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Purification and Characterization of Bovine Brain γ-Aminobutyraldehyde Dehydrogenase

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Summary: γ-Aminobutyraldehyde dehydrogenase was purified to homogeneity from bovine brain. The molecular weight of the native enzyme and subunit were 230,000 and 58,000, respectively. The Km value for γ-aminobutyraldehyde and NAD^+ were 154 μM and 53 μM, respectively. The optimum pH and temperature were 8.0 and 37°C, respectively. N-terminal sequence of the enzyme is as follows: NH_2-S-A-A-T-Q-A-V-P-T-P-N-Q-Q-COOH. The enzyme migrates on isoelectric focusing with pI=6.5. Enhancement of the enzyme activity by polyamine, Mn^{2+}, Mg^{2+} and inhibition by γ-aminobutyric acid and Zn^{2+} will enhance the limited information on regulation of the γ-ABALDH activity and GABA metabolism to some extent.

Putrescine (1,4-diaminobutane), one of the polyamines, degrades to γ-aminobutyric acid (GABA) via the intermediate γ-aminobutyraldehyde (γ-ABAL)(1,2). It thus provides ABAL substrate for the synthesis of GABA by γ-aminobutyraldehyde dehydrogenase (γ-ABALDH), which is well known to be the major inhibitory neurotransmitter in mammalian CNS. This pathway is an another pathway for GABA synthesis (3). In spite of studies on purification and biochemical characterization from rat brain (4) and human liver(5,6), no satisfactory explanation of the means by which the enzyme is regulated is yet available.

This paper reports studies intended to show N-terminal sequence of the enzyme from the bovine brain, which has not been previously reported. Most importantly, the data presented here also show that the effects of polyamine enhanced enzyme activity very significantly, suggesting it would be a novel regulation of GABA metabolism.

Materials and Methods

Materials: NAD^+ and SDS were purchased from Sigma. γ-ABAL was prepared by hydrolysis of 4-aminobutyraldehyde diethyl acetal. 4-Aminobutyraldehyde diethyl acetal was purchased from Aldrich Chemical Company, Inc. Acetaldehyde and propionaldehyde were from Fluka. Succinic semialdehyde was purchased from

ABBREVIATIONS: γ-ABAL, γ-aminobutyraldehyde; γ-ABALDH, γ-aminobutyraldehyde dehydrogenase; GABA, γ-aminobutyric acid; NAD^+, β-nicotinamide adenine dinucleotide; SDS, sodium dodesyl sulfate; FPLC, fast protein liquid chromatography.

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Sigma. DEAE-Sephacel, 5'-AMP-Sepharose 4B and Sephacryl S-300 were from Sigma. CM-Trisacryl and Mono-Q column were from Pharmacia Fine Chemicals. All other chemicals were reagent grade. All bovine brains were obtained from a butcher-house and maintained frozen at - 70 ° C.

Activity Assay: During purification, enzyme activity was assayed in a solution containing a 0.1 M K^+-phosphate buffer (pH 8.0), 5 mM NAD^+, 0.5 mM DTT, 2 mM γ-ABAL. All assays were performed spectrophotometrically at 340 nm (7). For the effect of polyamines, purified enzyme activities were assayed in a assay solution containing polyamines with different concentrations.

Protein Determination: Protein concentration was determined as described by Lowry et al.(8) with BSA as standard, or spectrophotometrically at 280 nm.

Enzyme Preparation: About 400 g of bovine brain tissues kept frozen at -70 ° C were homogenized in 4 vol. of 50mM K^+-phosphate buffer, pH 7.0, containing 1mM-2-mercaptoethanol. The homogenate was centrifuged at 10000 g for 50 min., and a clear supernatant was obtained.

Purification: All procedures were carried out at 0-4 ° C. Buffers used for enzyme purification were as follows. Buffer A: 5 mM K^+-phosphate buffer (pH 6.0) containing 1mM mercaptoethanol, Buffer B: 5 mM K^+-phosphate buffer (pH 7.0) containing 1mM mercaptoethanol. Crude enzyme was fractionated with 40%-75% satd.$(NH_4)_2SO_4$. The suspension was centrifuged at 10000 g for 50 min., the pellets were dissolved in buffer A, and the solution was dialysed against buffer A. The enzyme solution was loaded on a CM-Tris Acryl ion exchange column (4 x 15 cm) preequilibrated with buffer A. The column was washed with the same buffer. Fractions containing γ-ABALDH activity were pooled together, and the pooled fractions were dialysed against buffer B. The dialysed sample was applied on a DEAE-Sephacel ion exchange column (4 x 15 cm) preequilibrated with buffer B and washed with the same buffer. γ-ABALDH was eluted with a linear gradient of 0-200 mM KCl and fractions containing enzyme activity were collected. Collected fractions were concentrated with Amicon ultrafiltration membrane PM10, and the pH was adjusted to 6.0. The concentrated enzyme fractions were applied on a 5'-AMP-Sepharose column (microcolumn), which was previously equilibrated with buffer A. The column was washed with the same buffer and was eluted stepwise with buffer B containing 1mM NAD^+. Active fractions were pooled and then concentrated. The enzyme fractions were subjected to additional chromatography, fast protein liquid chromatography (FPLC) on a Mono-Q column. The column was equilibrated with a 20mM-potassium phosphate buffer (pH 7.0), and elution of the enzyme was via a linear salt gradient of 0-500 mM NaCl in the same buffer. The purified enzyme solution was collected and used for all other studies.

Homogeneity and Molecular Weight: Molecular weights of native enzymes were determined by the Sephacryl S-300 gel filtration and 5 - 30% polyacrylamide gradient gel electrophoresis. The molecular weight of subunits was obstained by the procedure of Laemmli (9) employing SDS polyacrylamide gel. Protein standards for the SDS-gel electrophoresis were purchased from Pharmacia.

Isoelectric Focusing: Isoelectric focusing was carried out on agarose plates composed of 1% (w/v) agarose, 12% (w/v) sorbitol, 0.063% of Pharmalyte, pH 3.5-pH 9.3 (Pharmacis Fine Chemicals), for 16 h at 125 V. The enzyme was rendered visiable by "protein stain" with Coomassie Brilliant Blue. For isoelectric point determination, an Isoelectric Focusing Calibration Kit, Broad Range (pH 3.5-9.3) was purchased from Pharmacia Fine Chemicals and employed according to the instructions of the manufacturer.

Kinetic Studies: Kinetic studies for different aldehyde substrate were performed spectrophotometrically at 340 nm and 37 ° C in 1 mL total volume: 0.1 M K^+-phosphate buffer (pH 8.0), 5 mM NAD^+. *Km* for NAD^+ were determined in 2 mM γ-ABAL.

Esterase Activity and Disulfiram Inhibition: Esterase activity was determined spectrophotometrically at 400 nm. The reaction mixture was 0.1 M K^+-phosphate buffer (pH 7.0), 0.3 mM p-nitrophenyl acetate and purified enzyme. The activity of the disulfiram-inactivated enzyme was determined (10).

N-terminal Sequencing: N-terminal sequencing was performed on the Applied Biosystems Model 477A Peptide Sequencer. An estimated 1 nmoles were loaded for sequencing.

Table 1. Purification of γ-aminobutyraldehyde dehydrogenase from bovine brain

Step	Total Prorein (mg)	Total Activity (unit)	Specific Activity (unit\mg)	Recovery (%)	Purification (fold)
Crude	785.8	105.2	0.1339	100	1
$(NH_4)_2SO_4$ (45-70 %)	497.3	73.3	0.1474	69.7	1.1
CM-Trisacryl	109.9	29.1	0.2648	27.7	2.0
DEAE-Sephacel	6.9	24.9	3.61	23.7	27.0
5'-AMP-Sepharose	0.32	8.9	27.81	8.5	207.7

Results and Discussion

The results of purification of γ-ABALDH are summarized in Table 1. Because the method employed by Abe et al (4) to purify the enzyme from rat brain was not suitable for purification of the enzyme from bovine tissues, we did not use DEAE-cellulose, phosphocellulose, Blue Agarose, and Sephacryl S-300 as they did; we instead used CM-Trisacryl and DEAE-Sephacel. The purified enzyme was subjected to additional chromatography, FPLC on a Mono Q column for amino acid composition and N-terminal sequencing. The molecular weight of the purified enzyme was determined to be 230,000 by Sephacryl S-300 gel filtration. And SDS gel electrophoresis showed a single band indicating that the subunit molecular weight of the enzyme was 58,000 (Fig. 1). These data suggest that the enzyme is a homo-tetrameric enzyme. Furthermore, the molecular weight of the bovine enzyme is larger than that of the rat brain enzyme with 210,000.

Unexpectedly, the bovine enzyme did obey typical Michaelis-Menten kinetics with a K_m value of 154 μM (Table 2), which is almost the same as that of the rat brain (151μM)(4). However, K_m values for other substrates used in our experiment were quite larger than those of the rat brain enzyme. Clearly, the rat brain enzyme has a lower specificity toward γ-ABAL than those toward acetaldehyde, propionaldehyde,

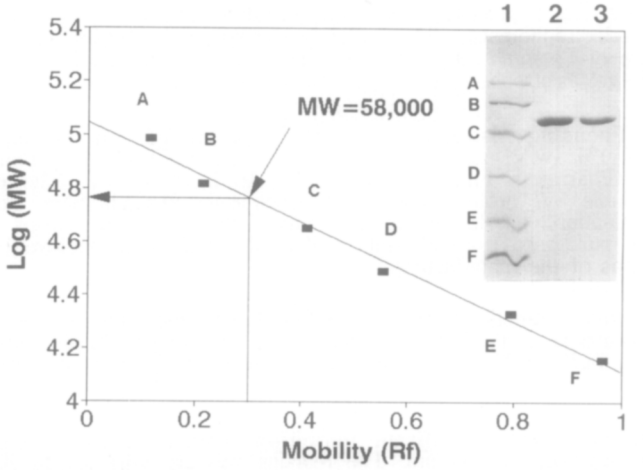

Fig. 1. Determination of the molecular weight of subunit by SDS-polyacrylamide gel electrophoresis (12.5 %). A, phosphorylase b (97,000); B, bovine serum albumin (66,000); C, ovalbumin (45,000); D, carbonic anhydrase (29,000); E, trypsin inhibitor (20,100); F, lysozyme (14,300); ←, γ-ABALDH. Inset: Lane 1, molecular weight markers; Lane 2, ABALDH (20μg) Lane 3, ABALDH (10μg).

Table 2. Kinetic properties of an enzyme

Varied substance (mM)	Constant Substrate (mM)	K_m (μM)
NAD^+(0.05-5)	γ-ABAL(2.0)	53
γ-ABAL(0.05-1)	NAD^+(5.0)	154
Acetaldehyde(0.7-17.7)	NAD^+(5.0)	625.0
Propionaldehyde(0.2-13.8)	NAD^+(5.0)	365.4
Succinic semialdehyde(0.07-1.4)	NAD^+(5.0)	241.1

The assay system contained 0.1 M-pottassium phosphate buffer, pH 8.0. Km and V values were respectively obtained from the slope and intercept of a first-order regression line fitted to Lineweaver-Burk plots.

and succinic semialdehyde. However, the bovine purified enzyme has a higher specificity toward γ-ABAL than any other substrates.

The bovine purified enzyme is very unstable when subjected to heat, and the enzyme activity completely disappeared at 50 °C for 20 min, and it was hard to store it even at -20 °C without severe loss of activity. The optimal temperature and optimal pH for activity were 37 °C and 8.0, respectively. The isoelectric point was determined to be 6.5 by gel isoelectric focusing(Figure not given).

The N-terminal sequencing of the bovine enzyme was made and turned out to be NH_2-S-A-A-T-Q-A-V-P-T-P-N-Q-Q-COOH, which is identical to that of human liver aldehyde dehydrogenase (11,12) except alanine substituted by threonine. Interestingly, the N-terminal of the rat brain enzyme was reported to be blocked, and data is not available for now.

The purified enzyme also has the esterase activities. Disulfiram known as an inhibitor of aldehyde dehydrogenase was used in different concentrations, and enzyme activity was decreased by 70% when 10 μM of disulfiram was applied. Enzyme activity was decreased by 83% when 50 μM was applied (Table 3).

γ-ABALDH was slightly activated by Mg^{2+} (13) and Mn^{2+} at 50μM - 100μM, whereas it was inhibited by Zn^{2+}. γ-ABALDH was remarkably activated by spermidine and spermine (Table 3), which have been reported to enhance enzyme activity such as S-adenosylmethionine decarboxylase (14). Unexpectedly, GABA was observed to inhibit the enzyme to some extent (Table 3). This is the first

Table 3. Effects of polyamines and other compounds on the activity of γ-Aminobutyraldehyde dehydrogenase from bovine brain. Purified enzyme was used. Enzyme activities were assayed under the standard assay conditions.

Addition	Conc.	Relative Activity(%)	Addition	Conc.	Relative Activity(%)
Control		100			
Putrescine	100 μM	94.4	$MgCl_2$	50 μM	119.2
	1 mM	96.9		100 μM	130.7
Spermidine	100 μM	260	$MnCl_2$	50 μM	124.1
	1 mM	280		100 μM	141.1
Spermine	100 μM	248	$ZnCl_2$	50 μM	77.1
	1 mM	266		100 μM	75.3
Cadavaline	100 μM	89.2	Disulfiram	10 μM	30.4
	1 mM	103.2		50 μM	17.0
γ-aminobutyrate	10 μM	89.8			
	100 μM	81.1			
	1 mM	66.7			

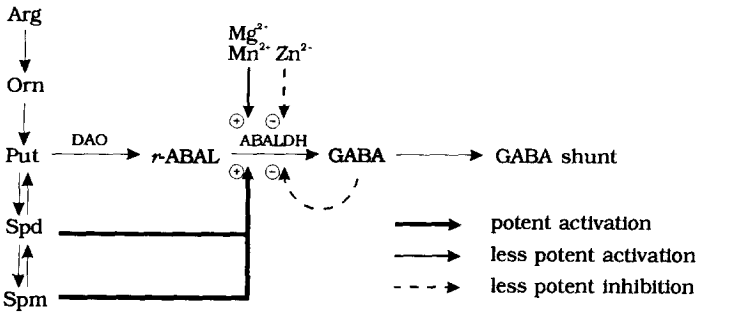

Fig. 2. Possible scheme of the regulation of γ-aminobutyraldehyde dehydrogenase by polyamines and others in bovine brain. Orn; ornithine, Put; putrescine, Spd; spermidine, Spm; spermine, γ-ABAL; γ-aminobutyraldehyde, GABA; γ-aminobutyric acid, DAO; diamine oxidase, γ-ABALDH; γ-aminobutyraldehyde dehydrogenase.

report of such activation and inhibition by polyamine and others, suggesting that γ-ABALDH could be regulated and the GABA level would be controlled, and in turn have an effect on GABA metabolism to some extent. Such cumulative results lead us to propose the following scheme (Fig 2).

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