Biochem. J. (1990) 269, 25–29 (Printed in Great Britain)

Purification and characterization of a rat brain aldehyde dehydrogenase able to metabolize γ-aminobutyraldehyde to γ-aminobutyric acid

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An enzyme which catalyses dehydrogenation of γ-aminobutyraldehyde (ABAL) to γ-aminobutyric acid (GABA) was purified to homogeneity from rat brain tissues by using DEAE-cellulose and affinity chromatography on 5'-AMP–Sepharose, phosphocellulose and Blue Agarose, followed by gel filtration. Such an enzyme was first purified from mammalian brain tissues, and was identified as an isoenzyme of aldehyde dehydrogenase. It has an M_r of 210000 determined by polyacrylamide-gradient-gel electrophoresis, and appeared to be composed of subunits of M_r 50000. The close similarity of substrate specificity toward acetaldehyde, propionaldehyde and glycolaldehyde between the enzyme and other aldehyde dehydrogenases previously reported was observed. But substrate specificity of the enzyme toward ABAL was higher than those of aldehyde dehydrogenases from human liver (E_1 and E_2), and was lower than those of ABAL dehydrogenases from human liver (E_3), *Escherichia coli* and *Pseudomonas* species. The M_r and relative amino acid composition of the enzyme are also similar to those of E_1 and E_2. The existence of this enzyme in mammalian brain seems to be related to a glutamate decarboxylase-independent pathway (alternative pathway) for GABA synthesis from putrescine.

INTRODUCTION

It is well known that γ-aminobutyric acid (GABA), an inhibitory neurotransmitter in the central nervous system, is synthesized from glutamate by glutamate decarboxylase (GAD; EC 4.1.1.15) [1]. We have reported that γ-aminobutyraldehyde (ABAL) was able to be oxidized rapidly to GABA both in mouse brain [2] and in synaptosomes isolated from mouse brain tissues [3].

An alternative pathway for GABA synthesis from putrescine via ABAL or acetylated ABAL has been suggested in neuronal tissues and their derived tumour cells [4–7]. In previous papers, we have suggested the presence of the activity oxidizing ABAL to GABA in rodent brains [8]. Kurys *et al.* [9] recently purified an enzyme which catalyses dehydrogenation of ABAL to GABA from human liver, and identified it as an isoenzyme of aldehyde dehydrogenase (EC 1.2.1.3 and 1.2.1.5) (E_3). More recently, some enzymes which oxidized ABAL to GABA in rat brain were also characterized, and one of these possessed quite similar substrate specificity to E_3 [10].

These facts suggest the presence of enzymes which catalyse dehydrogenation of ABAL to GABA in mammalian brain. The aim of this study was to purify and characterize such enzymes from rat brain.

MATERIALS AND METHODS

Materials

Acetaldehyde, propionaldehyde, glycolaldehyde, succinic semialdehyde and NAD^+ were obtained from Sigma, St. Louis, MO, U.S.A. ABAL was prepared by the method previously described [3]. DEAE-cellulose DE52 and phosphocellulose were obtained from Whatman, Clifton, NJ, U.S.A. 5'-AMP–Sepharose and Sephacryl S-300 were obtained from Pharmacia, Uppsala, Sweden. Blue Agarose was obtained from Amicon, Danvers, MA, U.S.A. All other chemicals were of reagent grade.

Enzyme assay

NAD^+-dependent dehydrogenase activity was assayed spectrophotometrically by measuring the increase in A_{340} at 37 °C. The assay mixture (final volume 1 ml) contained 0.1 M-potassium phosphate buffer, pH 8.0, 2.5 mM-NAD^+, 2.0 mM substrate and a sample from each step in the purification procedure. For determinations of kinetic properties, we used the most purified enzyme (sp. activity 45 nmol/min per mg, as shown in Table 1) at a concentration of 4.5 μg/ml in the assay mixture. The K_m and maximum specific activity (V) were estimated from a straight line fitted to Lineweaver–Burk plots [11] by least-squares analysis.

Protein determination

Protein concentration was determined as described by Lowry *et al.* [12] with BSA as standard, or spectrophotometrically at 280 nm.

Determination of M_r of enzyme and subunits

The native M_r was determined by electrophoresis in 4–17% (w/v)-polyacrylamide gradient gel by the original procedure of Davis [13]. The M_r was determined from the standard curve obtained with protein standards purchased from Sigma (thyroglobulin, 660000; β-amylase, 200000; alcohol dehydrogenase, 150000; BSA, 66000). The native M_r was also determined by Sephacryl S-300 gel filtration. The column (1.6 cm × 96 cm) was equilibrated with 10 mM-potassium phosphate buffer, pH 7.0, containing 200 mM-NaCl and 1 mM-2-mercaptoethanol. The calibration curve was obtained with protein standards purchased from Sigma (as for electrophoresis, plus: carbonic anhydrase, 29000; cytochrome *c*, 12400). The subunit M_r was estimated by electrophoresis in 10% (w/v)-acrylamide slab gel in 0.2% SDS as described by Laemmli [14]. Protein M_r standards for SDS/PAGE were purchased from Sigma (MW-SDS-200 kit; myosin, 203000; β-galactosidase, 116000; phosphorylase *b*, 97400; BSA, 66000; egg albumin, 45000; carbonic anhydrase,

Abbreviations used: ABAL, γ-aminobutyraldehyde; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase.

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29000). The gels were stained with silver-stain kit (Kanto Kagaku, Tokyo, Japan).

Amino acid composition

Amino acid analysis was performed with an automatic amino acid analyser (JLC 300). Gas-phase hydrolysis was performed in 6 M-HCl at 150° C for 1 h. Statistical analysis was performed as described by Cornish-Bowden [15].

N-Terminal determination

Sequencing was done on an Applied Biosystems 447A Protein Sequencer.

Purification procedure

All procedures were carried out at 0–4 °C unless otherwise stated. In each step, ABAL-oxidizing activity in the fractions was assayed by the method described above.

Extraction. About 1100 g of rat brain tissues kept frozen at −80 °C was homogenized in 4 vol. of 50 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-2-mercaptoethanol. The homogenate was then centrifuged at 10000 *g* for 20 min, and a clear supernatant was obtained.

$(NH_4)_2SO_4$ fractionation. The supernatant was fractionated with 60%-satd. $(NH_4)_2SO_4$. The suspension was centrifuged at 10000 *g* for 20 min, and the pellets were dissolved in about 550 ml of 5 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-2-mercaptoethanol (Buffer A). The solution was dialysed extensively against Buffer A.

DEAE-cellulose chromatography. The dialysed solution was applied to a DEAE-cellulose column (5 cm × 60 cm) equilibrated with Buffer A. After washing the column with Buffer A, it was eluted with a linear gradient of 0–300 mM-KCl and fractions containing enzyme activity were collected.

5′-AMP–Sepharose chromatography. DEAE-cellulose-column fractions containing enzyme activity were applied directly to a 5′-AMP–Sepharose column (1 cm × 24 cm), which was previously equilibrated with Buffer A. The column was washed with the same buffer and was eluted stepwise with 10 mM-NaCl, 10 mM-AMP, 20 mM-AMP and 1 M-NaCl in the same buffer.

Phosphocellulose chromatography. The 5′-AMP–Sepharose-column fraction containing enzyme activity was dialysed against 10 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol (Buffer B), and was applied to a phosphocellulose column (3.4 cm × 14 cm) equilibrated with the same buffer. After washing the column, it was eluted with 0.2 M-Tris/HCl buffer, pH 7.5, containing 2.5 mM-D-fructose 1,6-bisphosphate and 5 mM-EDTA.

Table 1. Purification of an enzyme from rat brain which catalysed dehydrogenation of ABAL to GABA

Experimental details are given in the text. The purification was monitored with 2 mM-ABAL as substrate in an assay mixture composed of 0.1 M-potassium phosphate buffer. pH 8.0, and 2.5 mM-NAD^+.

Step	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (fold)
0–60%-satd. $(NH_4)_2SO_4$	15600	1980	0.127	100	1
DEAE-cellulose	687	778	1.13	39.3	8.9
5′-AMP–Sepharose	515	730	1.42	36.9	11.2
Phosphocellulose	357	599	1.68	30.3	13.2
Blue Agarose	5.40	123	22.8	6.21	180
Sephacryl S-300	0.47	21	45	1.1	350

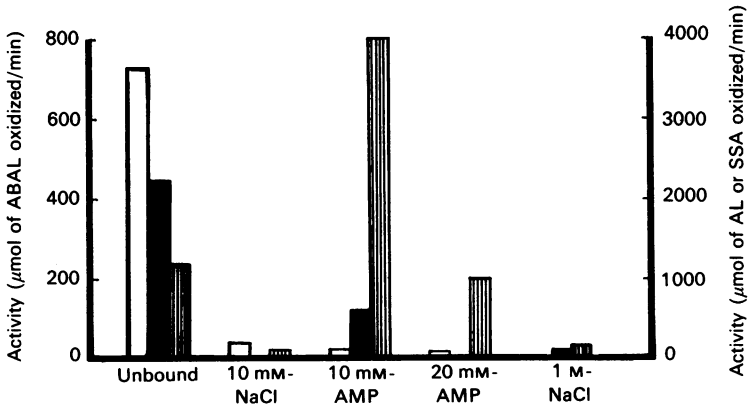

Fig. 1. Enzyme activities of each fraction from 5′-AMP–Sepharose

The active fractions from the DEAE-cellulose column were applied directly to a 5′-AMP–Sepharose column, which was previously equilibrated with 5 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-2-mercaptoethanol. The column was washed with the same buffer and was eluted stepwise with 10 mM-NaCl, 10 mM-AMP, 20 mM-AMP and 1 M-NaCl in the same buffer. Enzyme activities of each fraction toward ABAL (□), acetaldehyde (AL, ■) and succinic semialdehyde (SSA, ▥) were assayed as described in the Materials and methods section.

Blue Agarose chromatography. The phosphocellulose-column fraction containing enzyme activity was applied directly on a Blue Agarose column (3.4 cm × 11 cm), which was previously equilibrated with Buffer B. The column was washed with the same buffer (giving fraction 1) and then eluted with the same buffer containing 0.65 M-KCl (fraction 2), 0.2 M-KCl with 12.5 mM-NAD^+ (fraction 3) and 1 M-KCl (fraction 4).

Sephacryl S-300 gel filtration. Fraction 3 from the Blue Agarose column was concentrated by ultrafiltration (Amicon) and dialysed against 10 mM-potassium phosphate buffer, pH 7.0, containing 200 mM-NaCl and 1 mM-2-mercaptoethanol. The solution obtained was applied to a Sephacryl S-300 column (1.6 cm × 96 cm) equilibrated and eluted with the same buffer.

RESULTS

Purification

Table 1 summarizes the purification of an enzyme from rat brain which catalyses dehydrogenation of ABAL to GABA.

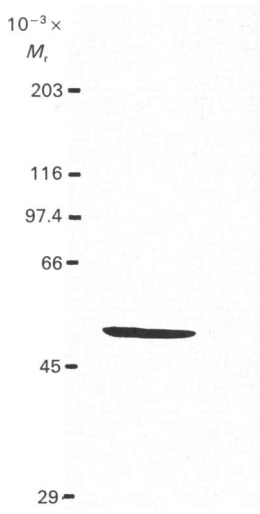

Fig. 2. SDS/PAGE of the purified enzyme

The gel was run by the method of Laemmli [14], and was stained with a silver-staining kit. A mixture of Sigma protein standards was used as M_r markers; myosin (203000), β-galactosidase (116000), phosphorylase *b* (97400), BSA (66000), egg albumin (45000) and carbonic anhydrase (29000).

On the first DEAE-cellulose column, ABAL-oxidizing activity appeared in the unbound fraction as well as in the eluted fraction. With regard to substrate specificity, the activity in the former was due to another aldehyde dehydrogenase (results not shown). The latter was applied to a 5′-AMP–Sepharose column. On this column, ABAL-oxidizing activity was found in the unbound fraction and was separated partially from the activities oxidizing both succinic semialdehyde and acetaldehyde (Fig. 1). To avoid contamination by aldolase, the unbound fraction from 5′-AMP–Sepharose was applied to a phosphocellulose column. Aldolase was bound tightly to the column, whereas the enzyme passed through unbound (results not shown). The unbound fraction from the phosphocellulose column was then applied to the Blue Agarose column. The enzyme activity was divided into two fractions, 2 and 3. The specific and total activities of fraction 2 were 0.3 nmol/min per mg and 42.1 nmol/min, and those of fraction 3 were 22.8 nmol/min per mg and 123 nmol/min respectively. Because the specific and total activities of fraction 3 were much higher than those of fraction 2, fraction 3 was subjected to Sephacryl S-300 gel filtration. From this, the enzyme activity was eluted between β-amylase (M_r 200000) and alcohol dehydrogenase (150000). The homogeneity of this peak fraction was assessed by both polyacrylamide-gradient-gel electrophoresis and SDS/PAGE, and a single band was obtained (Fig. 2). Thus

Table 2. Relative amino acid composition of various aldehyde dehydrogenases

Amino acid	Content (residues/100 residues) Rat brain (this work)	Human E_1*	Human E_2*
Asx	10.0	10.7	9.8
Ser	5.9	6.2	8.3
Gly	9.7	10.0	10.8
Glx	12.1	8.4	5.3
Pro	4.8	8.8	8.0
Thr	5.9	5.5	5.9
Ala	10.3	8.6	10.2
Val	9.0	8.1	10.8
Met	1.7	1.3	1.2
Tyr	3.2	2.8	2.1
Ile	4.1	6.1	4.7
Leu	7.5	7.1	6.5
Phe	4.5	4.3	4.5
His	1.3	1.7	2.7
Lys	6.3	7.4	6.5
Arg	3.7	2.9	2.6

* From ref. [16].

Table 3. Kinetic properties of an enzyme oxidizing ABAL

The assay system contained 0.1 M-potassium phosphate buffer, pH 8.0. K_m and V values were respectively obtained from the slope and intercept of a first-order regression line fitted to Lineweaver–Burk plots by least-squares analysis. All correlations between 1/[S] and $1/v$ were > 0.9, and were statistically significant ($P < 0.05$).

Varied substrate (concn. range)	Constant substrate (mM)	K_m (μM)	V (nmol/min per mg)	V/K_m
ABAL (66 μM–2 mM)	NAD^+ (2.5)	151	68.4	0.45
Acetaldehyde (22 μM–2 mM)	NAD^+ (2.5)	20	248	12
Propionaldehyde (10 μM–2 mM)	NAD^+ (2.5)	4.5	149	33
Glycolaldehyde (50 μM–2 mM)	NAD^+ (2.5)	40	525	13
Succinic semialdehyde (2 mM)	NAD^+ (2.5)	Inactive		
NAD^+ (66 μM–2 mM)	Acetaldehyde (2.0)	86.9	207	2.4

Table 4. K_m values of various aldehyde dehydrogenases

K_m values of aldehyde dehydrogenases from rat brain (the present work), human liver (E_1 [16,24]; E_2 [16,24], E_3 [9]), horse liver (F_1 [17]), human brain (Peak III [20]), and of ABAL dehydrogenases from *Escherichia coli* [21] and *Pseudomonas* species [22] are noted. V/K_m ratio is calculated from the individual values. Units: K_m, μM; V, nmol/min per mg.

Substrate	Rat brain		Human liver isoenzyme E_1		Human liver isoenzyme E_2		Human liver isoenzyme E_3		Horse liver isoenzyme F_1		Human brain isoenzyme Peak III		ABAL dehydrogenase	
	K_m	V/K_m	K_m	V/K_m	K_m	V/K_m	K_m	V/K_m	K_m	V/K_m	K_m	V/K_m	*E. coli* K_m	*Pseudomonas* sp. K_m
Acetaldehyde	20	12	30	9.3	3	133	50.4	5.5	70	1.86	48	9.4	–	–
Propionaldehyde	4.5	33	5	–	0.7	–	8	38.7	5	31.2	4	–	–	–
Glycolaldehyde	40	13	–	–	–	–	–	–	130	0.9	110	–	–	–
ABAL	151	0.45	760	0.17	512	0.29	13.8	145	–	–	–	–	31.3	28

0.47 mg of the purified enzyme was obtained from 1100 g of rat brain tissues, and the ABAL-oxidizing activity was concentrated about 350-fold relative to the $(NH_4)_2SO_4$-precipitation fraction.

Native and subunit M_r of the enzyme

The M_r was determined by polyacrylamide-gradient-gel electrophoresis. The plot of M_r versus mobility was a straight line, and the M_r of the purified enzyme was estimated to be 210000. The subunit M_r of the purified enzyme obtained on SDS/PAGE was estimated to be 50000 (Fig. 2).

Amino acid composition and *N*-terminal determination

Relative amino acid frequencies of the purified enzyme are compared with those of several aldehyde dehydrogenases in Table 2. In amino acid composition, the purified enzyme was very similar to aldehyde dehydrogenases derived from human liver (E_1 and E_2) [16] by statistical analysis (an index, $S\Delta n$, as described by Cornish-Bowden [15]). No identifiable *N*-terminal sequence was observed for the purified enzyme, showing clearly that the *N*-terminal of this protein was blocked.

Michaelis constants of the enzyme

Table 3 shows the kinetic properties of the purified enzyme for various aldehydes and NAD^+. Since both K_m and V vary with substrate, V/K_m ratios are also listed. The K_m value for ABAL (151 μM) was higher than those for propionaldehyde (4.5 μM), acetaldehyde (20 μM) and glycolaldehyde (40 μM). The V with glycolaldehyde (525 nmol/min per mg) was the highest. In contrast, the V with ABAL (68.4 nmol/min per mg) was lower than those with other aldehydes. V/K_m ratios of four substrates indicated that propionaldehyde was the best substrate for this purified enzyme.

DISCUSSION

There have been many reports about the purification of aldehyde dehydrogenase from many mammalian sources and species [9,16–20,23], but only one group was successful in the purification of the enzyme from brain without loss of activity [20].

In the present study, an aldehyde dehydrogenase which catalysed dehydrogenation of ABAL to GABA was purified to homogeneity from rat brain tissues. The kinetic properties of the purified enzyme indicated that substrate specificity toward ABAL was lower than those toward propionaldehyde, glycolaldehyde and acetaldehyde. Despite the close similarity of substrate specificity toward acetaldehyde, propionaldehyde and glycolaldehyde between the purified enzyme and other enzymes previously reported, each enzyme had its own K_m value as well as the V/K_m ratio for ABAL as substrate (Table 4). These results might indicate that substrate specificity toward ABAL of the purified enzyme was higher than those of E_1 [24] and E_2 [24], but was lower than those of E_3 [9] and ABAL dehydrogenases (EC 1.2.1.19) from *Escherichia coli* [21] and from *Pseudomonas* species [22]. The M_r and relative amino acid composition of the purified enzyme also resembled those of both E_1 and E_2 (Table 2). These properties allowed identification of the purified enzyme in this study as one of the isoenzymes of brain aldehyde dehydrogenase with relatively high substrate specificity toward ABAL compared with other aldehyde dehydrogenase isoenzymes which had been purified from mammalian tissues, except E_3.

The subunit M_r of the purified enzyme was 50000. The native M_r of the purified enzyme determined on polyacrylamide-gradient-gel electrophoresis was 210000. However, M_r of the purified enzyme determined by gel filtration was smaller than 210000. The same results were obtained in some previous reports

in which both polyacrylamide-gradient-gel electrophoresis and gel filtration were employed to determine M_r of aldehyde dehydrogenase [9,20,23]. From the data mentioned above, the purified enzyme might be a tetramer rather than a trimer.

This is the first report of purification of an aldehyde dehydrogenase from mammalian brain which catalyses dehydrogenation of ABAL to GABA. However, recent reports demonstrated that an aldehyde dehydrogenase isoenzyme which catalysed dehydrogenation of ABAL is present in human liver [9]. On a Blue Agarose column, total ABAL-oxidizing activity of fraction 3 was higher than that of fraction 2; therefore the purified enzyme from fraction 3 may be the major enzyme in rat brain which catalyses dehydrogenation of ABAL to GABA. However, the physiological importance of the conversion of putrescine into GABA via the intermediate biogenic aldehyde, ABAL, is not yet well understood; the existence of this enzyme in mammalian brain seems to be related to the GAD-independent pathway (alternative pathway) for GABA synthesis from putrescine.

We thank Dr. Y. Tsukada for his helpful advice and criticisms. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

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Received 7 December 1989/5 February 1990; accepted 14 February 1990