Two isozymes of branched-chain amino acid aminotransferase in submandibular gland of the monkey

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[Accepted for publication: May 21, 1984]

Key words: branched-chain amino acid / aminotransferase / submandibular gland

Abstract: Crude extract from the submandibular gland of a macaque monkey contained a high activity of branched-chain amino acid aminotransferase. Two isozymes of aminotransferase were detected in the crude extract and separated by DEAE-Sepharose CL-6B column chromatography. The isozymes were partially purified and the properties of the isozymes studied. These isozymes were different in their affinity for substrates and the velocity in reverse reaction beside their behaviors on the chromatographies. Distribution of the isozymes in other monkey tissues was also examined.

Introduction

Branched-chain amino acids, leucine, isoleucine and valine, are primarily degraded in extra-hepatic tissues, although most of the indispensable amino acids are catabolized in the liver^{1,2}. Skeletal muscle rapidly degrades these branched-chain amino acids, and muscle is probably the primary site of their degradation³⁻⁹. The oxidation of leucine supplies appreciable amount of ATP to muscle, especially in starvation⁴. Adipose tissue also actively degrades leucine: leucine can be utilized as a precursor of fatty acid and sterol and also serves as a source of nitrogen for synthesis of glutamine, glutamate and alanine¹⁰⁻¹².

The first step in the catabolic pathway of these amino acids is catalysed by a branched-chain amino acid: 2-oxoglutarate aminotransferase (EC 2.6.1.6). The aminotransferase activity is high in extra-hepatic tissues and low in the liver^{2,13}. Previous paper has reported that crude extracts from submandibular glands of several animals con-

tained high activities of branched-chain amino acid aminotransferase comparable to those from the heart *etc.*¹⁴ The enzyme of rat submandibular gland was partially purified; the properties of the purified enzyme suggested that it was the same type as that from the heart. The study also indicated the occurrence of two types of enzyme only in the macaque monkey submandibular gland among the animals examined. In the present study, two isozymes of aminotransferase were partially purified from the macaque monkey submandibular gland and characterized.

Materials and Methods

Animal

Monkeys (*Macaca irus*) were maintained on laboratory diet *ad libitum*.

Preparation of tissue extract

All procedures were carried out at 0-4°C. Monkeys were anaesthized with ethyl ether, and killed by bleeding. The various tissues were removed, minced and homogenized in a Ultra-Disperser (Model LK-21, Yamato

Inc., Japan) for 1 min with 5 vol. of 5 mM potassium phosphate buffer (pH 7.5)-0.2 mM pyridoxal phosphate. Each homogenate was sonicated for 30 s at 10 KHz, squeezed through gauze and then centrifuged at $100,000 \times g$ for 40 min. The resulting supernatants were referred to as the crude tissue extracts.

Separation of isozymes I and III of aminotransferase

All manipulations were carried out at 0-4°C. The crude extract from submandibular glands (7.0 g) was prepared as above (Step 1) and treated with ammonium sulphate. A fraction precipitated between 25 and 75% $(\text{NH}_4)_2\text{SO}_4$ saturation was collected by centrifugation, dissolved in 5 mM potassium phosphate buffer (pH 8.2)-1 mM EDTA-2 mM 2-mercaptoethanol-0.01 mM pyridoxal phosphate, and dialysed against the same buffer (Step 2). These concentrations of EDTA and 2-mercaptoethanol were added to all buffers used during the purification and pyridoxal phosphate was contained only in dialysis buffers. The enzyme solution was applied to a column (2.0 $\times$ 18 cm) of DEAE-Sephacrose CL-6B equilibrated with the same

buffer (Fig. 1). The column was washed with 180 ml of the same buffer and eluted with a linear concentration gradient of 5-500 mM phosphate (680 ml). Fractions of 18-ml were collected. The activity of aminotransferase was eluted from the column in two fractions. The first fraction was eluted at about 80 mM phosphate and the second fraction at about 280 mM phosphate. The first fraction (fractions 12-17) was pooled, concentrated by ultrafiltration, dialysed against 5 mM potassium phosphate buffer, pH 7.5 and used as isozyme I fraction in further purification (Step 3). On the other hand, the second fraction (fractions 28-35) was also pooled, concentrated by ultrafiltration, dialysed against 10 mM potassium phosphate buffer, pH 6.8 and used as isozyme III fraction in further purification (Step 3').

Purification of isozyme I

The isozyme I fraction obtained from DEAE-Sephacrose CL-6B column chromatography was subjected to isoelectric focusing on a 110 ml column (LKB) according to the method of Vesterberg and Svensson¹⁵⁾. Focusing was carried out on a pH 3-10 Pharmalyte gradient for 40 h at 500 V. The

Fig. 1 Elution profile of aminotransferase on DEAE-Sephacrose. (○), Aminotransferase activity; (●), protein monitored by the absorbance at 280 nm; (---), potassium phosphate concentration.

eluate was collected in 2-ml fractions and the activity and pH of each fraction were measured. Enzyme activity was detected at pH 7.1 in one peak. The active fractions 28–31 were pooled, concentrated by ultrafiltration and applied to a column (2.5×100 cm) of Sephacryl S-200 in 50 mM potassium phosphate buffer (pH 7.5)–10% glycerol. The column was eluted at flow rate of 25 ml/h with the same buffer and 3-ml fractions were collected. The active fractions of enzyme emerging in a single peak at fractions 99–114 were pooled, concentrated by ultrafiltration and dialysed against 10 mM potassium phosphate buffer, pH 6.8 (Step 4). The dialysed solution was applied to a column (1.0×8.8 cm) of hydroxyapatite equilibrated with the same buffer. The column was washed with 20 ml of the same buffer and then eluted with a linear concentration gradient of 10–400 mM phosphate, pH 7.8 (100 ml). Fractions of 2.6 ml were collected. The activity was eluted from the column at about 130 mM phosphate. The active fractions 18–29 were pooled, concentrated by ultrafiltration (Step 5) and used as the partially purified preparation of isozyme I from the monkey submandibular gland.

Purification of isozyme III

The isozyme III fraction resulting from DEAE-Sephacryl column chromatography was applied to a column (2.0×9.0 cm) of hydroxyapatite equilibrated with 10 mM potassium phosphate buffer, pH 6.8. After washing with 90 ml of the same buffer, the enzyme was eluted with a 180-ml linear gradient of 10–400 mM phosphate, pH 7.8. Fractions of 5.7 ml were collected. The activity was eluted from the column at about 80 mM phosphate. The active fractions 23–29 were pooled and concentrated by ultrafiltration (Step 4'). The concentrated solution was applied to a column (2.5×100 cm) of Sephacryl S-200 in 50 mM potassium phosphate buffer (pH 7.5)–10% glycerol. The column was eluted with the same buffer and 3-ml fractions were collected. A single aminotransferase activity peak was obtained. The active fractions 81–97 were pooled,

concentrated by ultrafiltration (Step 5') and used as the partially purified preparation of isozyme III.

Assay of enzyme activity

For the assay of aminotransferase activity, a standard reaction mixture, containing 6 μ mol of L-amino acid, 1 μ mol of α -ketoglutarate, 0.02 μ mol of pyridoxal phosphate, 20 μ mol of 2-mercaptoethanol, 10 μ mol of potassium pyrophosphate buffer (pH 8.2) and enzyme in a total volume of 0.3 ml, was incubated for 30 min at 37°C and the branched-chain α -keto acids formed were measured as previously described¹⁴⁾. One enzyme unit is defined as that amount of enzyme which catalyses the formation of 1 μ mol of α -keto acid/min under standard assay conditions. For routine assay, leucine was used as an amino acid unless otherwise specified. In a reverse reaction, the α -ketoglutarate formed was determined as previously described¹⁴⁾.

Other materials and methods

DEAE-Sephacryl CL-6B, Sephacryl S-200 and Pharmalyte were purchased from Pharmacia Fine Chemicals. Hydroxyapatite was obtained from Seikagaku Kohgyo (Tokyo, Japan). Protein was assayed by the method of Lowry *et al.*¹⁶⁾ All chemicals were obtained from commercial sources.

Results and Discussion

Table 1 shows the distribution of branched-chain amino acid aminotransferase activity in various tissues of the monkey. Extract from the submandibular gland exhibited a high activity of aminotransferase comparable to those from stomach, heart and kidney. The same result was obtained in the estimation using isoleucine or valine as a substrate.

The aminotransferase activity in the crude extract from submandibular gland was separated into two fractions by means of chromatography on a DEAE-Sephacryl CL-6B column as shown in Fig. 1. The activity which was eluted at lower concentration of phosphate was tentatively designated as isozyme I; the activity eluted at higher concen-

Table 1 Aminotransferase distribution in various tissues of the monkey

Tissue	Activity ^a (units/g wet tissue)	Distribution of isozyme ^b		
		I	II ^c (%)	III
Submandibular gland	2.49	55		45
Brain	0.43	10		90
Liver	0.08	15	85	
Lung	0.71	50		50
Spleen	0.47	75		25
Stomach	3.00	85		15
Heart	2.14	100		
Kidney	2.10	100		
Tongue	0.79	100		
Skeletal muscle	0.39	100		

^a The crude extracts obtained from the various tissues were incubated under the standard assay conditions.

^b The crude extracts were fractionated by ammonium sulphate and applied to DEAE-Sephacryl CL-6B column chromatography. The procedures were the same as those in the purification of enzyme from submandibular gland.

^c Isozyme II was eluted from the column at about 250 mM phosphate and specific for leucine only.

tration of phosphate, isozyme III. The both isozyme fractions were further purified. The result of the purification is summarized in Table 2. In each isozyme preparation, the ratios among the specific activities for the three branched-chain amino acids remained

constant during enzyme purification.

The activity of isozyme III fraction obtained from DEAE-Sephacryl CL-6B column chromatography was inactivated during isoelectric focusing which availed in the purification of isozyme I. When the crude extract was directly subjected to isoelectric focusing, two activity peaks at pH 4.3 and 7.1 were detected, suggesting that the isoelectric point of isozyme III is 4.3.

The optimum pH for both aminotransferase activities in 33.3 mM potassium pyrophosphate was near 8.2 for all three amino acids. The apparent *K_m* values for substrates of the two isozymes were different (Table 3). Both enzyme reactions were accelerated by SH-reagent. The maximum activity of isozyme I was achieved in about 15 mM 2-mercaptoethanol or about 4 mM dithiothreitol. Activation of isozyme III by 2-mercaptoethanol or dithiothreitol reached a maximum at about 10 mM or 3 mM, respectively. The activities of both isozymes were completely inhibited in the presence of 0.1 mM *p*-chloromercuribenzoate or 1 mM iodoacetamide. The above results indicate that both isozymes are SH-enzymes.

Table 4 shows that there was considerable formation of ketodicarboxylic acid from L-glutamate as the keto acids corresponding to the branched-chain amino acids were incubated with glutamate and enzyme. This indicates that the transamination of each isozyme is reversible. Then, the ratios of the formation of ketomonocarboxylic acid and

Table 2 Purification of isozymes I and III

Isozyme	Step	Fraction	protein (mg)	Total activity (units)	Specific activity (units/mg)
I	1.	Crude extract	1335.2	17.36	0.013
	2.	Ammonium sulphate	695.4	15.30	0.022
	3.	DEAE-Sephacryl	38.7	7.00	0.181
	4.	Isoelectric focusing and Sephacryl S-200	5.7	2.65	0.465
	5.	Hydroxyapatite	2.0	2.02	1.010
III	3'	DEAE-Sephacryl	42.3	5.86	0.139
	4'	Hydroxyapatite	24.3	4.89	0.201
	5'	Sephacryl S-200	5.8	3.88	0.669

Table 3 Apparent K_m values

Substrate	K_m (mM)	
	Isozyme I	Isozyme III
L-Leucine	0.65-0.70	0.30-0.35
L-Isoleucine	0.65-0.70	0.30-0.35
L-Valine	1.80-1.85	1.05-1.10
α -Ketoglutarate	1.40-1.45	1.40-1.45

ketodicarboxylic acid in the two aminotransferase reactions were apparently different; the velocity of reverse reaction of isozyme III was larger than that of isozyme I. The lower formation of ketodicarboxylic acid from D- α -keto- β -methylvalerate than those from two other branched-chain keto acids, which was observed in the study on aminotransferase from the rat submandibular gland, may be due to use of a D and L stereoisomeric mixture as a substrate.

Distribution of isozymes of branched-chain amino acid aminotransferase in other various

tissues of the monkey was also examined. The result is shown in Table 1. Isozyme I was contained in all the tissues examined. On the other hand, isozyme III was located in a narrower range. Isozyme II in the liver was specific for leucine only and its properties are on study.

Ogawa, Yokojima and Ichihara showed that there are three isozyme types of branched-chain amino acid aminotransferase in the rat tissues and that they are separated by DEAE-cellulose column chromatography¹⁷⁾. Isozyme I is widely distributed in various tissues and isozyme II is present only in the liver. Isozyme III occurs in the brain, ovary, placenta and hepatomas cell. In the rat submandibular gland, type of isozyme I only have been detected¹⁴⁾. Distribution of isozymes, especially isozyme III, is variable among animal species. Study on distribution and physiological roles of isozymes in submandibular gland seems be interesting.

Table 4 Reversibility of the reaction

Substrate		Activity (nmol/30 min)			
		α -Ketomonocarboxylate formed		α -Ketodicarboxylate formed	
Amino acid	Keto acid	Isozyme I	Isozyme III	Isozyme I	Isozyme III
L-Leucine	α -Ketoglutarate	74	67		
L-Isoleucine	α -Ketoglutarate	81	77		
L-Valine	α -Ketoglutarate	67	77		
L-Glutamate	α -Ketoisocaproate			31	50
L-Glutamate	D- α -Keto- β -methylvalerate ^a			9	12
L-Glutamate	α -Ketoisovalerate			28	50

^a D-L Isomeric mixture.

抄録: サル顎下腺の粗抽出液中に分岐鎖アミノ酸アミノ基転移酵素の高い活性が存在していた。粗抽出液中には分岐鎖アミノ酸アミノ基転移酵素の二種のアイソザイムが検出され、それらは DEAE-セファローズカラムクロマトグラフィーにより分離された。各々のアイソザイムは部分精製され、その諸性質が比較検討された。両アイソザイムは分岐鎖アミノ酸いずれにも作用し似たような性質を示すが、クロマトグラフィー上の挙動の相違に加えて、基質に対する親和性および逆反応の際の速度が異なることが認められた。

サルの他の組織におけるアイソザイムの分布についても検討が加えられた。

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