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PURIFICATION AND PROPERTIES OF 4-AMINOBUTYRATE 2-KETOGLUTARATE AMINOTRANSFERASE FROM PIG LIVER

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Summary

4-Aminobutyrate-transaminase (4-aminobutyrate : 2-oxoglutarate aminotransferase, EC 2.6.1.19) from pig liver has been purified to electrophoretic homogeneity. It has a molecular weight of about 110 000 and is composed of two subunits of the same molecular weight but of different charges. Two forms of pig liver 4-aminobutyrate-transaminase were isolated by DEAE-cellulose chromatography and designated as 4-aminobutyrate-transaminase I and 4-aminobutyrate-transaminase II, corresponding to a cationic and anionic form. Some physical and kinetic properties of liver enzyme were compared to those of brain enzyme and no significant differences were found, except for their sedimentation coefficients and the charges of their subunits. The role of 4-aminobutyrate-transaminase in liver remains a matter of speculation, but could be related to a metabolic function.

Introduction

It is now well established that 4-aminobutyrate is a major inhibitory transmitter in many invertebrate systems and in the vertebrate central nervous system [1—3]. Since the discovery of 4-aminobutyrate as a constituent of rodent brain [4], much work has been published about 4-aminobutyrate and the two enzymes directly involved in the metabolism of 4-aminobutyrate, namely, L-glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) and 4-aminobutyrate : 2-oxoglutarate aminotransferase (EC 2.6.1.19). Most of this work has been done on nervous tissues. However, 4-aminobutyrate, glutamate decarboxy-lase, and 4-aminobutyrate-transaminase are present in non-neuronal tissues of man and other mammals [5—10], where they may have a metabolic function; an outline of this metabolism is shown in Fig. 1. The 4-aminobutyrate shunt constitutes an alternate pathway to the portion of the tricarboxylic acid cycle that leads from 2-ketoglutarate to succinate. In mammalian kidney,

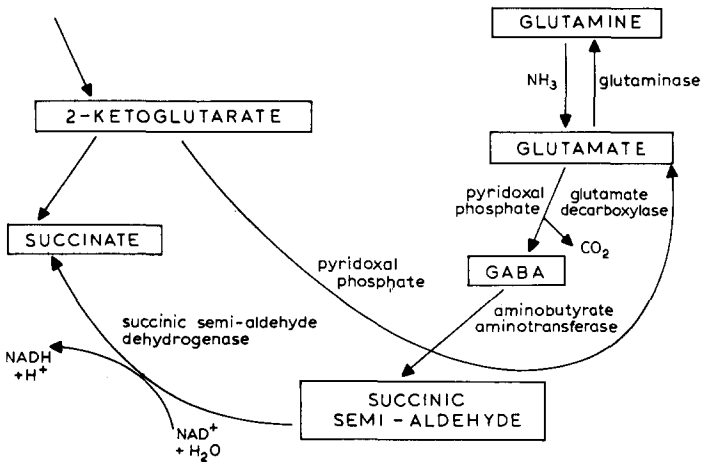

Fig. 1. The pathway of synthesis and degradation of 4-aminobutyrate, and its relation to the tricarboxylic acid cycle.

the 4-aminobutyrate pathway represents an efficient route that eliminates glutamate during glutamine-dependent ammoniagenesis [7—8]. There is also good evidence that 4-aminobutyrate, glutamate decarboxylase and 4-aminobutyrate-transaminase occur in mammalian liver, although at much lower concentrations than in nervous tissues [11] (6 and 3 units/mg protein, respectively, for pig brain and liver 4-aminobutyrate-transaminase). It seems of interest to carry out studies on hepatic 4-aminobutyrate-transaminase in order to establish whether brain and hepatic enzymes are related, to compare their properties, and to try to elucidate the function of the 4-aminobutyrate shunt in hepatic tissue.

Materials and Methods

Materials. The following reagent grade chemicals and proteins were purchased from Sigma Chemical Co.: Pyridoxal phosphate, 2-ketoglutaric acid, 4-aminobutyric acid, dithiothreitol, bovine serum albumin, cytochrome *c*, pig-heart soluble aspartate aminotransferase and yeast alcohol dehydrogenase. Glutamate decarboxylase from *Escherichia coli* was purchased from Fluka. L-Tryptophane was obtained from BDH Chemicals Ltd. L-Histidine and L-glutamic acid were purchased from Nutritional Biochemicals Corp. Other amino acids, EDTA and $(NH_4)_2SO_4$ were obtained from Merck. Hydroxyapatite was obtained from Bio-Rad Lab., CM- and DEAE-cellulose, from Whatman, Sephadex G 100 and Dextran blue from Pharmacia Fine Chemicals. γ-Vinyl 4-aminobutyrate (RMI 71-754-07) and γ-acetyl 4-aminobutyrate (RMI 71-645-14) were donated by Merrell Laboratories (Strasbourg), β-(4-chlorophenyl)-4-aminobutyric acid (Lioresal) by Ciba-Geigy Laboratories and 2-oxo-1-pyrrolidine acetamide (cyclo 4-aminobutyrate) (Piracetam) by UCB. All other chemicals were of analytical grade. [1-^{14}C]2-Ketoglutarate (specific activity: 64 μCi/μmol) was obtained from New England Nuclear.

Protein determination. Protein was assayed by the method of Lowry et al.

[12], using bovine serum albumin as the standard, or by measuring the ultraviolet absorbance at 280 nm.

Enzyme assays. Two method were used to measure the aminotransferase activity. The first one was a radioisotopic assay which has been developed in our laboratory [13]. In the first step, [1-^{14}C]2-ketoglutarate and unlabeled 4-aminobutyrate were used as the substrates; in the second step, glutamate decarboxylase, commercially available, from *E. coli*, was added to decarboxylate [1-^{14}C]glutamate formed by the transaminase reaction. The rate of $^{14}CO_2$ evolved corresponded to the rate of glutamate formed during the 4-aminobutyrate transamination, i.e., to the enzyme activity. Radioactivity was counted by liquid scintillation. A second method, less sensitive than the radioisotopic assay, was also used as a standard assay method in purification procedures in order to identify enzyme activity in the column eluates. It consists in a spectrophotometric assay, developed by Sytinsky et al. [14] who measured the quantity of succinic semi-aldehyde formed during the transamination through the formation of a colorimetric complex which absorbed at 660 nm. The enzyme activity was expressed as U/ml of enzyme solution and specific activity as U/mg of protein. 1 U represents the activity catalysing the formation of 1 μmol of glutamate per min at 37°C. Unless otherwise stated, the enzyme assays were carried out at 37°C.

Determination of the sedimentation coefficient. Sedimentation velocity analyses were performed in a Beckman Spinco Model L I preparative centrifuge, using an SW 50 L Rotor, for 8 h at 45 000 rev./min and 4°C. The sedimentation velocity coefficient ($s_{20,w}$ value) was evaluated by density gradient centrifugation of three marker proteins: Cytochrome *c* [15], $s_{20,w}$ = 1.9 S; pig-heart soluble aspartate aminotransferase (EC 2.6.1.1) [16], $s_{20,w}$ = 5.5 S; yeast alcohol dehydrogenase (EC 1.1.1.1) [17] $s_{20,w}$ = 7.2 S. A 0.2 ml sample of each preparation containing 60—80 μg protein was added in identical sucrose density gradients (5—15% in 0.01 M phosphate buffer, pH 6.8), containing 10^{-5} M pyridoxal phosphate and dithiothreitol. After centrifugation, 4 drop fractions were collected and both enzyme activity and protein concentration were determined.

Sephadex gel filtration for molecular weight determination. Molecular weight was estimated by gel filtration on a Sephadex G 100 column (2.5 × 95 cm); the column was equilibrated with 0.1 M phosphate acetate buffer (pH 6.8) containing 10^{-5} M dithiothreitol and pyridoxal phosphate at 4°C. Elution was carried out with the equilibration buffer at a flow rate of 11 ml/h, collected in fractions of approx. 2 ml. The column was calibrated with cytochrome c, yeast alcohol dehydrogenase and egg ovalbumin. Dextran blue was used to determine the void volume (V_o) of the column.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed according to the original method of Davis [18]. The sample was applied on a 7% polyacrylamide separating gel column (1.3 × 8 cm, pH 8.9) with a 3% concentrating gel (1.3 × 0.5 cm, pH 6.7). Ammonium persulfate was used as the polymerizing agent. A Tris-glycine buffer (0.6 g of Tris and 0.58 g of glycine per l, pH 8.3) was used as the electrophoresis buffer. Electrophoresis was carried out at 4°C for 3 h using a current of 10 mA/column. The samples consisted of 10—40 μg of enzyme in Tris-glycine buffer, pH 8.3, containing

one drop of Bromphenol Blue to mark the front and two drops of glycerol. Each protein sample was run in duplicate. For protein staining, one gel was placed in 0.12% Coomassie Blue R-250 in 30% acetic acid overnight and then destained in 10% acetic acid. For enzyme assay, immediately after electrophoresis, the second gel was cut into 5-mm slices; the slices were homogenised individually in 1 ml of 0.2 M Tris · HCl buffer, pH 8.8, containing substrates and cofactors as described in isotopic assay, agitated for 2 h, frozen overnight and then tested for their activity.

Polyacrylamide SDS- and urea- disc-gel electrophoresis. Polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (SDS) and urea was carried out according to the procedure of Weber and Osborn [19]. An aliquot of 100 μl enzyme solution was incubated either 1 h at 45°C in 0.1 M sodium phosphate (pH 7.2) containing urea 4 M and 0.1% β-mercaptoethanol, or for 15 min at 60°C in the same buffer containing 1% SDS and 0.1% β-mercaptoethanol. One drop of neutral glycerol and a trace of Bromophenol Blue were added to the sample. The sample was layered on to a 5% polyacrylamide gel column (0.5 × 10 cm) in a 0.1 M sodium phosphate buffer (pH 7.2). Electrophoresis was carried out at room temperature with a constant current of 6 mA/tube for 4 h. Bovine serum albumin, egg ovalbumin and cytochrome *c* were used as standards. After electrophoresis the gels were stained with Coomassie Blue G-250 in 5% trichloroacetic acid overnight and destained with 7% acetic acid.

Isoelectric focusing on polyacrylamide gel. Electrofocusing in the pH range 5—7 was performed using LKB Ampholines with a M 137-A analytical unit (Medical Research Apparatus). The technique of Righetti et al. [20] was utilized with some modifications. The pH gradient was established in about 15 min. A 200 μl enzyme aliquot was mixed with 20 μl of 2% Ampholine solution and one drop of 10% glycerol. A 100 μl sample of this mixture was layered at the top each column. A current intensity of 1 mA/column (500 V) was applied for 5 h. The gels were cut into 2-mm slices and eluted by agitation with 0.5 ml of 0.9% NaCl for 1 h at 4°C. The fractions were then tested for pH and enzymatic activity.

Determination of pH optimum. 0.2 M Tris · HCl buffers (pH 7—9) containing 25 mM of 4-aminobutyrate, 10 mM of 2-ketoglutarate and the usual protectors were used. The purified enzyme preparation was mixed with the buffer and the enzymatic activity was determined by the radioisotopic assay.

Substrate specificity. The specificity of the enzyme toward various amino acids as substrates was studied using the radioisotopic assay; amino acids were assayed at three different concentrations (10, 25, 50 mM).

Purification

The purification procedure was very similar to that used previously for pig-brain 4-aminobutyrate transaminase, with slight modifications [21]. All steps were performed at 4°C unless specified otherwise. After each step, samples were taken for determination of protein concentration and enzyme activity. The preparations were protected from light.

Step I: Extraction. Pig livers were obtained from local slaughter houses and immediately treated. After removal of connective tissue, blood vessels and fat,

a 150 g fraction of liver was minced and homogenized (Waring Blendor, slow speed, 30 s) in 450 ml of 0.01 M phosphate acetate buffer containing 10^{-3} M EDTA, pyridoxal phosphate, dithiothreitol and 2-ketoglutarate. The solution was centrifuged at 15 000 × *g* for 30 min in an MSE SP 50 centrifuge.

Step II: Heat precipitation. The supernatant was adjusted to pH 5.2 by addition of 10 ml of 0.5 M propionic acid and an appropriate volume of 0.5 M 2-ketoglutaric acid. The solution was placed in a 56° C water bath and brought up to 56° C within 10 min. The protein solution was removed immediately, cooled to 4° C and its pH brought to 6.8. Heat-denatured proteins were removed by centrifugation at 17 500 × *g* for 30 min.

Step III: Ammonium sulfate precipitation. The enzyme was precipitated with a buffered $(NH_4)_2SO_4$ solution between 55 and 80% saturation. The precipitate obtained after a centrifugation of 60 min at 17 000 × *g* was dissolved in a minimum of 0.1 M phosphate acetate buffer, pH 6.8, containing 10^{-4} M pyridoxal phosphate and dithiothreitol.

Step IV: Sephadex G 100 gel filtration. A Sephadex G 100 column (5 × 100 cm) was equilibrated with 0.1 M phosphate acetate buffer (pH 6.8) containing pyridoxal phosphate and dithiothreitol. The enzyme was dialysed overnight against the same buffer and applied to the column. Chromatography was performed in the same buffer with an elution rate of 20 ml · h^{-1}; the eluate was collected in 5-ml aliquots. The fractions showing enzyme activity were pooled.

Step V: CM-cellulose chromatography. The dialysed solution was applied to a CM-cellulose column (1.5 × 30 cm) equilibrated with 0.01 M acetate buffer (pH 5.4) containing 10^{-3} M EDTA. The enzyme was eluted with an 0.01—0.2 M

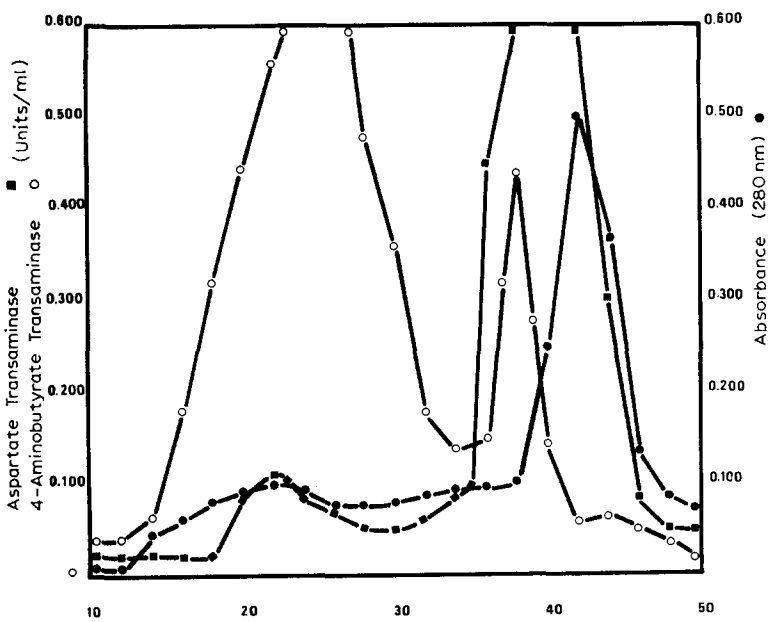

Fig. 2. DEAE-cellulose chromatograph of the CM-cellulose active fraction. Chromatography conditions are given in detail under Purification. The elution patterns for protein (●), 4-aminobutyrate-transaminase (○) and aspartate transaminase (■) are shown.

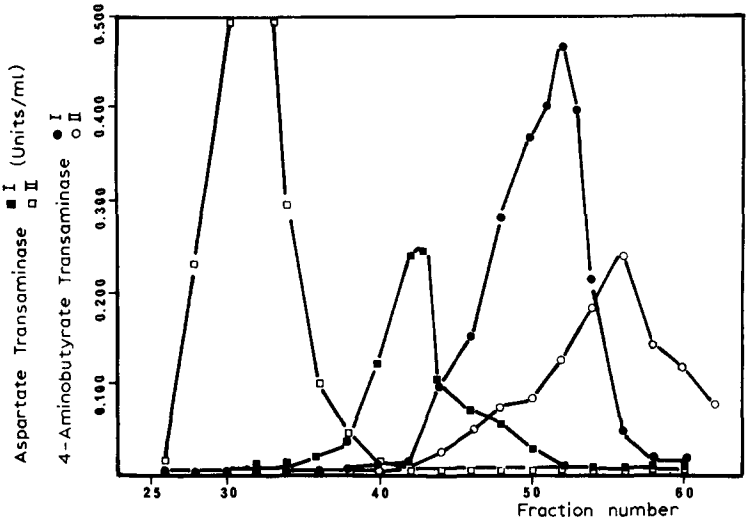

Fig. 3. Calcium phosphate gel chromatograph of DEAE-I and DEAE-II peak. Chromatography conditions are given in detail under Purification. The elution profile of 4-aminobutyrate-transaminase I (●), II (○) and aspartate transaminase associated to form I (■) and II (□) are shown.

sodium acetate linear gradient (pH 5.7). The flow rate was maintained at 20 ml · h^{-1} and the eluate collected in 2.3-ml fractions. The fractions containing 4-aminobutyrate-transaminase were combined and dialysed against the equilibration buffer of the next column.

Step VI: DEAE-cellulose chromatography. A column (1.5 × 30 cm) was packed with DEAE-cellulose and equilibrated with 10 mM Tris-maleate buffer (pH 7.2), containing 10^{-3} M EDTA. The dialysed sample was added on the column and eluted with a linear pH gradient of 200 ml of 0.09 M Tris-maleate, pH 7.2, in the mixing chamber and 200 ml of the same buffer, pH 5.2, in the reservoir. The elution profile showed two peaks of enzymatic activity; the first one was designated as 4-aminobutyrate-transaminase I and the second one as 4-aminobutyrate-transaminase II (Fig. 2).

Step VII: Calcium phosphate gel chromatography. Each dialysed enzyme solution was applied separately on a column of calcium phosphate, previously equilibrated with 0.01 M sodium phosphate buffer (pH 6.8) containing 10^{-5} M pyridoxal phosphate and dithiothreitol. The column was eluted with a linear gradient of 0.05 M to 0.25 M sodium phosphate buffer (pH 7.2). Aspartate aminotransferase was separated from the 4-aminobutyrate-transaminase by this procedure (Fig. 3).

Results

Enzyme purification. The purification procedure is summarized in Table I. A purification of 300- to 600-fold was obtained. The recovery of enzyme activity was about 7%. The specific activity was between 1.8 and 3.5 U/mg of protein. The preparation could be stored at 4°C for a few days.

Molecular weight. There was a linear correlation between the log of the

TABLE I

PURIFICATION OF 4-AMINOBUTYRATE-TRANSAMINASE FROM PIG LIVER

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Homogenate	89	15 400	0.006	100	1
Heat precipitation	83	4 350	0.019	92.4	3
Ammonium sulfate (55—80%)	48	1 140	0.042	54.1	7
Sephadex G-100	27	320	0.083	29.7	14
CM-Cellulose	12	79	0.16	13.8	27
DEAE-Cellulose (peak I)	10	20	0.54	12	90
DEAE-Cellulose (peak II)	1.3	2.35	0.57	1.5	95
Hydroxyapatite (peak I)	6	3.25	1.80	6.5	300
Hydroxyapatite (peak II)	0.6	1.75	3.33	0.65	555

molecular weights of the proteins and the ratio of V to V_o, where V_o was the void volume on the column and V was the elution volume of the individual proteins. The molecular weight of 4-aminobutyrate transaminase was estimated to be 110 000 ± 5000.

Homogeneity test and subunit molecular weight determination. Polyacrylamide gel electrophoresis showed a single protein band containing all the enzyme activity. Electrophoresis in the presence of SDS was carried out on the homogeneous enzyme and revealed a single band of protein. Estimation of molecular weight for this band gave a value of 55 000 ± 3000. Electrophoresis in the presence of urea showed two bands of protein. These results suggest that 4-aminobutyrate-transaminase was a dimer consisting of two subunits of identical molecular weight but of different charges. 4-aminobutyrate-transaminase I and II showed same profiles in the three conditions (Fig. 4).

Sedimentation coefficient. The sedimentation coefficient; $s_{20,w}$ was deter-

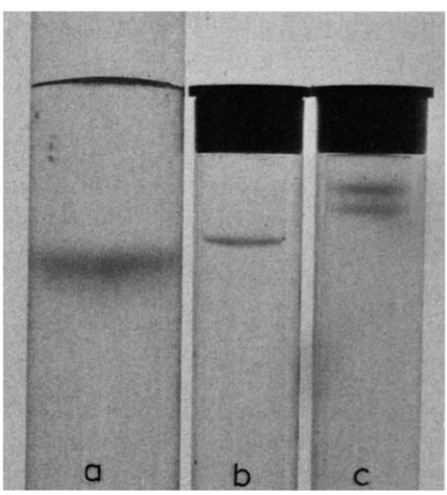

Fig. 4. Polyacrylamide gel electrophoresis of purified 4-aminobutyrate-transaminase: a, According to the method of Davis [18]; b, in the presence of 0.1% SDS and 0.1% β-mercaptoethanol.

mined by density gradient centrifugation. The sedimentation coefficient was 6.75 for 4-aminobutyrate-transaminase I and 6.85 for 4-aminobutyrate-transaminase II.

pH Optimum. When 4-aminobutyrate aminotransferase was assayed at 37°C in buffer solutions of various pH values, 4-aminobutyrate-transaminase I had maximum activity at pH 8.75 and 4-aminobutyrate-transaminase II at pH 8.65.

Determination of pH_i. Fig. 5 shows the pattern of electrofocusing; each form of 4-aminobutyrate-transaminase gave two active peaks. 4-aminobutyrate-transaminase I was found at pH 6.10 and 6.30, 4-aminobutyrate-transaminase II at pH 5.90 and 6.35.

Determination of K_m values. Apparent K_m values for the substrates 2-ketoglutarate and 4-aminobutyrate were determined either with varying concentrations of 2-ketoglutarate at several fixed concentrations of 4-aminobutyrate, or with varying concentrations of 4-aminobutyrate at fixed concentrations of 2-ketoglutarate. The experimental data were plotted as Lineweaver-Burk plots. The lines obtained in double reciprocal plots were nearly parallel at low concentrations of the fixed substrate. The series of parallel lines obtained indicated a ping-pong bi-bi-like mechanism for the enzyme. The reciprocal K_m values for substrate S1 obtained from these parallel lines were plotted against the reciprocal concentrations of S2 and vice-versa. At the intercepts of the ordinates, these straight lines gave the apparent reciprocal K_m value for one substrate at infinite concentration of the other substrate (Fig. 6a). The K_m value for 2-ketoglutaric acid was evaluated to be 0.70 mM and for 4-aminobutyric acid, 1.10 mM (Fig. 6b).

Inhibition by excess substrate. At high concentrations of 2-ketoglutaric acid, 4-aminobutyrate-transaminase activity was inhibited, whereas no inhibition

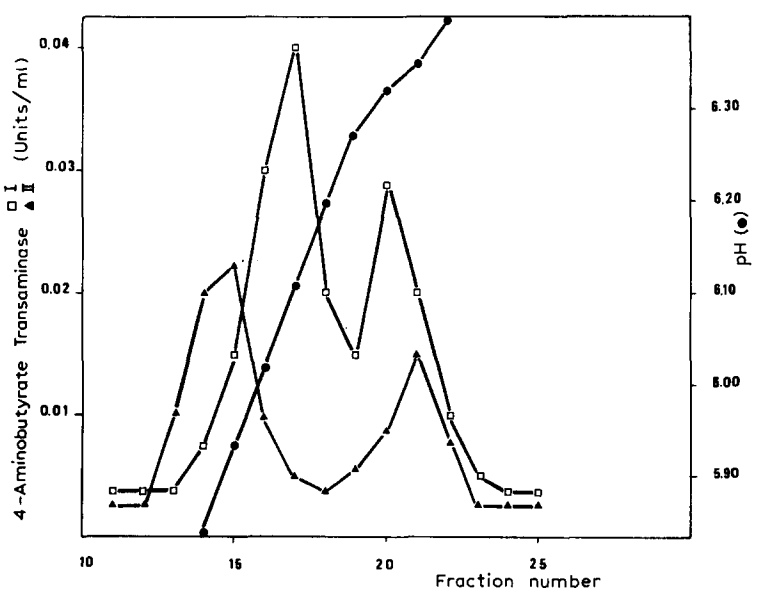

Fig. 5. Electrofocusing of the purified forms of the enzyme. The cathode is at the bottom. For details, see text. □, 4-Aminobutyrate-transaminase I; ▲, 4-aminobutyrate-transaminase II; ●, pH.

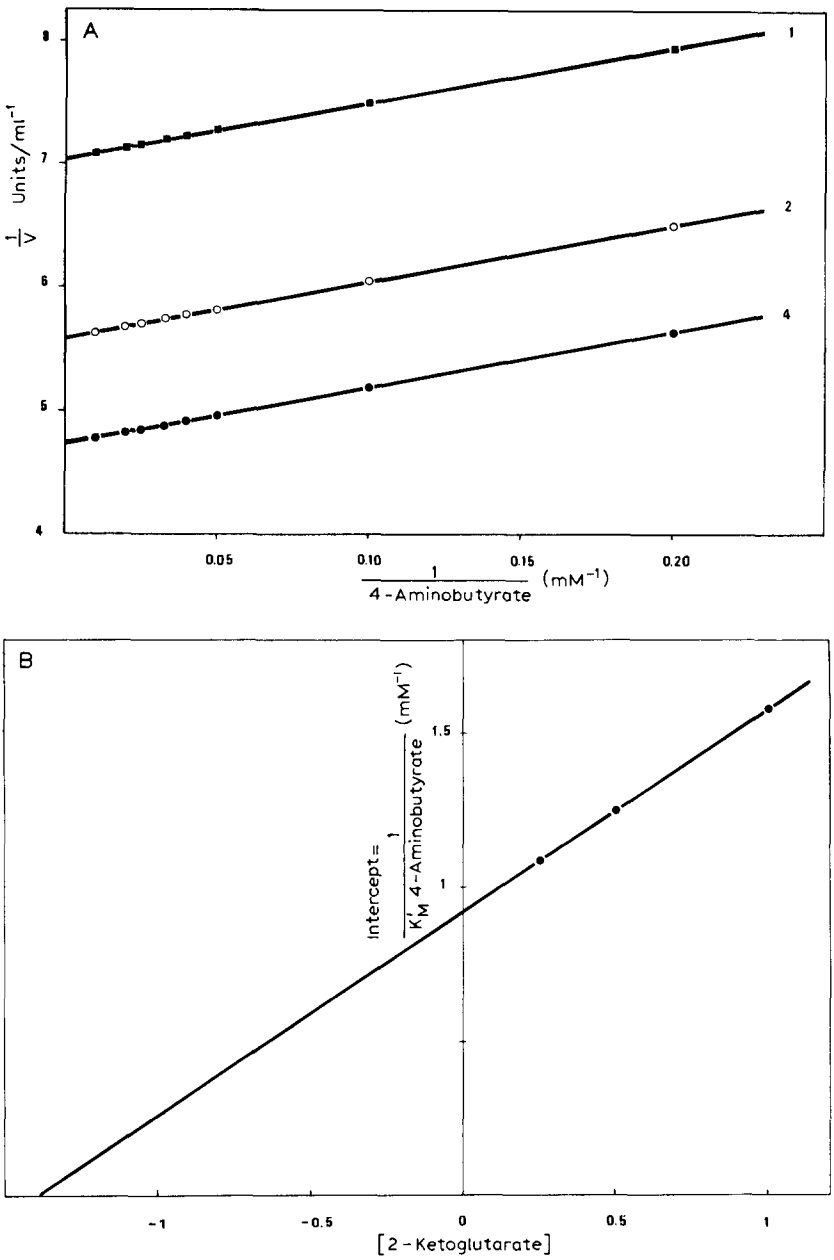

Fig. 6. a. Lineweaver-Burk plot of liver 4-aminobutyrate-transaminase I. 4-Aminobutyrate concentrations were varied from 5 mM to 100 mM. ■, 2-ketoglutarate at 1 mM; ○, at 2 mM; ●, at 4 mM. b. Secondary plot of intercepts on x axis (K_m/[4-aminobutyrate]) from a against the reciprocal of 2-ketoglutarte concentrations.

could be observed with 4-aminobutyrate concentrations up to 100 mM (Fig. 7).

Substrate specificity. With respect to the substrate specificity of 4-aminobutyrate-transaminase I, none of the following L-α-amino-acids gave any activity

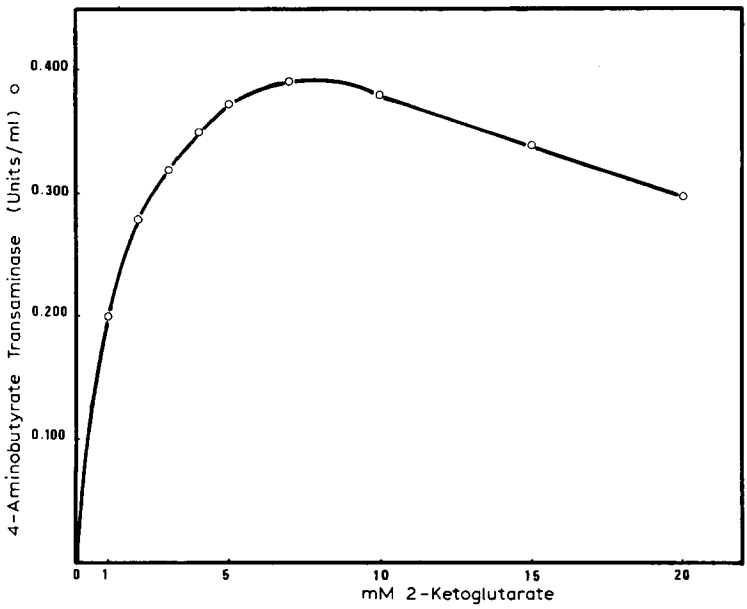

Fig. 7. Inhibition of pig-liver 4-aminobutyrate-transaminase by excess 2-ketoglutarate, 2-Ketoglutarate concentrations were varied from 1 mM to 25 mM. Reaction mixtures contained 25 mM 4-aminobutyrate and the usual protectors.

when substituted for 4-aminobutyric acid: Glycine, alanine, leucine, proline, histidine, arginine, threonine, methionine, tryptophane, phenylalanine, aspartic acid. Glutamic acid was the only L-α-amino-acid which was transaminated by the 4-aminobutyrate-transaminase I. Some amino-acids containing amino groups in other than the α position were tested (Table II). β-Alanine was transaminated to the same extent as 4-aminobutyrate. Among 4-aminobutyrate analogues, 5-amino-*n*-valeric acid was found to be a good substrate and β-aminoisobutyric a relatively good substrate; however, ϵ-aminocaproic and β-amino-isobutyric were found to be only moderately good substrates. Taurine, hypotaurine, γ-acetyl 4-aminobutyrate, γ-vinyl 4-aminobutyrate, β-(4 chloro-

TABLE II

SUBSTRATE SPECIFICITY OF 4-AMINOBUTYRATE-TRANSAMINASE I

Relative activity is defined as the percentage of the activity compared to that with 4-aminobutyrate as amino group donor.

Amino group donor	Relative activity (%)
Glycine	0
Glutamic acid	100
β-Alanine	100
DL-β-Amino-isobutyric acid	51
γ-Aminobutyric acid	100
DL-β-Hydroxy-γ-aminobutyric acid	20
S-Aminovaleric acid	85
ϵ-Aminocaproic acid	14
L-α-β-Diaminotyric acid	0

phenyl)-4-aminobutyric acid, cyclo 4-aminobutyrate could not be transaminated.

Discussion

Pig-brain and pig-liver 4-aminobutyrate-transaminases were purified by nearly identical procedures; the two enzymes showed similar properties during purification. However, some differences appeared: The hepatic enzyme precipitated at higher $(NH_4)_2SO_4$ concentrations than those needed for precipitated of the brain enzyme and elution patterns of the chromatographies showed some differences. Like the brain enzyme, the chromatography of liver enzyme on DEAE-cellulose separated the enzymatic activity into two peaks. The first one, which was homogeneous, was designated as 4-aminobutyrate-transaminase I; the second one, 4-aminobutyrate-transaminase II, was strongly bound to aspartate transaminase. Chromatography on hydroxyapatite succeeded in dissociating aspartate transaminase activity from 4-aminobutyrate-transaminase activity, yielding a purified 4-aminobutyrate-transaminase II.

According to the specific activity of the original liver homogenate, 4-aminobutyrate-transaminase I was purified 600-fold and 4-aminobutyrate-transaminase II 300-fold. Polyacrylamide gel electrophoresis gave a single band containing all the enzyme activity for each form of 4-aminobutyrate-transaminase. Electrophoresis in the presence of SDS revealed a single band of protein, whereas electrophoresis in the presence of urea showed two bands. Like the brain enzyme, liver transaminase is constituted of two subunits of identical molecular weight; however, the two subunits of the hepatic enzyme have different charges, whereas the brain subunits have the same charge [21].

Gel filtration on Sephadex G-100 yielded a molecular weight of 110 000 ± 5000 for liver enzyme which agreed with the molecular weight of 105 000 ± 7000 reported for the brain enzyme [21]. The $s_{20,w}$ values calculated for liver transaminases I and II are higher than those obtained for these forms of the enzyme from the brain [24]. Determination of the pH optimum, the isoelectric point, affinities of the enzyme for its substrates and for other amino-acids failed to show significant differences between brain and liver enzymes.

The existence of different forms of 4-aminobutyrate-transaminase reported in mouse and rat brain [22], in guinea-pig brain [23] and in pig brain [21] has also been found in pig liver; the liver enzyme has been separated into anionic and cationic forms according to their electrophoretic mobilities and their adsorption onto DEAE-cellulose. 4-Aminobutyrate-transaminase I is probably the cationic form, while 4-aminobutyrate-transaminase II could be the anionic form. It is interesting to note that these two forms exist in the liver, since one of the questions about brain 4-aminobutyrate-transaminase is whether the different forms of the enzyme play a significant role in the regulation of 4-aminobutyrate levels in the nervous system. In liver, 4-aminobutyrate has no transmitter function, and its physiological significance is not clear. The enzyme could have a metabolic function, either in the shunt of 4-aminobutyrate or in the catabolism of β-alanine.

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