Purification and properties of rabbit brain and liver 4-aminobutyrate aminotransferases isolated by monoclonal-antibody immunoabsorbent chromatography

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(Received 5 March 1985/24 April 1985; accepted 10 May 1985)

The use of a monoclonal-antibody immunoaffinity column for the rapid isolation of 4-aminobutyrate aminotransferases (EC 2.6.1.19) from rabbit brain and liver is described. Homogeneous enzyme protein is eluted from the immunoabsorbent with 100 mM-citrate buffer, pH 5, and remains stable at 4°C for several days. One such column (bed volume 8 ml) has been used 40 times in a 9-month period to isolate 10–15 units of enzyme activity (specific activity approx. 3.5–7.5 units/mg) per extraction. Kinetic and spectral analysis of the enzymes from the two tissues revealed a close similarity. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed the isolated enzyme to have a monomeric M_r of 52 000, and this was confirmed by h.p.l.c. gel exclusion at pH 5.0. The results of Sephadex G-100 chromatography at different pH values are taken to indicate that the enzyme behaves as a dimer at pH 7.0 and above, but as a monomer at pH 5.0. 4-Aminobutyrate aminotransferase isolated from the brain by the procedure of Fowler & John [(1981) *Biochem. J.* **197**, 149–152] is more stable than the immunoaffinity-purified material, and has been shown to contain a contaminant protein of M_r 84 000 that exhibits succinic semialdehyde dehydrogenase activity.

4-Aminobutyrate:2-oxoglutarate aminotransferase (GABA-T, EC 2.6.1.19) in mammalian brain is responsible for the degradation of the inhibitory neurotransmitter 4-aminobutyrate. This enzyme has been isolated from several species, including rat (Vasil'ev & Eremin, 1968; Maitre *et al.*, 1975), mouse (Schousboe *et al.*, 1973), rabbit (John & Fowler, 1976), pig (Beeler & Churchich, 1978) and man (Cash *et al.*, 1974). A typical purification procedure followed in these cases would include heat and pH treatment, precipitation with $(\text{NH}_4)_2\text{SO}_4$ or acetone, followed by chromatography on cellulose anion-exchange and cation-exchange columns. The yield of purified enzyme from these treatments has been low and its character was possibly altered during isolation.

We report in this paper the purification of GABA-T from rabbit brain and liver by immunoabsorbent chromatography employing a monoclonal antibody to rabbit brain enzyme (Edwardson *et al.*, 1985). This purification procedure involves no pretreatment before the single rapid chromatographic step. Homogeneous enzyme can be prepared quickly and in high yield, allowing the prospect that the elucidation of the structure and mechanism of the enzyme will be possible.

An enzyme catalysing the same reaction as brain GABA-T has been identified in extracts of pig kidney and liver (Vasil'ev *et al.*, 1973; Buzenet *et al.*, 1978), rat kidney (Lancaster *et al.*, 1973) and human kidney and liver (White & Sato, 1978), but it is not clear whether the enzyme in brain is the same as that in other tissues.

Abbreviations used: GABA-T, 4-aminobutyrate:2-oxoglutarate aminotransferase; SSA-DH, succinic semialdehyde dehydrogenase.

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Experimental

Materials

Rabbit brain and liver were obtained from Buxted-Olac Co., Buxted, Sussex, U.K., the organs

being removed immediately after death and stored at -20°C until required.

The immunoabsorbent column containing monoclonal antibody (C8) was prepared as described elsewhere (Edwardson *et al.*, 1985).

2-Oxoglutaric acid, 3,5-diaminobenzoic acid, 4-aminobutyric acid and $\beta\text{-NAD}^+$ were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Affigel-15 and low- M_r markers were purchased from Bio-Rad Laboratories, Watford, Herts., U.K.

Enzyme and protein assays

GABA-T was assayed as previously described (Salvador & Albers, 1959). For kinetic experiments, 4-aminobutyrate concentration was varied in the range 2–25 mM and that of 2-oxoglutarate in the range 2–10 mM, again in 100 mM-boric acid, pH 8.2, at 37°C . One unit of activity is defined as the conversion of $1\ \mu\text{mol}$ of substrate/min.

Succinic semialdehyde dehydrogenase (SSADH) was assayed as previously described (De Boer & Bruinvels, 1977).

Protein was assayed by the method of Bradford (1976), with bovine serum albumin as standard.

Purification of rabbit brain GABA-T

Immunoaffinity purification of brain GABA-T was performed as previously described (Edwardson *et al.*, 1985) except that the homogenate was prepared in 4 vol. of ice-cold distilled water without additions. Briefly, unfractionated rabbit brain supernatant was applied to the immunoabsorbent column at a rate of 1 ml/min. Binding was increased by passing the supernatant over the

column twice; not all of the enzyme activity present in the supernatant, however, was retained by the column. After the column had been thoroughly washed with 50 mM-sodium phosphate buffer, pH 7.0, GABA-T activity was eluted at room temperature with a linear gradient made with 15 ml of 100 mM-sodium citrate buffer, pH 7.5, and the same volume of the same buffer adjusted to pH 2.5. The active fractions were kept separate and stored at 4°C .

After each use the immunoabsorbent column was washed with 50 ml of 100 mM-glycine/HCl buffer, pH 2.0, and re-equilibrated in 50 mM-sodium phosphate buffer, pH 7.0. Between uses the column was stored in this buffer containing 0.02% NaN_3 at 4°C . One such column has been used for enzyme purification on 40 separate occasions over a period of 9 months. During this period an approx. 30% loss of binding capacity occurred.

Purification of rabbit liver GABA-T

This was identical with the purification of the brain enzyme. Before application to the column, however, the glycogen layer was removed from the surface of the supernatant.

Polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/15%-(w/v)-polyacrylamide-gel electrophoresis was conducted as described previously (Edwardson *et al.*, 1985).

H.p.l.c.

The column used in all h.p.l.c. analyses was a TSK 3000 SW (LKB Produkter, Bromma, Sweden)

Fig. 1. Protein (○) and GABA-T activity (●) profiles in the immunoaffinity purification of rabbit brain GABA-T. Unfractionated rabbit brain supernatant (150 ml) was passed twice over a column of monoclonal antibody (C8) coupled to Affigel-15, over a period of approx. 5 h. At point A the column was washed with 50 mM-sodium phosphate buffer, pH 7.0, until the absorbance at 280 nm fell to 0. At point B a 100 mM-citrate pH gradient was applied (----). Other experimental details are given in the text.

column (600 mm $\times$ 7.5 mm) with pre-column (100 mm $\times$ 7.5 mm). The mobile phase was 100 mM-sodium acetate adjusted to pH 5.0; the flow rate was 1 ml/min. The calibration of the M_r values of the different fractions separated by the TSK 3000 column was achieved by the construction of standard curves by using proteins of known M_r (γ -globulin, 160 000; bovine serum albumin, 68 000; ovalbumin, 45 000; cytochrome *c*, 12 000).

Results

Enzyme purification

It has proved possible to purify rabbit brain and liver GABA-T to homogeneity in a single immuno-adsorbent step by using monoclonal-antibody immunoaffinity chromatography. Fig. 1 shows the eluted protein and enzyme activity profile for a typical separation (the brain and the liver enzymes gave exactly the same elution profile).

The fractions containing GABA-T activity were eluted in the pH range 5.5–4.5. An overall purification of 760-fold was achieved with a yield of 27% for the brain enzyme, and 900-fold purification and 50% yield for the liver enzyme (Table 1; mean for four preparations).

Both liver and brain GABA-T purified in this manner were very unstable to freezing and thawing. On thawing a heavy white precipitate was observed, and all GABA-T activity was shown to be lost. Fractions were, in consequence, stored at 4°C, at which their activity decreased to zero over a 4-day period. The addition of EDTA and 2-mercaptoethanol had no effect on this loss of activity. However, 100 μ M-pyridoxal 5'-phosphate had a small stabilizing effect on the fractions. Fig. 2 shows that the enzymes prepared by immunoaffinity chromatography are homogeneous on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The subunit M_r was estimated to be 52 000 for both enzymes, six proteins of known M_r being used as a reference.

High-speed supernatants prepared from rat and mouse brain, and also mouse liver, were subjected to the same procedure. No GABA-T activity present in these tissues was retained by the immunoaffinity column.

Spectral properties

Figs. 3(a) and 3(b) show the absorption spectra of the brain and liver enzymes respectively. Two absorption peaks associated with the cofactor can be seen, one at 415 nm and the other at 330 nm; the 415 nm peak has been ascribed to the aldimine

Fig. 2. Sodium dodecyl sulphate/15%-polyacrylamide-gel electrophoresis of immunoaffinity-purified GABA-T. Tracks (from left to right): 1, marker proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor, lysozyme); 2, immunoaffinity-purified rabbit brain GABA-T (40 μ g); 3, immunoaffinity-purified rabbit brain GABA-T (20 μ g); 4, immunoaffinity-purified rabbit liver GABA-T (20 μ g).

Table 1. Purification of GABA-T from rabbit brain and liver

Details of the purification are given in the Experimental section. Typically, 1.6 mg of brain GABA-T and 3.3 mg of the liver enzyme were purified from 50 g of starting material.

	Brain				Liver			
	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Homogenate	44	0.01	(100)	1	22	0.004	(100)	1
Supernatant	30	0.042	68	4.2	15	0.016	68	4
Immunoaffinity chromatography	12	7.6	27	760	12	3.6	50	900

Fig. 3. Absorption spectra of immunoaffinity-purified GABA-T

Spectra: (a) rabbit brain GABA-T; (b) rabbit liver GABA-T. Spectra were recorded with use of a 1 cm-light-path quartz cuvette in a Cecil 505 spectrophotometer.

Fig. 4. Substrate kinetics for immunoaffinity-purified rabbit brain GABA-T

(a) Replot of the reciprocal of the apparent K_m for 2-oxoglutarate (2-OG) versus the reciprocal of the concentration of 4-aminobutyrate. (b) Replot of the reciprocal of the apparent K_m for 4-aminobutyrate (GABA) versus the reciprocal of the concentration of 2-oxoglutarate. (c) Replot of the reciprocal apparent V_{max} versus the reciprocal of the concentration of 4-aminobutyrate (Δ) and versus the reciprocal of the concentration of 2-oxoglutarate ($\circ$).

form of the enzyme (John & Fowler, 1976). The peak at 330 nm can be attributed either to an inactive form of the enzyme or to a different form of bound pyridoxal phosphate. It may be noted that this peak at 330 nm is still seen after such a rapid enzyme purification, indicating that unless the 330 nm peak arises in the homogenization itself it is a normal part of the enzyme *in vivo*. The ratios of the absorbance at 280 nm to the absorbances due

to the cofactor, i.e. at 330 nm and 415 nm, were found to be similar for both conventionally purified GABA-T and immunopurified enzyme; in both cases the A_{280}/A_{415} ratio was approx. 10:1.

Enzyme kinetics

Substrate curves were prepared for each enzyme in the usual manner. The patterns of parallel lines obtained in such plots supported a Ping Pong Bi Bi

Fig. 5. Substrate kinetics for immunoaffinity-purified rabbit liver GABA-T
(a) Replot of the reciprocal of the apparent K_m for 2-oxoglutarate (2-OG) versus the reciprocal of the concentration of 4-aminobutyrate. (b) Replot of the reciprocal of the apparent K_m for 4-aminobutyrate (GABA) versus the reciprocal of the concentration of 2-oxoglutarate. (c) Replot of the reciprocal of the apparent $V_{\max}$ versus the reciprocal of the concentration of 4-aminobutyrate (Δ) and versus the reciprocal of the concentration of 2-oxoglutarate ($\circ$).

Table 2. Kinetic properties of GABA-T

Source	K_m (2-oxoglutarate) (mM)	K_m (4-aminobutyrate) (mM)	$V_{\max}$ (units/mg)
Rabbit brain	5.4 ± 0.5	11.5 ± 1.2	3.0 ± 0.3
Rabbit liver	9.3 ± 0.7	10.5 ± 1.0	4.8 ± 0.8

type of enzyme mechanism (Cleland, 1963) for both forms of the enzyme. Figs. 4(a), 4(b) and 4(c) and Figs. 5(a), 5(b) and 5(c) show secondary plots used to calculate kinetic parameters. All lines were drawn by using the weighted-least-squares-fit procedure of Wilkinson (1961) with a BBC model B microcomputer. Table 2 summarizes these results.

H.p.l.c.

H.p.l.c. analysis of rabbit brain GABA-T purified by the method of Fowler & John (1981) gave an elution profile shown in Fig. 6(a). This

pattern was seen in the analysis of over 20 different batches of the enzyme. Only the peak at M_r 52000 (20min elution) carried GABA-T enzymic activity. The 84000- M_r (18min elution) peak was assayed for SSA-DH activity and was found to have a specific activity of $5.8 \mu\text{mol/min per mg}$ at 24°C . Sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of this peak revealed two bands corresponding to M_r 58000 and 26000. On thawing of the isolated 52000- M_r peak after storage at -20°C a white precipitate was seen and GABA-T activity shown to be lost. This situation is similar to that seen with the fractions from the

Fig. 6. *H.p.l.c.* analysis of rabbit brain GABA-T (a) Elution profile of GABA-T (0.8 mg) purified by the method of Fowler & John (1981). (b) Elution profile of immunoaffinity-purified rabbit brain GABA-T (0.3 mg). (c) Elution profile of supernatant from immunoaffinity-purified brain GABA-T after precipitation by freeze-thawing. All *h.p.l.c.* analyses were performed on a TSK 3000-SW column, with 100 mM-sodium acetate buffer, pH 5; the flow rate was 1 ml/min.

immunoaffinity column. It was therefore decided to perform a similar *h.p.l.c.* analysis of the immunopurified brain enzyme, this analysis being carried out immediately after elution from the column to minimize any effects due to the instability of this enzyme. The result of such an analysis is shown in Fig. 6(b). Only one peak is present on the chromatogram, occurring at M_r 52000. Again this peak has GABA-T activity associated with it. When a similarly sized portion of the same fraction from the immunoaffinity column was precipitated by freeze-thawing and *h.p.l.c.* analysis was performed on the supernatant, the result shown in Fig. 6(c) was obtained. The elution profile is similar to that in Fig. 6(a), but the two peaks, however, are present in only very small amounts (<0.5% original peak height) and could

only be seen at a high-sensitivity setting on the u.v. detector. As a result it was not possible to detect which, if any, of the peaks carried GABA-T or SSA-DH activity. The addition of the 84000- M_r protein to immunoaffinity-purified brain GABA-T prevented the precipitation seen normally on freezing and thawing, and 85% of the enzyme activity was also retained.

All *h.p.l.c.* runs were performed at pH 5.0, at which pH GABA-T activity is eluted at M_r 52000. John & Fowler (1976) proposed that the enzyme is a dimer composed of two such subunits having a total M_r of approx. 110000. Further studies with Sephadex G-100 chromatography have shown that pH has an effect on the oligomeric form of the enzyme. The results of these experiments are shown in Fig. 7. At pH 5 the enzyme occurs as a 52000- M_r monomer, and it is dimeric at pH 7 and above. At the intermediate pH of 6.2 a mixture of monomer and dimer occurred.

Discussion

This paper describes the rapid isolation under mild conditions of GABA-T from rabbit brain and liver by using an affinity column prepared from a monoclonal antibody to rabbit brain enzyme. Active enzyme is eluted from the column with the use of particularly mild conditions compared with those used with other monoclonal-antibody columns (Denney *et al.*, 1982; Le *et al.*, 1984; Oida *et al.*, 1984). It is possible that this ready elution is a consequence of dimer-monomer dissociation on changing the pH from 7.0 to 5.0, but we have no evidence to substantiate this hypothesis. This antibody is of the IgG₁ subclass and would appear to be species-specific. Since it recognizes GABA-T in both liver and brain, the enzymes from these sources might be expected to be very similar, and this has been confirmed by the studies on their physical, spectral and kinetic properties.

However, despite this similarity, we must presume that the enzyme in liver performs a different function from that in the brain, since it has not been shown that 4-aminobutyrate is a neurotransmitter in the liver. Roberts & Bregoff (1953) demonstrated that 4-aminobutyrate-2-oxoglutarate transamination could occur in the liver as well as in the brain, and that β -alanine could be used as an alternative substrate to 4-aminobutyrate in the reaction. GABA-T could equally well be termed β -alanine aminotransferase, and could be primarily involved in β -alanine metabolism, or it could be that the enzyme found in the liver is a relatively non-specific ω -amino acid aminotransferase dealing with dietary ω -amino acids.

Preliminary studies (N. Kirby, unpublished work) have shown that enzyme-activated inhibi-

Fig. 7. Sephadex G-100 chromatography of GABA-T

A 1 ml solution of rabbit brain GABA-T purified by the method of Fowler & John (1981), containing 3.1 units (0.75 mg) of enzyme activity, was chromatographed on Sephadex G-100 in 50 mM-sodium acetate adjusted to pH 5.4 (Δ) and pH 6.2 (●) and 50 mM-Tris/HCl buffer adjusted to pH 8.0 (○).

tors such as 4-aminohex-5-enoate (Lippert *et al.*, 1977) and ethanolamine *O*-sulphate (Fowler & John, 1972) have similar effects on the liver enzyme and on brain GABA-T. A question arises, therefore, about the possible effects of side reactions to such drugs as a consequence of their peripheral site of action.

The h.p.l.c. findings were of great interest, indicating that we are isolating a different form of GABA-T on immunoaffinity purification. The existence of 'enzyme clusters' composed of GABA-T and SSA-DH in the mitochondria was proposed by Hearl & Churchich (1984). These clusters would prevent the build-up of toxic succinic semialdehyde within the cell. It could be argued that the two peaks given by conventionally purified GABA-T could be accounted for by such 'enzyme clusters', since it has been shown that the 84000- M_r protein has SSA-DH activity. The M_r of this protein is not in agreement with previously published reports for mammalian SSA-DH. Blaner & Churchich (1979) calculated the M_r of pig brain SSA-DH to be 160000, and later confirmed that this was in fact a tetramer of M_r 40000 subunits (Blaner & Churchich, 1980). It remains a possibility that the

84000- M_r protein that we have isolated is a dimeric form of the enzyme.

The question remains whether the 'enzyme clusters' are an artifact of the purification method, due to heat and pH treatment, or whether they are a true reflection of the naturally occurring enzyme. Immunoaffinity purification of the enzyme is a much more rapid procedure, and the vast excess of the 52000- M_r protein may indicate the true state of the enzyme *in vivo*; however, it has been shown that the 'enzyme clusters' indicative of GABA-T purified by conventional techniques are dissociated by immunoaffinity chromatography, yielding only one protein of 52000- M_r on h.p.l.c. and sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis, and it may be for this reason that we see only the 52000- M_r protein after immunoaffinity purification of the enzymes. In both cases, however, it can be said that the presence of the protein with SSA-DH activity has the effect of stabilizing GABA-T *in vitro* and that there is a possibility of a physical interaction between the two proteins. As yet it is not possible to decide if the enzyme *in vivo* exists bound to SSA-DH in an 'enzyme cluster', or whether GABA-T

normally exists alone, and the presence of such clusters is merely a result of the lengthy purification procedure.

We thank Dr. Alistair Aitken for help with the h.p.l.c. experiments. N. K. and N. I. P. are Medical Research Council Scholars.

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