

Aldehyde dehydrogenase from adult human brain that dehydrogenates γ -aminobutyraldehyde: purification, characterization, cloning and distribution

Alexandra KIKONYOGO and Regina PIETRUSZKO*

Center of Alcohol Studies and Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ 08855-0969, U.S.A.

Enzyme purification and characterization, cDNA cloning and Northern blot analysis were the techniques utilized during this investigation to determine the identity and occurrence of the aldehyde dehydrogenase that metabolizes γ -aminobutyraldehyde in adult human brain. The purification yielded one major protein which was active with γ -aminobutyraldehyde. It had the physico-chemical and kinetic properties of the human liver E3 isoenzyme of aldehyde dehydrogenase (EC 1.2.1.3), and also interacted with an anti-(liver E3 isoenzyme) antibody. Tryptic peptides derived from the purified brain protein matched the amino acid sequence of the liver E3 isoenzyme. Employing liver E3 cDNA, a human cerebellar cDNA library was screened and a 2.0 kb cDNA fragment was isolated. The cerebellar cDNA yielded a derived

primary structure which differed from the liver E3 amino acid sequence by a single serine-to-cysteine substitution at position 88 (position 84 in the liver sequence). Thus the γ -aminobutyraldehyde-metabolizing enzyme from human brain can be identified as E3', a variant of the E3 isoenzyme. The catalytic properties of the brain variant were indistinguishable from those of E3, and so the functional importance of this variant is at present unknown. The distribution of this enzyme in brain was investigated by Northern blot analysis, which demonstrated the presence of E3' mRNA in all regions of the human brain. mRNA levels were variable in the different brain areas, with the highest levels in the spinal cord and the lowest in the occipital pole.

INTRODUCTION

γ -Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the central nervous system. It is also present in significant amounts in mammalian peripheral tissues [1]. The major pathway for GABA synthesis is by the decarboxylation of L-glutamate by L-glutamate decarboxylase [2]. GABA can also be formed from putrescine by two pathways. The first involves the direct oxidative deamination of putrescine, by diamine oxidase, to give γ -aminobutyraldehyde [3]. The γ -aminobutyraldehyde is then converted into GABA by an aldehyde dehydrogenase. The second pathway involves the acetylation of putrescine to *N*-acetylputrescine, which is converted into *N*-acetyl- γ -aminobutyraldehyde by monoamine oxidase [4], and then to *N*-acetyl-GABA by an aldehyde dehydrogenase. *N*-Acetyl-GABA is deacetylated to form GABA. Levels of diamine oxidase in the mammalian brain are reportedly low [5], and so the main pathway of GABA formation from putrescine in the brain is believed to be through monoamine oxidase.

Aldehyde dehydrogenase (EC 1.2.1.3) catalyses the NAD⁺-linked oxidation of aldehydes to acids. It has a broad substrate specificity, with substrates ranging from straight-chain and branched aliphatic and aromatic aldehydes to the aldehyde metabolites of naturally occurring compounds, such as biogenic amines and corticosteroids [6]. Three isoenzymes of aldehyde dehydrogenase have been purified from the human liver [7,8]. They are the cytoplasmic E1 and E3 isoenzymes, and the mitochondrial E2 isoenzyme. The E3 isoenzyme was identified as the enzyme that catalyses the conversion of γ -aminobutyraldehyde into GABA in the liver, with high affinity (K_m 5–14 μ M) and high catalytic efficiency [8].

More recently, aldehyde dehydrogenases which metabolize γ -aminobutyraldehyde were purified from rat and bovine brain [9,10]. The reported K_m values for γ -aminobutyraldehyde of the rat and bovine enzymes (151 and 154 μ M respectively) were an order of magnitude higher than that of the E3 isoenzyme. K_m values of the brain enzymes for other substrates also differed from those of E3. These differences in kinetic properties, together with the reportedly low levels of brain diamine oxidase, suggested that the brain enzyme may be different from the liver E3 isoenzyme. Here we report the purification of an enzyme with γ -aminobutyraldehyde dehydrogenase activity from human brain. The kinetic, protein chemistry and molecular biology approaches used to characterize this enzyme are described.

EXPERIMENTAL

Materials

Acetaldehyde and γ -aminobutyraldehyde diethyl acetal were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Propionaldehyde, from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.), was redistilled before use. Ammonium sulphate was from Schwarz/Mann Co. (Orangeburg, NY, U.S.A.). CM-Sephadex C-50, DEAE-Sephadex A-50, 5'-AMP-Sepharose 4B, Blue Sepharose CL-6B, FPLC Mono-P column, Pharmalyte and the Isoelectric Focusing Calibration Kit (pH range 3–10) were from Pharmacia Biotech. Co. (Piscataway, NJ, U.S.A.). Formamide and tetramethylammonium chloride were from Fisher Scientific (Pittsburgh, PA, U.S.A.). NAD⁺ was from Boehringer-Mannheim Co. (Indianapolis, IN, U.S.A.). The Sequenase Version 2.0 DNA Sequencing Kit was from United States Biochemical (Cleveland, OH, U.S.A.). The Multiprime

Abbreviations used: GABA, γ -aminobutyric acid; PVDF, poly(vinylidene difluoride); SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; SSPE, 0.15 M NaCl, 0.001 M EDTA, 0.01 M sodium phosphate.

* To whom correspondence should be addressed.

The nucleotide sequence displayed in Figure 3 has been deposited with the GenBank Nucleotide Sequence Database under the accession number BRAINE3 U50203.

DNA Labeling System, Hybond-N nylon membranes and all radioactive nucleotides were from Amersham Co. (Arlington Heights, IL, U.S.A.). Immobilon poly(vinylidene difluoride) (PVDF) membranes were from Millipore Corp. (San Francisco, CA, U.S.A.). Nitrocellulose filters were from Schleicher and Schuell (Keene, NH, U.S.A.). Betaine aldehyde chloride, glycol-aldehyde, *N*-acetylputrescine, catalase, monoamine oxidase, β -mercaptoethanol, *p*-Nitro Blue Tetrazolium chloride, phenazine methosulphate, salmon testes DNA, SDS and Fuji RX film were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The human cerebellar cDNA library was from Stratagene (La Jolla, CA, U.S.A.). The human brain Multiple Tissue Northern blots and the human fetal brain cDNA library were from Clontech Laboratories (Palo Alto, CA, U.S.A.). E1, E2 and E3 aldehyde dehydrogenase isoenzymes from human liver were purified using previously described procedures [7,8].

Preparation of aldehyde substrates

γ -Aminobutyraldehyde was prepared by the acid hydrolysis of fractionally redistilled diethyl acetal, as described by Ambroziak and Pietruszko [11]. *N*-Acetyl- γ -aminobutyraldehyde was generated from *N*-acetylputrescine by the action of monoamine oxidase, as described in [11].

Enzyme activity assay

γ -Aminobutyraldehyde-metabolizing activity was assayed in 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, 500 μ M NAD⁺ and 500 μ M γ -aminobutyraldehyde. Activity towards propionaldehyde was assayed in 0.1 M sodium pyrophosphate buffer, pH 9.0, containing 1 mM EDTA, 500 μ M NAD⁺ and 1 mM propionaldehyde. Reactions were initiated by the addition of enzyme. All assays were carried out at 25 °C. NADH formation was monitored at 340 nm using a Gilford spectrophotometer. A molar absorption coefficient of 6.22 mM⁻¹·cm⁻¹ for NADH was used. K_m values were determined by the single reaction curve method of Yun and Suelter [12], and also by the Lineweaver–Burk method [13].

Protein determination

Protein concentration was determined as described by Goa [14], using BSA as a standard. Protein was also assayed spectrophotometrically at 280 nm, using a molar absorption coefficient of 1.0 (mg/ml)⁻¹·cm⁻¹ [8].

Isoelectric focusing

Agarose plates composed of 1% (w/v) agarose, 12% (w/v) sorbitol and 0.063% (v/v) Pharmalyte (pH 3–10) were used for isoelectric focusing. Gels were run for 16–18 h at 120 V and stained for activity with γ -aminobutyraldehyde (500 μ M) using 0.1 M sodium phosphate buffer, pH 7.4, and for activity with propionaldehyde (13 mM) using 0.1 M Tris/HCl buffer, pH 8.5. The staining solution also contained NAD⁺ (10 mg/100 ml), *p*-Nitro Blue Tetrazolium (10 mg/100 ml), phenazine methosulphate (1 mg/100 ml) and 1 mM EDTA. The Pharmacia Isoelectric Focusing Calibration Kit (range pH 3–10) was used, according to the manufacturer's instructions, to determine isoelectric points.

Subunit molecular mass determination and Western blotting

Subunit molecular mass was determined by SDS/PAGE according to the procedure of Laemmli [15]. The standards were

from Sigma Chemical Co. Protein bands were transferred from the gel to an Immobilon PVDF membrane using a Milliblot Graphite Electroblotter System (Millipore Corp.). Western blot analysis was then carried out using the polyclonal anti-E3 antibody described by Kurys et al. [16].

Primary structure analysis

Approx. 2 μ g of the main protein band was transferred from an SDS/PAGE gel to a nitrocellulose filter using the Milliblot Graphite Electroblotter System. The filter was then stained with 0.1% Ponceau S in order to visualize the protein bands, and the sample band was cut out. *In situ* tryptic digestion, MS and microsequencing of the band were carried out by the Microchemistry Core Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A., as described by Erdjument-Bromage et al. [17].

Screening of a human brain cDNA library

A human cerebellar cDNA library constructed in λ ZAP was screened (1×10^6 plaque-forming units) using a 1.6 kb human liver E3 cDNA [16] and a 24-mer oligonucleotide (5' GCTATCACTCGGCCGGTTGCTGGC 3') complementary to the 5'-end region of the E3 cDNA (nt 3–26). Filters were prehybridized for 4 h at 65 °C in a solution containing 6 \times SSC (1 \times SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 \times Denhardt's solution, 0.5% (w/v) SDS, 0.05% (w/v) sodium pyrophosphate and 50 μ g/ml denatured salmon testes DNA [18]. The cDNA probe was ³²P-labelled by the random priming method using [α -³²P]dCTP, and the oligonucleotide probe was labelled at the 5' end with [γ -³²P]ATP. The specific radioactivities of the cDNA and oligonucleotide probes were 3×10^9 and 2×10^8 d.p.m./ μ g respectively. Hybridization with the cDNA probe was carried out in the same solution as above, containing 50% formamide, at 37 °C for 12 h, whereas hybridization with the oligonucleotide probe was carried out in 6 \times SSC, 10 \times Denhardt's solution, 0.05% sodium pyrophosphate and 20 μ g/ml yeast tRNA at 45 °C for 12 h [18]. cDNA-probed filters were washed four times for 15 min in 2 \times SSC/0.1% SDS at room temperature. Oligonucleotide-probed filters were washed twice for 30 min in 6 \times SSC/0.05% sodium pyrophosphate and twice for 20 min in 3 M tetramethylammonium chloride, 50 mM Tris, pH 8.0, 2 mM EDTA and 0.1% SDS at 50 °C. Three rounds of screening yielded five plaque-purified positive clones which, after *in vivo* excision of the λ vector, formed phagemids containing the cloned inserts. The clones were then characterized by restriction enzyme analysis and DNA sequencing [19].

Northern blot analysis

Two Multiple Tissue Northern blots (Clontech Laboratories) containing 2 μ g aliquots of poly(A)⁺ mRNA obtained from various regions of the human brain were probed using the 1.6 kb human liver E3 cDNA [16], and also with a 2.0 kb β -actin control cDNA provided with the blots by Clontech. Each blot was prehybridized for 3 h at 65 °C in 10 ml of a solution containing 5 \times SSPE (1 \times SSPE = 0.15 M NaCl, 0.001 M EDTA and 0.01 M sodium phosphate, pH 7.4), 10 \times Denhardt's solution, 2% (w/v) SDS and 100 μ g/ml denatured salmon testes DNA. The cDNA probes were labelled as described for the cDNA library screen, and hybridization was carried out in 10 ml of the above solution at 65 °C for 24 h. The blots were washed three times for 15 min in 2 \times SSC/0.05% SDS at room temperature, and twice for 20 min in 0.1 \times SSC/0.1% SDS at 50 °C. They were then exposed to Fuji RX film at –70 °C for various

periods of time. The specific radioactivity of all the probes used in these hybridizations was 3×10^9 d.p.m./ μ g.

Purification procedure

Human brains obtained at autopsy (6–24 h after death) were stored at -70°C until use. Four purifications were attempted, and the sources of the brains used were: (1) a 47-year-old Caucasian male (death by hanging); (2) a 20-year-old Caucasian male (motor vehicle accident); (3) a 72-year-old Caucasian female (myocardial infarction); and (4) a 44-year-old Caucasian male (cause of death unknown). The purification procedure was based on those described for rat brain [9] and bovine brain [10] γ -aminobutyraldehyde dehydrogenases.

During purification the following buffers were used: buffer A, 5 mM sodium phosphate buffer, pH 6.0, 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol; buffer B, 5 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol. These buffers were the same as those described by Abe et al. [9] and Lee and Cho [10]. Buffer C consisted of 100 mM sodium phosphate buffer, pH 8.0, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. All buffers were de-aerated and nitrogenated exhaustively. All operations were carried out at 4°C .

Extraction

About 500 g of brain tissue was homogenized in 4 vol. of buffer A. The homogenate was left to extract at 4°C for 90 min and then centrifuged at 15000 *g* for 70 min. The supernatant was collected.

$(\text{NH}_4)_2\text{SO}_4$ fractionation

The supernatant was fractionated with $(\text{NH}_4)_2\text{SO}_4$. γ -Aminobutyraldehyde dehydrogenase activity was in the 40–70% satd. fraction. The suspensions were centrifuged at 15000 *g* for 50 min. The pellet was resuspended in a minimal volume of buffer A, and the sample was dialysed extensively against buffer A.

CM-Sephadex

The dialysed sample was applied on to a CM-Sephadex column (8 cm \times 14 cm) equilibrated with buffer A [10]. The column was washed with buffer A. The enzyme did not bind to the column. Active fractions were pooled, and the sample pH was adjusted to 7.0 using a saturated solution of Tris base.

DEAE-Sephadex

The CM-Sephadex flow-through sample was then applied on to a DEAE-Sephadex column (6.5 cm \times 20 cm) equilibrated with buffer B [10]. The column was washed with buffer B and then eluted with a linear gradient of 0–0.4 M NaCl. Fractions containing the enzyme activity were pooled and the pH was adjusted to 6.0 using a saturated solution of monobasic sodium phosphate.

5'-AMP-Sepharose

The active sample from the DEAE-Sephadex column was applied on to a 5'-AMP-Sepharose column (1.5 cm \times 7 cm) equilibrated with buffer A [10]. The enzyme did not bind to the column, so the column was washed with buffer A until no more enzyme came off. The active fractions were pooled and the pH was adjusted to 7.0.

Blue Sepharose

The active sample from the 5'-AMP-Sepharose column was applied on to a Blue Sepharose column (3.5 cm \times 11 cm) equilibrated with buffer B [9]. The column was washed with buffer B and then with buffer C. The enzyme was eluted with buffer C containing NAD^+ (1 mg/ml).

FPLC Mono-P HR 5/20

Active fractions from the Blue Sepharose column were pooled and concentrated. The sample was then applied on to an FPLC Mono-P column equilibrated with 10 mM sodium phosphate buffer, pH 6.8, 1 mM EDTA [8]. The column was washed with the same buffer and the enzyme was eluted with a linear 0–1.0 M NaCl gradient. Active fractions were pooled.

RESULTS

Purification of the γ -aminobutyraldehyde-metabolizing enzyme

The purification from brain 1 is summarized in Table 1. The procedure attempted to follow those already published for the rat and bovine enzymes [9,10]. However, this was not always possible. For example, the bovine enzyme became attached to the 5'-AMP-Sepharose 4B column whereas the rat and human enzymes did not. Thus the 5'-AMP column could only be used for the removal of other nucleotide-binding enzymes. The procedure represents one of four purifications attempted, using four different brains, none of which yielded homogeneous enzyme in the final Blue Sepharose step. The material obtained from this step was, however, analysed by both isoelectric focusing and SDS/PAGE (see below). It was also electroblotted on to a nitrocellulose filter in order to obtain pure material for protein sequence analysis.

Isoelectric focusing

The determination of the isoelectric point is shown in Figure 1. It can be seen that the major band, visualized either by protein staining (Figure 1A, lane 2) or by activity towards γ -aminobutyraldehyde (lane 1), has a pI of about 5.3. Although the enzyme appears homogeneous (lane 2), there were several minor protein bands visualized on the gel which are not visible on the photograph.

Subunit molecular mass and Western blot analysis

The main protein component had a subunit molecular mass of 55000 Da (Figure 2, lanes 1 and 3). A PVDF membrane blotted

Table 1 Purification of the γ -aminobutyraldehyde-metabolizing enzyme from human brain

Enzyme was purified from 500 g of brain. For enzyme activity measurement the assay contained 0.1 M sodium phosphate buffer, pH 7.4, 1 mM EDTA, 500 μ M NAD^+ and 500 μ M γ -aminobutyraldehyde. Protein was determined by the method of Goa [14].

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)
$(\text{NH}_4)_2\text{SO}_4$ pptn. and dialysis	4600	6490	1.4	100
CM-Sephadex	1100	2660	2.4	40
DEAE-Sephadex	330	1590	4.8	24
5'-AMP-Sepharose	190	1000	5.3	15
Blue Sepharose	3.5	480	137	7.4

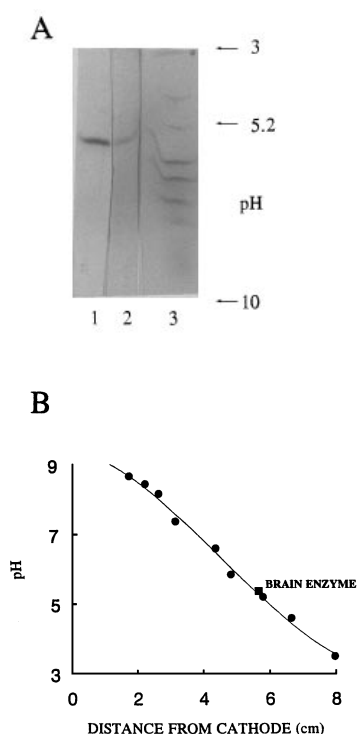


Figure 1 Isoelectric focusing of the human brain enzyme

(A) Isoelectric focusing gel: development for activity towards γ -aminobutyraldehyde (lane 1), and staining with Coomassie Brilliant Blue (lanes 2 and 3). Lanes 1 and 2, brain enzyme; lane 3, standards. (B) Isoelectric point determination. The Pharmacia standards (●) used were: amyloglucosidase (pI 3.5), soybean trypsin inhibitor (pI 4.55), β -lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 7.35), lentil lectins (pI 8.15, 8.45 and 8.65). The brain enzyme (■) is indicated.

with samples of the brain enzyme, liver E3 isoenzyme, a mixture of liver E1 and E2 isoenzymes and standards was probed with anti-E3 polyclonal antibody. The antibody interacted with only the major 55 000 Da component of the brain sample and the liver E3 isoenzyme.

A PVDF membrane blotted with human cerebellar, putamen and hippocampal extracts, following SDS/PAGE, was also probed with anti-E3 antibody. The antibody interacted with a 54 000 Da component in all three extracts, the most intense interaction being with the hippocampal extract.

Specific activity determination

In order to determine the specific activity of the brain enzyme, the purification was repeated and an additional chromatographic step, using an FPLC Mono-P column, was performed. The specific activity of the eluted enzyme was found to be 1.4 μ mol/min per mg. The E3 isoenzyme is extremely unstable and sensitive to atmospheric oxygen. The large increase in specific activity in the FPLC step is due to the removal of inactive enzyme.

Kinetic properties

All the K_m values determined for the human brain enzyme were comparable with those of the liver E3 isoenzyme (Table 2). K_m values for the human brain enzyme were also determined under the conditions used by Abe et al. [9] and Lee and Cho [10] for the

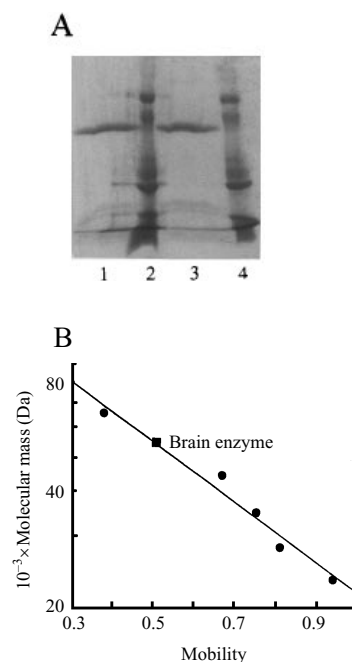


Figure 2 Determination of homogeneity and molecular mass by SDS/PAGE

(A) SDS/polyacrylamide gel. Lanes 1 and 3, brain enzyme; lanes 2 and 4, mixture of Sigma standards: BSA (66 000 Da), egg albumin (45 000 Da), glyceraldehyde-3-phosphate dehydrogenase (36 000 Da), carbonic anhydrase (29 000 Da) and trypsinogen (24 000 Da). The polyacrylamide gel (10%, w/v) was stained with Coomassie Brilliant Blue. (B) Plot of molecular mass versus mobility. The Sigma standards are identified by ● and the brain enzyme by ■.

Table 2 Comparison of the Michaelis constants for the γ -aminobutyraldehyde-metabolizing enzymes from human brain and liver

K_m values were determined at 25 °C using 0.1 M sodium phosphate buffer, pH 7.4, 1 mM EDTA and 500 μ M NAD⁺, conditions previously employed by Kurys et al. [8]. The K_m value for *N*-acetyl- γ -aminobutyraldehyde was also determined at 25 °C, in 0.05 M sodium phosphate buffer, pH 7.4, containing 1 mM EDTA and 500 μ M NAD⁺. Values were obtained from the following references: *, [28]; †, [8]; ‡, [23].

Substrate	K_m (μ M)	
	Human brain	Human liver
γ -Aminobutyraldehyde	7	5*
NAD ⁺	4	14†
Acetaldehyde	40	50†
Glycolaldehyde	200	240
Betaine aldehyde	140	260‡
<i>N</i> -Acetyl- γ -aminobutyraldehyde	110	100

determination of rat and bovine brain enzyme K_m values. Comparison of these K_m values (Table 3) showed that only the rat enzyme K_m value for acetaldehyde was comparable with that of the human brain enzyme.

Protein sequence analysis

Tryptic digestion and amino acid sequencing of the main SDS/PAGE gel protein band (see Figure 2A) resulted in the determination of the primary structure of one peptide, T44. Its sequence matched that of the liver E3 isoenzyme at residues

Table 3 Comparison of the Michaelis constants for the γ -aminobutyraldehyde-metabolizing enzymes purified from human, rat and bovine brain

All K_m values were determined under the conditions described by Abe et al. [9], which were: 0.1 M potassium phosphate buffer, pH 8.0, at 37 °C, containing 2.5 mM NAD⁺. The K_m value for NAD⁺ was determined at 2 mM acetaldehyde. The acetaldehyde K_m value for human liver E3 was 32 μ M. The values for the rat brain enzyme are from [9], and those for the bovine brain enzyme from [10].

Substrate	K_m (μ M)		
	Human brain	Rat brain	Bovine brain
γ -Aminobutyraldehyde	70	151	154
NAD ⁺	4	87	53
Acetaldehyde	22	20	625
Glycolaldehyde	200	40	—

Table 4 Identification of tryptic peptides obtained from the purified brain enzyme that metabolizes γ -aminobutyraldehyde

Amino acid residues are numbered according to the human liver E3 isoenzyme sequence reported by Kurys et al. [16].

Peptide	Method of identification	Sequence	Amino acid positions
T35	MS	SPLIFSDCDMNNNAVK	227–242
T20	MS	VLGGDIYVPEDPK	321–334
T36	MS	ANDTTFGLAAGVFTR	380–394
T44	Amino acid sequencing	VVAELQAGT(C)FINNYNVXPV	402–421

402–421 (Table 4) [16]. Three other peptides, T20, T35 and T36, were identified by MS as matching predicted liver E3 tryptic fragments at residues 321–334, 227–242 and 380–394 respectively (Table 4) [16,17].

Primary structure derived from cDNA

Screening of the cerebellar cDNA library resulted in the isolation of five clones of 2.0 kb, all of which appeared to be identical as determined by restriction enzyme analysis. Sequencing of one of the clones yielded the DNA sequence, and derived amino acid sequence, shown in Figure 3. Comparison of this sequence with that of the published liver E3 cDNA [16] showed that the two cDNAs were almost identical. The brain cDNA was longer at the 5' end by 12 nucleotides and at the 3' end by 221 nucleotides plus the poly(A) tail (Figure 3, underlined). There were also six nucleotide differences (Figure 3, marked by *) and one nucleotide insertion (Figure 3, double underlined). Nucleotide 246 (234 in the liver cDNA) changed from a C to a T. This base change did not result in an amino acid change at position 82 (position 78 in the liver sequence); it remained an isoleucine. A three-base change at positions 263–265 (251–253 in the liver cDNA), CGT to GCC, resulted in the presence of cysteine at position 88 (compared with serine at the equivalent position 84 in the liver sequence), but leucine-89 did not change. Nucleotide 1395 (1383 in the liver cDNA) changed from a G to a T but did not result in an amino acid change, with an alanine in both liver and brain sequences. Nucleotide 1460 (1448 in liver cDNA), in the 3' non-coding region, also changed from a G to a T. There was also a T insertion in the brain cDNA 3' non-coding region, at nucleotide 1630. In order to determine that the nucleotide changes observed

in the cerebellar cDNA were real, another human brain cDNA library (fetal brain; Clontech) was screened using the same probes as those used to screen the cerebellar library. With the exception of nucleotide 246, all of the above-mentioned nucleotide changes were observed in a 2.2 kb cDNA isolated from the fetal brain library. The reason for the difference at nucleotide 246 between the cerebellar and fetal brain cDNAs is not known. The T insertion observed in the cerebellar cDNA at nucleotide 1630 was also present in the fetal brain cDNA 3' non-coding region. Thus the sequence derived from the brain cDNA differed from that of the liver E3 cDNA by one amino acid: a cysteine at position 88 (position 84 in liver cDNA) instead of a serine. As in the case of the liver E3 cDNA [16], the start codon of the brain cDNA was never encountered.

Distribution of E3 mRNA in the brain

The 1.6 kb E3 cDNA probe hybridized to mRNA in all regions of the brain represented on the Multiple Tissue Northern blots (Figure 4). Only one 2.9 kb band was seen in all lanes, demonstrating the presence of only one species of E3 mRNA in the human brain. On re-probing the blots with the control β -actin cDNA, the expected 2.0 kb band was observed in each lane (not shown). As can be seen in Figure 4, the E3 mRNA levels were not uniform. Autoradiographs of the two blots were scanned using a Mustek MFS 6000 scanner and the SigmaGel gel analysis software (Jandel Scientific), in order to compare band intensities and thus determine the distribution of E3 mRNA in the brain. The order of distribution, starting with the highest levels of mRNA, was as follows: spinal cord > corpus callosum > subthalamic nucleus > medulla > thalamus > amygdala > cerebral cortex > substantia nigra > hippocampus > caudate nucleus > temporal lobe > cerebellum > putamen > frontal lobe > whole brain > occipital pole.

DISCUSSION

Enzyme purification, cDNA cloning and Northern blot analysis were three major approaches utilized during this investigation in an attempt to determine the identity and occurrence of the enzyme that metabolizes γ -aminobutyraldehyde in the adult human brain. The purification (Table 1; Figures 1 and 2) did not result in complete homogeneity, but the γ -aminobutyraldehyde-metabolizing enzyme was the major component. In three human brains, only one protein band with activity towards γ -aminobutyraldehyde was observed on the isoelectric focusing gel (Figure 1, lanes 1 and 2). Its pI value of 5.3 (Figure 1) was identical with that previously determined for the major component of the human liver E3 isoenzyme [8]. The minor component of the liver enzyme (pI 5.45) was observed in only one human brain (brain 4). One major protein band was also visualized by SDS/PAGE (Figure 2, lanes 1 and 3). Its subunit molecular mass of 55000 Da was comparable with that of the liver E3 isoenzyme (54000 Da) [8]. The major SDS/PAGE band also interacted with a polyclonal anti-E3 antibody on a Western blot. Tryptic digestion of this band yielded a 20-amino-acid peptide whose sequence completely matched part of that of the E3 isoenzyme, and three other peptides which were matched to the E3 sequence by MS (Table 4).

The 2.0 kb cerebellar cDNA was longer at the 3' end than the liver E3 cDNA [16] and included a poly(A) tail. Six nucleotide differences were also observed between the two cDNAs and an insertion was found in the brain cDNA 3' non-coding region. These differences were not due to sequencing error and, because almost all the nucleotide substitutions in the cerebellar cDNA

cdNA	<u>GAGAAAGCTTTCGAGCCGCAACCGCCGAGTGATGCTACTTTCACATGTTCCAGAGAAAAGGAAATTTGGCTGTTCAAAATGCA</u> 0090 <u>E K A F E P A T G R V I A T G T C S G E K E V N L A V Q N A</u>
cdNA	AAGGCTGCTTTAAAAATATGGAGTCAAAAACTGGCATGGAGCGTTGCCGAATCCTTTGGAGGCTGCCAGGATAAATAGGGAAACGGGAG 0180 K A A F K I W S Q K S G M E R C R I L L E A A R I I R E R E
cdNA	GATGAAATGCTACTATGGAGTGCATCAACAATGGCAAGTCCATCTTTGAGGCCCGCTGGCAATTGACATTTCTCTGGCAGTGCCTGGAG 0270 D E I A T H E C I N N G K S I F E A R L D I D I S W Q C L E
cdNA	TATTATCGGGCTGGCTGCATCCATGGCTGGTGAACACATCCAGCTCCAGGTGGATCGTTGGTTATACAGAAAGAGAACCCTTGGG 0360 Y Y A G L A A S M A G E H I Q L P G G S F G Y T R R E P L G
cdNA	GTATGTGGGAAATAGGAGCATGGAACACTCCCTTTTCAGATTGCCTCTTGAAGTGGCTCCAGCATTAGCCTGGTAAATGCCATGGTC 0450 V C V G I G A W N Y P F Q I A S W K S A P A L A C G N A M V
cdNA	TTTAAACCTTCCCTTTACACCTGTTTCTGCATGCTACTGGCTGAAATCTACAGTGAGGCTGGTGTACTCTCTGGGCTCTTCAATGTG 0540 F K P S P F T P V S A L L L A E I Y S E A G V P P G L F N V
cdNA	GTCCAGGGAGGGCTGCCACAGGCCAGTTTCTGTGTCAGCATCCCGATGTGGCCAAAGTCTCCTTCACTGGAAAGTGGCCACTGGCATG 0630 V Q G G A A T G Q F L C Q H P D V A K V S F T G C S V P T G
cdNA	AAGATCATGGAGTGTGACATAAAGAACTCAAACCTGTTACCTTGGAACTGGAGCCAAATCTCCACTCATCTTCTCAGACTGTGAT 0720 K I M E M S A K G I K P V T L E L G G K S P L I I F S D C D
cdNA	ATGAACAATGCTGTAAGGGGGCGCTGATGGCCAACTTCTCACACAAGCCAGGTTTGCCTGTAATGGCACAAGAGTATTTGTGCAGAAA 0810 M N N A V K G A L H A N F L T Q G Q V C C N G T R V F V Q K
cdNA	GAAATCTTGATAAAATTTACAGAGGAAGTGGTGAACAAGCCAAAGGATTAATAATGGAGATCCCTTCTGGAAGATACAAGGATGGGT 0900 E I L D K F T E E V V K Q T Q R I K I G D P L L E D T R H G
cdNA	CCACTCATCAACCGACCAACCTGGAGCGATCCTTGGGTTTGTCAAAGTGGCAAAGGAGCAGGGTCTAAAGTGTATGTGGTGGAGAT 0990 P L I N R P H L E R V L G F V K V A K E Q G A K V L C G G D
cdNA	ATATATGTACCTGAAGATCCCAAAATTAAGGATGGATATTACATGAGACCTTGTGTATTAACATAATTCAGAGACGATGACCTGTGTG 1080 I Y V P E D P K L K D G Y Y M R P C V L T N C R D D M T C V
cdNA	AAGGAAGAGATCTTTGGCCCTGTATGTCCATTTTATCATTTGACACTGAAGCTGAGGTTCTAGAAAGGCCAATGATACCACTTTTGA 1170 K E E I F G P V M S I L S F D T E A E V L E R A N D T T F G
cdNA	CTAGCAGCTGGGCTCTTTACAGGGACATCCAAAGGGCTCATAGAGTGGTAGCTGAGCTTCAGGCTGGGACGTGCTTCATTAACAATAT 1260 L A A G V F T R D I Q R A H R V V A E L Q A G T C F I N N Y
cdNA	AACGTCAGCCAGTGGAGTTCGCCCTTTGGTGGATATAAGAAGTCAAGATTTGGCAGAGAGAAGCCCTGTGACAATCGAATATTATPCA 1350 N V S P V E L P F G G Y K K F G F G R A N G R V T I E Y Y S
cdNA	CAGCTGAAGACTGTGTGTGGAGATGGGTGATGGAACTGCTTTTGAACCTGCAGTGAACCTATTGACATGGCAGCTGTGGA 1440 Q L K T V C V E H G D V E S A F ***
cdNA	ATGATGTGAATTTGGCCCTGTTTACAGAGGAGTACAACTGAATGTTATTTTACATCCAGAAATTTGGCCTTCAGTATAAGAGAAATGGTTC 1530
cdNA	ATGTACTCTTTCTCTCCATCAGCTTCTCCTCACTGAAAATGTGCATTAAGTGCCTTGTAGATACTAATCAAGAAAGCTGTGAAATCTCCT 1620
cdNA	CAAAGCGTATTTTGTGAAATCTTTAAGAGCCAGTAACTACTCTAGAGAACAGGAAAAGAGACTAGAATAATACATCTTCCACACAT 1710
cdNA	<u>TTGGCCACTGATAATGTTAATCTCTGGCCTATTTCAAAGAAGTCTGTTCTCTGGCTGATCCAAAGTGCAGTGGTATTTCAAACTAATTTGAT</u> 1800
cdNA	<u>CACAACCAAGTTTGTAGATTTCTTTGTTCTCTCCATCCCACTGCTTCACTTGGCTGATGTAAGAAAAAACAACAAAAA</u> 1890
cdNA	<u>AA</u>

Figure 3 cDNA isolated from a human cerebellar library and derived amino acid sequence

The cerebellar cDNA is longer than the published liver E3 cDNA [16] at the 5' and 3' ends (underlined regions). Nucleotide differences from the liver E3 cDNA are marked by *, and the nucleotide insertion is doubly underlined.

were also observed in the fetal brain cDNA, they were most probably not due to reverse transcription errors during the making of the cerebellar cDNA library. Of the five nucleotide substitutions found in the coding region of the brain cDNA, only one resulted in an amino acid substitution. As a result, the brain enzyme amino acid sequence differs from the liver E3 sequence by one amino acid, a cysteine at position 88 (position 84 in liver E3) instead of a serine. The presence of serine at position 84 in liver E3 was corroborated by both cDNA and protein sequencing [16]. Thus the human brain contains a variant of the E3 isoenzyme, designated E3'.

Like the liver E3 isoenzyme, the brain enzyme catalysed the dehydrogenation of betaine aldehyde, acetaldehyde, propionaldehyde and other aldehydes as well as that of γ -

aminobutyraldehyde. Its K_m values for these substrates were similar to those determined for the liver E3 isoenzyme (Table 2). Interestingly, at 37 °C the K_m values for acetaldehyde of both the brain and liver enzymes decreased to about 50% of their values at 25 °C (compare Tables 2 and 3), which suggests that E3 may be important in alcohol metabolism. Following gradient elution from the FPLC Mono-P column, the specific activity of the brain enzyme (1.4 $\mu\text{mol}/\text{min per mg}$) and its γ -aminobutyraldehyde/propionaldehyde activity ratio (3.3) were also similar to those of the E3 isoenzyme (1.7 $\mu\text{mol}/\text{min per mg}$ and 3.2 respectively [8]). All of the above results suggest that the catalytic properties of the brain enzyme and the liver E3 isoenzyme are similar.

Comparison of the properties of the human brain γ -aminobutyraldehyde-metabolizing enzyme with those of the

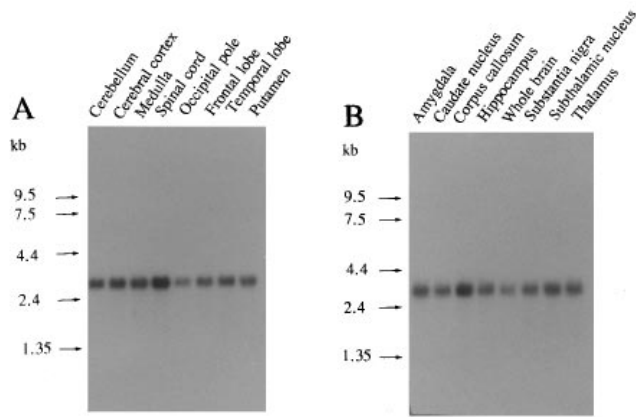


Figure 4 Northern blot analysis of RNA from various human brain regions

Shown are autoradiographs obtained following hybridization of the multiple tissue Northern blots (Clontech) with a radiolabelled 1.6 kb liver E3 cDNA and exposure for 18 h. The positions of the RNA molecular mass standards (in kb) are indicated for each blot.

similar enzymes from rat and bovine brain [9,10] revealed some similarities and some differences. All three enzymes have a subunit molecular mass of 50000–58000 Da, and a similar and broad substrate specificity. However, the K_m value for γ -aminobutyraldehyde appeared to be higher for the rat and bovine enzymes than for the human brain enzyme. These differences could have been due to the assay conditions used. Therefore K_m value determinations for the human brain enzyme were repeated at pH 8.0 and 37 °C, the conditions used to determine the rat and bovine enzyme K_m values (Table 3). The human brain enzyme K_m value for γ -aminobutyraldehyde increased 10-fold to 70 μ M, but it was still half that of the rat and bovine enzymes. At high pH and high temperature, γ -aminobutyraldehyde is likely to cyclize more rapidly into Δ^2 -pyrroline, which could result in a higher apparent K_m value for γ -aminobutyraldehyde. K_m values for the human brain enzyme for NAD⁺ and glycolaldehyde remained the same (compare Tables 2 and 3); only the K_m value for acetaldehyde agreed with that of the rat enzyme under these conditions. Thus it appears that, while the γ -aminobutyraldehyde-metabolizing enzyme from human brain is similar to the liver E3 isoenzyme [8], it may be different from the γ -aminobutyraldehyde-metabolizing enzymes from rat and bovine brain [9,10].

The E3 isoenzyme has been postulated to play a role in the formation of GABA from putrescine [8]. The reportedly low levels of diamine oxidase in mammalian brain [5] point to the formation of GABA from acetylated putrescine via monoamine oxidase. It was, therefore, expected that the brain enzyme would reflect this in its K_m value for *N*-acetyl- γ -aminobutyraldehyde. However, there is no difference in the K_m values for *N*-acetyl- γ -aminobutyraldehyde between the human brain E3' and liver E3 enzymes. Thus the brain enzyme may be involved in the formation of GABA from putrescine either directly via diamine oxidase, or indirectly through acetylated putrescine via monoamine oxidase. Since GABA is synthesized mainly by glutamate decarboxylase in the central nervous system, the physiological role of the E3' isoenzyme in the brain is still uncertain. Recently the E3 isoenzyme has been identified as a human betaine aldehyde dehydrogenase [20], which suggests a role for it in choline metabolism. Choline is converted into betaine aldehyde by choline dehydrogenase, and then into betaine by betaine aldehyde

dehydrogenase. Betaine appears to be necessary for osmo-regulation in all living organisms [21,22]. In mammals it has been implicated in the alleviation or even prevention of fatty and cirrhotic liver [23]. It also functions as a methyl donor and a nitrogen source [24,25].

The occurrence of different isoenzymic forms in different tissues has been known for a long time. A prime example is lactate dehydrogenase, a tetramer made up of two types of subunits (H and M); the H type predominates in the heart and the M type predominates in liver and skeletal muscle [26]. Both forms of the enzyme have become subtly adapted to the different metabolic requirements of these tissues. For the brain E3' enzyme, the serine-to-cysteine substitution does not appear to have any effect on those catalytic constants that have been determined so far. During purification, however, it was noted that the brain enzyme appeared to be more stable than the liver E3 isoenzyme. It is, therefore, possible that the serine-84-to-cysteine substitution leads to some structural rearrangement, perhaps the introduction of a disulphide bond, which renders the cysteine-substituted E3' enzyme more stable. However, the functional significance of the serine-to-cysteine replacement in the brain enzyme is as yet unknown.

During the purification of the brain enzyme it was observed that the amount of γ -aminobutyraldehyde-metabolizing activity was not proportional to the weight of brain used for enzyme purification. This suggested that different brain regions may vary in enzyme content. Western blot analysis of cerebellar, putamen and hippocampal extracts demonstrated the presence of anti-E3-antibody-interacting protein in all three brain areas, with the strongest interaction in the hippocampus. Northern blot analysis (Figure 4) showed that a single 2.9 kb band of E3' mRNA was present in all regions of the brain that were tested. This suggested that E3' mRNA is localized throughout the whole brain. Comparison of E3' mRNA levels in the cerebellum, putamen and hippocampus by autoradiograph scanning showed that the hippocampus had the highest levels, confirming the results from Western blotting, and also suggesting that E3' mRNA levels reflect the amount of expressed E3' protein in the brain. When all brain areas were compared, E3' mRNA levels were highest in the spinal cord. Interestingly, it has been reported that betaine levels are higher in the spinal cord than in the brain of guinea pigs [27]. This could result from the presence of higher levels of an enzyme metabolizing betaine aldehyde. Thus the E3' enzyme may also be involved in the synthesis of betaine in the central nervous system.

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