.1 mM pyridoxal phosphate. In one experiment, initial velocities were obtained with varying concentrations of α-ketoglutarate at several fixed concentrations of L-aspartate. α-Ketoglutarate was held then at a series of constant concentrations, while L-aspartate concentration was varied. The data obtained were plotted as Lineweaver–Burk plots[23]. In both cases, the lines obtained in the double reciprocal plots were nearly parallel at

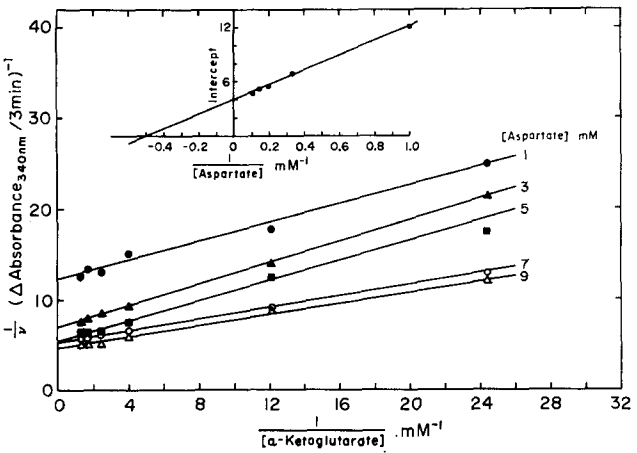

Fig. 2. Lineweaver–Burk plot of beef brain cytoplasmic aspartate aminotransferase. α-Ketoglutarate concentrations were varied from 0.041 mM to 0.75 mM. Reaction mixtures contained 67 mM Tris–HCl (pH 7.4), 0.1 mM pyridoxal phosphate, 0.236 mM NADH, 25 μg malate dehydrogenase, 3 μg aspartate aminotransferase and L-aspartate as indicated, in a total volume of 3 ml. L-Aspartate at 1 mM (●); 3 mM (▲); 5 mM (■); 7 mM (○); and 9 mM (△). The inset is a plot of the intercept values *vs* the reciprocal of aspartate concentrations.

low concentrations of the fixed substrate (Fig. 2). The intercept values from the ordinate were replotted against the reciprocal of the concentration of the nonvaried substrate (inset graph of Fig. 2). The replots were linear in both cases and were used to calculate the apparent K_m for α-ketoglutarate and L-aspartate. The apparent K_m values obtained were 2.0 mM for L-aspartate (Fig. 2) and 0.17 mM for α-ketoglutarate.

It has been shown previously that beef heart aspartate aminotransferase is inhibited by high concentrations of α-ketoglutarate, and that this inhibition is most

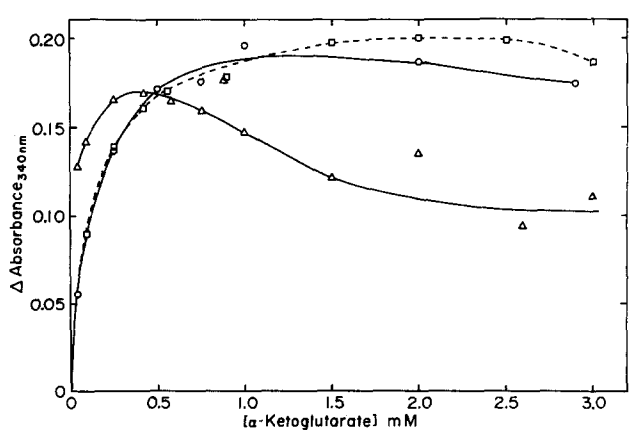

Fig. 3. Inhibition of beef brain cytoplasmic aspartate aminotransferase by excess α-ketoglutarate. α-Ketoglutarate concentrations were varied from 0.041 mM to 3 mM. Reaction mixtures contained 9 mM L-aspartate, 0.1 mM pyridoxal phosphate, 0.236 mM NADH, 25 μg malate dehydrogenase, 3 μg aspartate aminotransferase, and buffer as indicated, in a total volume of 3 ml. The buffer systems used were 60 mM cacodylate, pH 6.0 (△); 67 mM Tris–HCl, pH 7.4 (○); and 67 mM Tris–HCl, pH 8.0 (□).

noticeable below pH 8.0 (ref. 9). It was of interest, therefore, to study the effect of α-ketoglutarate on the reaction catalyzed by brain aspartate aminotransferase. The reactions were followed spectrophotometrically at 340 nm, and were conducted in 60 mM cacodylate (pH 6.0), 67 mM Tris–HCl (pH 7.4), and 67 mM Tris–HCl (pH 8.0). L-Aspartate was maintained at 9 mM, and α-ketoglutarate concentrations were varied from 0.041 mM to 3 mM.

Fig. 3 shows that aspartate aminotransferase activity is not inhibited at pH 8.0 by high concentrations of α-ketoglutarate, may be somewhat inhibited (5–10%) at pH 7.4 with 3 mM α-ketoglutarate, and is significantly inhibited by concentrations greater than 0.4 mM α-ketoglutarate in cacodylate (pH 6.0). Wada and Morino[9] reported approximately 80% inhibition of beef liver cytoplasmic aspartate aminotransferase by 3 mM α-ketoglutarate in arsenate buffer (pH 6.0). Beef liver aspartate aminotransferase also showed 25% inhibition by 3 mM α-ketoglutarate at pH 8.0, whereas no inhibition occurred with the beef brain enzyme at the same pH.

Possible inhibition of beef brain aspartate aminotransferase by γ-aminobutyrate was determined at pH 7.4 using 5.0 mM L-aspartate and 6.7 mM α-ketoglutarate and also using 2 mM L-aspartate and 1 mM α-ketoglutarate. At both substrate concentrations, no significant inhibition of enzyme activity was observed with γ-aminobutyrate concentrations up to 71 mM.

Physical properties

The spectra of beef brain and beef heart cytoplasmic aspartate aminotransferase in 50 mM acetate (pH 5.4) were recorded on a Cary Model 14 recording spectrophotometer at 25 °C. Fig. 4 shows that beef brain aspartate aminotransferase absorbs maximally between 430 and 440 nm, and the beef heart enzyme absorbs maximally at 440 nm. The 440-nm absorbance maximum differs somewhat from the 430-nm peak reported for pig heart α-aspartate aminotransferase[11]. However, the ratio of $A_{430\text{ nm}}/A_{340\text{ nm}}$ of 3.4 calculated for beef heart aspartate aminotransferase agrees with that of 3.7 reported previously for the α-subform of the pig heart enzyme[24].

Polyacrylamide disc gel electrophoresis was used to investigate the number

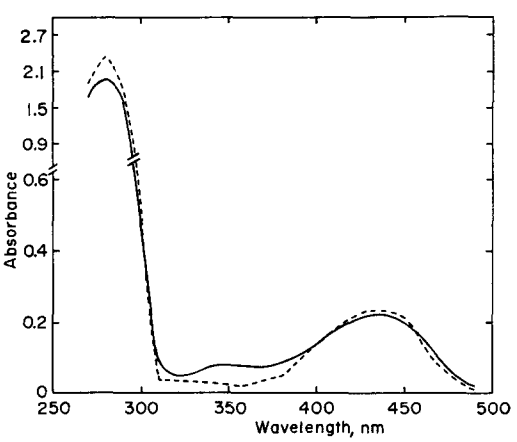

Fig. 4. Absorbance spectra of beef heart (———) and beef brain brain (– – –) cytoplasmic aspartate aminotransferase in 50 mM acetate (pH 5.4).

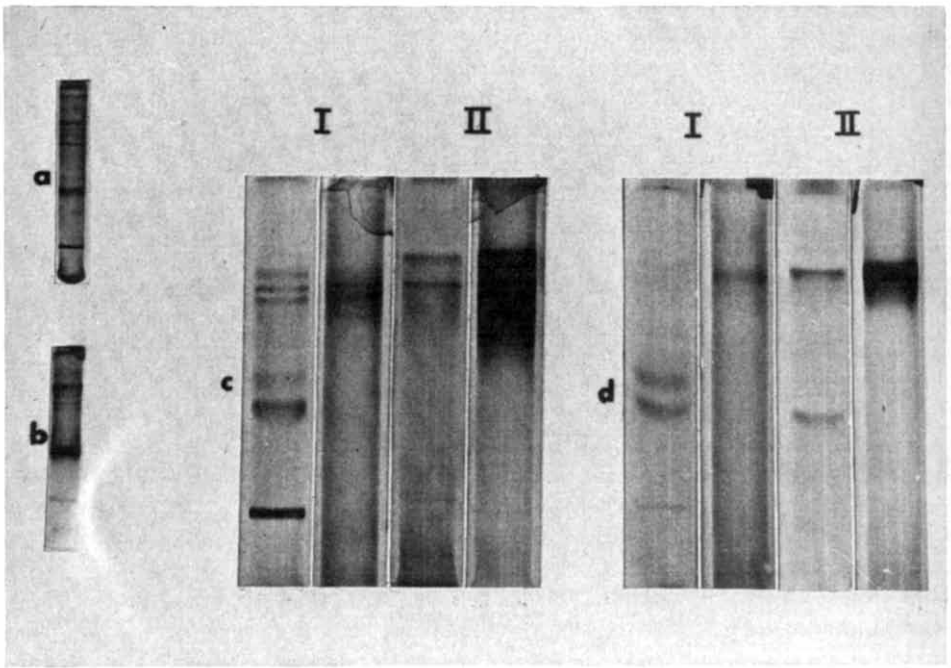

Fig. 5. Polyacrylamide disc gel electrophoresis of brain and heart cytoplasmic aspartate aminotransferase on 7% gels at pH 9.0. Gels in a and b are stained with coomassie blue. Gels in c and d are stained alternately with coomassie blue (1st and 3rd tubes) and fast violet B salt (2nd and 4th tubes). (a) 200 μg of beef brain aspartate aminotransferase before purification on Sephadex CM-50. (b) 200 μg of beef heart enzyme before purification on Sephadex CM-50. (c) 10 μg (Peak I) and 5 μg (Peak II) of beef brain cytoplasmic aspartate aminotransferase eluted from Sephadex CM-50. (d) 10 μg (Peak I) and 5 μg (Peak II) of beef heart enzyme eluted from Sephadex CM-50.

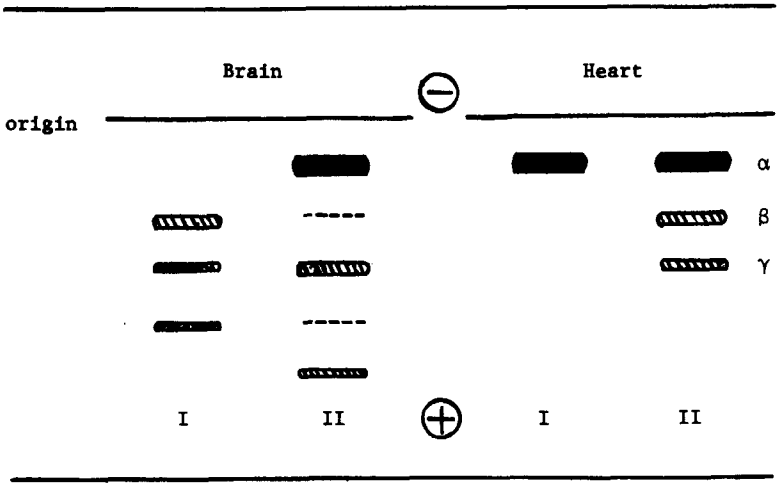

Fig. 6. Representation of multiple forms of brain and heart aspartate aminotransferase obtained on polyacrylamide disc gel electrophoresis.

and types of subforms of beef brain and heart aspartate aminotransferase present in Peaks I and II obtained from the Sephadex CM-50 column and compare them to the electrophoretic patterns for pig heart aspartate aminotransferase[11].

Fig. 5c shows a comparison of Peaks I and II of the brain aspartate aminotransferase preparation. Peak I appears to contain none of the α subform, but does contain faster-moving subforms (Fig. 6). The most dense band in Peak I seems to correspond to the middle band, perhaps γ, in Peak II of brain aspartate aminotransferase. The α-band (top band) of brain Peak II (Fig. 5c) aligns closely with the first dark band below the stacking gel in the pattern obtained with unpurified beef brain homogenate (Fig. 5a). All of the other aspartate aminotransferase subforms observed in Peaks I and II of the brain (Fig. 5c) also have corresponding bands in the electrophoresis of the unpurified brain homogenate (Fig. 5a). Peak II (Fig. 5c) appears to contain only aspartate aminotransferase.

Fig. 5d shows that the α-form is the major form of aspartate aminotransferase present in both Peaks I and II of the beef heart preparation. Referring to Fig. 5b, the uppermost dense band is probably the α-form in the unpurified heart preparation.

The molecular weight of brain aspartate aminotransferase was estimated by chromatography on a Sephadex G-200 column (1.5 cm × 43 cm), equilibrated in 40 mM sodium phosphate buffer (pH 8.0) with 5 mM EDTA. Samples were applied to the column in the same buffer system with 0.1% dextrose added. Void volume of the G-200 column was determined from the elution volume of blue dextran (2 mg/ml).

TABLE II

PARAMETERS OBTAINED BY GEL FILTRATION ON SEPHADEX G-200

Sample	*Elution volume (ml)*	*Calculated K_d**	*Stokes radius (Å)*	*Molecular weight*
Blue dextran	27	—	—	—
Ovalbumin	53	0.557	28**	45.000
Serum albumin (bovine)	48	0.451	35***	67.000
Beef brain aspartate aminotransferase	46	0.407	39.5†	103.000
Alcohol dehydrogenase (yeast)	43	0.332	46***	150.000

* Values for K_d were calculated according to the method of Siegel and Monty[25].
** Ackers[26].
*** Siegel and Monty[25].
† Value obtained from Sephadex G-200 data plotted as Stokes radius (Å) *vs* $(K_d)^{\frac{1}{3}}$ in this paper.

After the column was calibrated with bovine serum albumin (1 mg/ml), yeast alcohol dehydrogenase (3 mg/ml), and egg ovalbumin (1 mg/ml), beef brain aspartate aminotransferase (1 mg/ml) was applied. A linear relationship was obtained for the elution volumes of the standard proteins (Table II) and log molecular weights. A mol. wt of approximately 96 000 was determined from the elution volume for brain aspartate aminotransferase. Fig. 7 shows the plot of $(K_d)^{1/3}$, a distribution coefficient[25,27], *vs* the Stokes radius of the standard proteins. The Stokes radius for beef brain cytoplasmic aspartate aminotransferase was approximately 39.5 Å. A mol. wt of approxi-

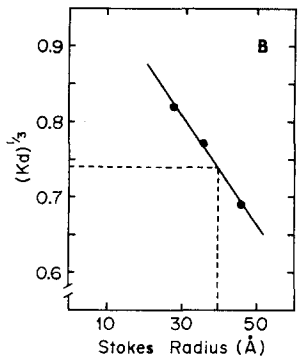

Fig. 7. Plot of $K_d^{\frac{1}{3}}$ obtained from chromatography on Sephadex G-200 *vs* Stokes radius of ovalbumin, bovine serum albumin, and yeast alcohol dehydrogenase. The $K_d^{\frac{1}{3}}$ and resulting Stokes radius of beef brain aspartate aminotransferase is indicated (— — —).

mately 103 000 was calculated for beef brain aspartate aminotransferase from its Stokes radius and sedimentation coefficient of 5.62 (see Table III).

Sedimentation velocity coefficients ($s_{20,w}$ values) were determined for cytoplasmic aspartate aminotransferase from beef brain and beef heart. Fig. 8 reveals symmetrical schlieren patterns which indicate the presence of only one component in each of the aspartate aminotransferase preparations. Observed s values were converted to $s_{20,w}$ values using a $\bar{v}$ of 0.745[8]. As shown in Table III, an $s_{20,w}$ of 5.20 for beef heart cytoplasmic aspartate aminotransferase and an $s_{20,w}$ of 5.62 for the beef brain enzyme were calculated.

From the Archibald approach to equilibrium method, average mol. wts of 90 000 ± 6000 for beef brain cytoplasmic aspartate aminotransferase and 93 000 ± 8000 for the beef heart enzyme were determined (Table III).

DISCUSSION

Beef brain and beef heart aspartate aminotransferase, purified by almost identical procedures, showed very similar properties during initial purification. The

TABLE III

SEDIMENTATION VALUES ($s_{20,w}$) AND MOLECULAR WEIGHTS OF CYTOPLASMIC ASPARTATE AMINOTRANSFERASE

Source	$s_{20,w}$ *(S)*	*Molecular weight* × 10^{-3}	*References*
Beef liver and beef heart (crystalline form)	5.40	120	Wada and Morino[9]
Pig heart	5.50*	110 ± 3**	Jenkins *et al.*[8]
	5.70	≤90	Martinez-Carrion *et al.*[30]
Rat brain	5.40*	79 ± 3	Magee and Phillips[10]
Beef heart	5.20	93 ± 8***	
Beef brain	5.62	90 ± 6***	

* Value determined after extrapolation of data to zero concentration.
** Molecular weights determined by the Archibald approach to equilibrium.
*** Molecular weights determined in this laboratory by the Archibald approach to equilibrium.

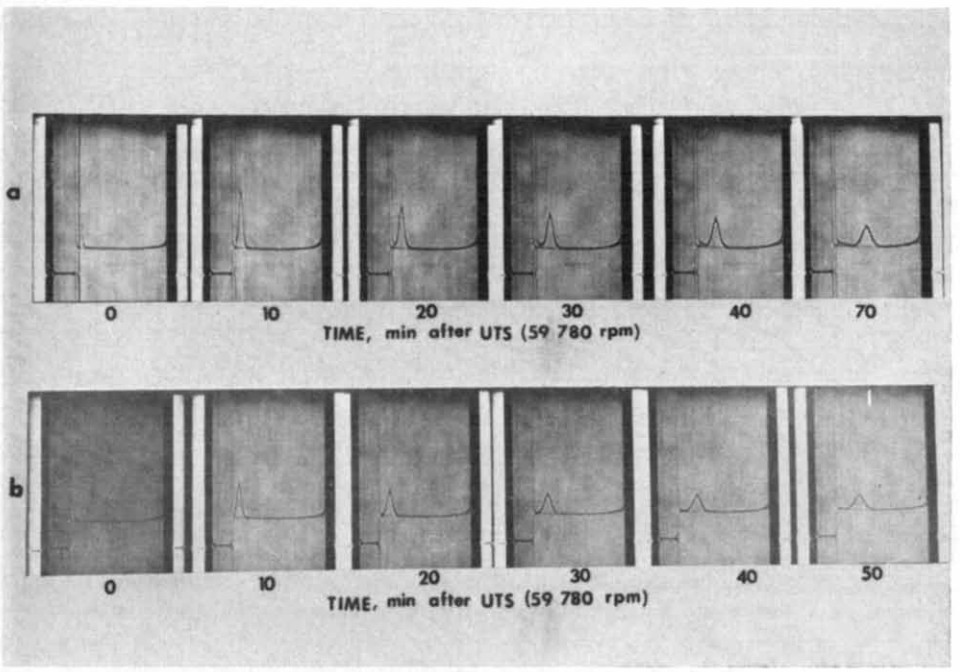

Fig. 8. Sedimentation velocity patterns of beef heart and beef brain cytoplasmic aspartate aminotransferase. Both samples were photographed at a 60° schlieren analyzer angle after reaching 59 780 rev./min. Runs were performed at 5 °C. (a) Beef brain aspartate aminotransferase at 7.5 mg/ml in 126 mM sodium acetate (pH 5.4). (b) Beef heart enzyme at 7.5 mg/ml in 50 mM sodium acetate (pH 5.4). The buffers also contained 0.1 mM EDTA, 0.1 mM pyridoxal phosphate, and 2.5 mM α-ketoglutarate.

only apparent difference between the two tissues was that heart aspartate aminotransferase precipitated at lower $(NH_4)_2SO_4$ concentrations than those needed for the brain enzyme.

During purification on Sephadex CM-50 with sodium acetate at pH 5.4, beef brain cytoplasmic aspartate aminotransferase separated into two peaks. The first peak contained more anionic subforms of aspartate aminotransferase (probably β, γ, and others), while the second, more active peak contained more of the α subform. Malate dehydrogenase has been reported to be copurified with cytoplasmic aspartate aminotransferase from pig heart[28]. Similarly, malate dehydrogenase was eluted from the CM-Sephadex columns with the first peak or the cytoplasmic β and γ subforms of aspartate aminotransferase of beef brain and beef heart.

The existence of multiple forms of aspartate aminotransferase from beef brain and beef heart was shown further by spectral analysis and disc gel electrophoresis. Spectrally, the enzymes purified from beef heart and brain absorbed maximally between 430–440 nm. The identical absorbance maximum for cytoplasmic α-aspartate aminotransferase of pig heart was attributed to the protonated Schiff base formed with pyridoxal phosphate and a lysine residue of the apoenzyme[11]. The small shoulder at 340 nm observed with the beef brain and beef heart enzymes is probably absorption by γ-aspartate aminotransferase. Marino *et al.*[29] proposed that pig heart cytoplasmic

γ-aspartate aminotransferase, which absorbs at 340 nm, contains pyridoxal phosphate bound to the apoenzyme through a substituted aldimine bond.

The relative amounts of α, β, and γ subforms of aspartate aminotransferase and their behavior upon disc gel appear to differ in the brain and heart preparations. After electrophoresis of beef heart aspartate aminotransferase, gels stained in fast violet B salt revealed one intensely stained band produced by α-aspartate aminotransferase activity (Fig. 5d). Minute amounts of β and γ subforms are indicated also in heart Peak II. Since only trace amounts of β- and γ-aspartate aminotransferase are present, they may be formed as breakdown products of the α subform. This observation supports the proposal of Martinez-Carrion *et al.*[30] that one gene codes for only one parent isozyme, *i.e.*, α-aspartate aminotransferase. Furthermore, it has been shown that the more anionic forms (β, γ, δ) can be generated *in vitro* from α-aspartate aminotransferase[29]. Although these multiple forms of cytoplasmic aspartate aminotransferase have an identical molecular weight, amino acid composition, and peptide map[30], they differ in isoelectric points[29].

Disc gel electrophoresis of the beef brain enzyme indicated no α-aspartate aminotransferase activity in Peak I. Only more anionic subforms, possibly β and γ, were present. Brain Peak II appeared to contain mostly α-aspartate aminotransferase. However, significant amounts of two subforms with greater anionic character were also present. One of these migrated the same as the γ subform of the heart aspartate aminotransferase preparation, and the other migrated faster than γ.

Magee and Phillips[10] observed the presence of three subforms of beef heart cytoplasmic aspartate aminotransferase and one form of the rat brain enzyme after both enzymes were subjected to column chromatography. As the rat brain aspartate aminotransferase preparation aged, an additional, faster migrating subform appeared. This subform was thought to be an altered state of the protein which occurred as the enzyme was modified to less enzymatically active forms. Thus, Magee and Phillips[10] reported that beef heart aspartate aminotransferase appeared to degenerate more rapidly than the rat brain enzyme. In contrast, we observed more subforms of beef brain than of beef heart cytoplasmic aspartate aminotransferase in the preparations after electrophoresis. This may be due to a more rapid *in vivo* rate of α-aspartate aminotransferase turnover in the brain or to a greater instability of the brain enzyme during purification. However, both unpurified brain and unpurified heart aspartate aminotransferase electrophoretically separated into what appeared to be several active subforms.

Gel filtration on Sephadex G-200 yielded a mol. wt of 103 000 for beef brain cytoplasmic aspartate aminotransferase which agreed with the mol. wts of 90 000–100 000 reported for the pig heart enzyme also determined by gel filtration[30–32].

The $s_{20,w}$ value calculated for beef heart aspartate aminotransferase is slightly lower than that obtained for the beef brain enzyme; however, both are similar to the previously reported sedimentation coefficients given in Table III. Molecular weights determined by ultracentrifugation for aspartate aminotransferase purified from both tissues are identical and fall within the molecular weight range noted for the pig heart enzyme. Thus, the Sephadex G-200 gel filtration and ultracentrifugation studies indicate that the beef brain and beef heart cytoplasmic aspartate aminotransferases have the same shape and molecular weight. Using the molecular weights and sedimentation coefficients determined by ultracentrifugation, a frictional ratio (f/f_0) of

1.38 for beef brain aspartate aminotransferase and an f/f_0 of 1.35 for the beef heart enzyme were determined. An f/f_0 of 1.4 was calculated for pig heart aspartate aminotransferase[8].

Possible substrate inhibition of beef brain aspartate aminotransferase activity was noted at high concentrations of L-aspartate. Nevertheless, the apparent Michaelis constant of beef brain cytoplasmic aspartate aminotransferase for L-aspartate is identical to the values (2–2.5 mM) reported for the beef heart and liver enzymes[9]. As with cytoplasmic aspartate aminotransferase isolated from other mammalian sources[9,10,33], the apparent Michaelis constant of the brain enzyme for α-ketoglutarate was less than that for L-aspartate. The parallel lines obtained in the Lineweaver–Burk plots for transaminase activity in the presence of varying low concentrations of L-aspartate or α-ketoglutarate are consistent with a Ping Pong reaction mechanism.

Pig heart, beef liver, and beef heart aspartate aminotransferases showed inhibition by high concentrations of α-ketoglutarate[9,33]. Beef brain activity was inhibited significantly only in pH 6.0 cacodylate, but not at pH 7.4 and 8.0 when excess α-ketoglutarate (1–3 mM) was added. Notable inhibition of beef liver aspartate aminotransferase activity was reported at pH 8.0 and 6.0, with greater inhibition at pH 6.0 (ref. 9). Because beef brain aspartate aminotransferase appears to (1) have a lower K_m for α-ketoglutarate (that reported for the beef liver and heart enzymes is 0.3–0.4 mM (ref. 9)) and (2) show less substrate inhibition by α-ketoglutarate than does the beef liver or heart enzyme, the formation of glutamate by transamination of α-ketoglutarate may be more favorable in the brain than in other tissues. These results may reflect a greater metabolic need for glutamate by the brain.

With the exception of the differences in subform distribution and possibly the difference in substrate inhibition by α-ketoglutarate, the results presented in this paper indicate no difference between beef heart and brain aspartate aminotransferase. Wada and Marino[9] had previously shown that antiserum to crude beef liver cytoplasmic aspartate aminotransferase inhibited a crude homogenate of the beef brain enzyme up to 80%. While this work was in progress, Shrawder and Martinez-Carrion[34] reported on substrate specificity and affinity, inhibition, and immunological studies that indicated that cytoplasmic pig heart aspartate aminotransferase was identical to cytoplasmic pig brain phenylalanine transaminase, or, as they suggest, aspartate aminotransferase.

Some means of control of glutamate production in the brain might be expected since glutamate is a neurotransmitter, while its decarboxylation product, γ-aminobutyrate, is a potent inhibitory neurotransmitter. If aspartate aminotransferase were a control point for glutamate production in the brain, the type of control is apparently not allosteric. The Lineweaver–Burk plots for the substrates, α-ketoglutarate and L-aspartate, were linear. Furthermore, γ-aminobutyrate does not appear to be a feedback inhibitor of beef brain aspartate aminotransferase. The existence of multiple subforms of brain aspartate aminotransferase may reflect a means of control of activity. Since the brain enzyme appears to have a greater number and a greater concentration of the more anionic, or less active subforms, than does the heart enzyme, brain aspartate aminotransferase may have a more rapid turnover rate or destruction rate. Another means of control of aspartate aminotransferase activity would be genetic, since transaminases have been demonstrated to be regulated at the genetic level in mammalian systems[35]. Moreover, in chick embryo[36] and mouse[37],

there is evidence that glutamate decarboxylase, the enzyme which catalyzes the formation of γ-aminobutyrate from glutamate, can be reduced in amount by artificially elevated levels of γ-aminobutyrate.

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