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A variant of ornithine aminotransferase from mouse small intestine

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Abbieviations: OAT, ornithine aminotransferase; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane

Abstract

The ornithine aminotransferase (OAT) activity of mouse was found to be highest in the small intestine. The mitochondrial OAT from mouse small intestine was purified to homogeneity by the procedures including heat treatment, ammonium sulfate fractionation, octyl-Sepharose chromatography, and Sephadex G-150 gel filtration. Comparing to the amino acid sequence of mouse hepatic OAT, six N-terminal amino acid residues have been deleted in intestinal OAT. However, the subsequent sequence was identical with that of hepatic OAT. The molecular weights of both intestinal and hepatic OAT were estimated as 46 kDa by SDS-gel electrophoresis and as 92 kDa by gel filtration, indicating that both native OATs are dimeric. Biochemical properties of intestinal OAT, such as molecular weight, pH optimum and K_m values for L-ornithine and α-ketoglutarate, were similar to those of hepatic OAT. However, intestinal OAT was more labile than hepatic OAT to tryptic digestion.

Keywords: ornithine aminotransferase; mouse; small intestine

Introduction

Ornithine aminotransferase (OAT; L-ornithine:2-oxo-acid aminotransferase; EC 2.6.1.13) is a pyridoxal phosphate-requiring enzyme which catalyzes the reversible transamination of L-ornithine and a-ketoglutarate to glutamate and glutamic-γ-semialdehyde (its cyclized form being Δ^1-pyrroline-5-carboxylate), and the latter product can be reversibly converted to no undeeline (Peraino and Pitot, 1963). OAT is expressed in nearly all mammalian tissues, including liver, kidney, brain, skeletal muscle, and eyes. OAT plays a role in arginine catabolism, proline biosynthesis, or *de novo* ornithine biosynthesis, depending on the tissue and the physiological circumstances (Mestichelli *et al*., 1979; Merril and Pitot,1983). Liver OAT is suggested to be involved in the ornithine synthesis for urea cycle while the kidney OAT participates in ornithine degradation (Herzfeld and Knox, 1968; Volpe *et al*., 1969). Matsuzawa *et al*. (1994) suggested that the intestinal OAT may be involved in the ornithine supply to the liver, with the reversal of OAT reaction. In human, a genetic deficiency of OAT causes gyrate atrophy, an autosomal recessive degenerative disease of the choroid and retina of the eye that leads to blindness (Valle *et al*., 1977; Kobayashi *et al*., 1995).

OAT is known as a mitochondrial matrix enzyme and has been purified from various tissue sources, including rat liver, kidney, and brain (Matsuzawa *et al*., 1968; Sanada *et al*., 1970; Deshmukh, 1984) as well as human liver (Ohura *et al*., 1982). The mitochondrial OAT has been shown to be synthesized as a large precursor molecule with N-terminal leader peptide on cytoplasmic ribosome, which is then processed and becomes associated with the mitochondrion (Mueckler and Pitot, 1985). The amino acid sequence of OAT precursor protein was predicted from the nucleotide sequence of cDNA (Mueckler and Pitot, 1985; Inaga *et al*., 1986; Ramesh *et al*., 1986; Giometti *et al*., 1992). Recently it was reported that human kidney OAT had the same nucleotide sequences as human liver OAT (Kobayashi *et al*., 1989), and rat kidney OAT had the identical amino acid sequence with that of rat liver OAT (Oyama *et al*., 1990).

In the present study, we purified OAT from mouse small intestine, and found that N-terminal amino acid sequence of the OAT purified from mouse small intestine was different from that of the mouse liver OAT.

Materials and Methods

Enzyme purification

Preparation of crude extract

Eighty male ICR mice weighing approximately 25 g were killed by cervical dislocation and their small intestines were rapidly removed, chilled, and homogenized in 4 volumes of ice-cold homogenization buffer (300 mM mannitol, 0.2 mM pyridoxal phosphate, 0.2 mM EDTA

and 5 mM Hepes, pH 7.4) with a Teflon pestle homogenizer. The homogenate was centrifuged at 600 *g* for 10 min and the supernatant was removed. The resultant sediment was resuspended in the homogenization buffer and rehomogenized. To solublize the OAT, the suspension was then exposed to ultrasonic oscillations and frozen at -90˚C overnight.

Heat treatment

The solubilized fraction was heated to 65˚C for 5 min and rapidly cooled by immersion in ice-water. Heat-denatured proteins were removed by centrifugation at 12,000 *g* for 20 min.

Ammonium sulfate fractionation

Solid ammonium sulfate was added to the heat-treated supernatant to a concentration of 30% (w/v). After 10 min of stirring, the suspension was centrifuged at 40,000 *g* for 20 min and supernatant was discarded. The sediment was triturated with 10 ml of 10 mM Tris buffer containg 0.2 mM pyridoxal phosphate, pH 8.0 (TB). After centrifugation at 40,000 *g* for 1 h, the sediment was suspended in 100 ml of TB and centrifuged at 40,000 *g* for 20 min. The supernatant was obtained, and solid ammonium sulfate was added to the supernatant to a 30% concentration. The same procedure was repeated exactly as described above.

Octyl-Sepharose chromatography

The enzyme solution treated with ammonium sulfate was applied to a column (2.6 × 6 cm) of octyl-Sepharose CL-4B equilibrated with TB containing 25% ammonium sulfate. The column was washed with TB buffer containing 25% ammonium sulfate until the absorbance at 280 nm of the washings had decreased to the initial value. Then the enzyme was eluted in a linear gradient of ammonium sulfate (25-5%) in TB (total volume of 300 ml) at a flow rate of 1 ml/min. The fractions containing the enzyme activity were concentrated by Centriprep-30 concentrator (Amicon).

Sephadex chromatography

The concentrated enzyme was chromatographed on a Sephadex G-150 column (1.5 × 95 cm) in TB at a flow rate of 15 ml/h. The active fractions were pooled and concentrated by Centriprep-30 concentrator. The OAT from mouse liver was also purified by the same procedure.

OAT activity assay

OAT activity was determined by the ninhydrin method of Kim *et al*. (1994). The reaction mixture contained 10 mM L-ornithine, 10 mM α-ketoglutarate, 0.05 mM pyridoxal phosphate, 50 mM Tris-HCl buffer, pH 8.0, and enzyme in a total volume of 1 ml. The reaction was carried out at 37˚C for 30 min, and terminated by the addition of 0.4 ml of 3 N perchloric acid and 0.2 ml of 2% ninhydrin. After heating for 10 min in a boiling water bath, a water-insoluble pigment was precipitated by centrifugation and dissolved in 1.5 ml of ethanol. The absorbance of the reddish supernatant was measured at 510 nm. The millimolar extinction coefficient of Δ^1-pyrroline-5-carboxylate was 16.5 at 510 nm. One unit of OAT is defined as the amount of the enzyme required to form 1 μmole of Δ^1-pyrroline-5-carboxylate per hour under the above assay conditions.

Estimation of molecular weight by gel filtration

The molecular weight of the native OAT was determined by gel filtration on Superose 6 column (Pharmacia) using the following standard proteins; thyroglobulin (670 kDa), gamma-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa).

Analysis of N-terminal amino acid sequence

The purified OATs from mouse small intestine and liver were desalted using Centricon-30 concentrator (Amicon). The OATs were electrophoresed on 10% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore). The transferred OAT bands were cut, and sent to Korean Basic Science Institute, Seoul Branch for analysis of N-terminal amino acid sequences of intestinal and hepatic OATs.

Tryptic hydrolysis

Digestion with trypsin was carried out at 37˚C in 10 mM Tris-HCl, pH 8.0, containing 1 mg/ml of intestinal or hepatic OAT and 0.1 mg/ml of trypsin. The hydrolysis was stopped at various times by adding an equivalent amount of aprotinin. After digestion with trypsin, the supernatants were used for OAT activity assay and electrophoresis.

Electrophoresis and protein determination

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970), and the proteins were stained with Coomassie Brilliant Blue R 250. Protein was measured by the procedure of Bradford (1976) with bovine serum albumin as a standard.

Results and Discussion

Tissue distribution and purification of OAT from mouse

Table 1 shows OAT activity in various tissues from mouse. The small intestine showed the highest activity. The activities in the liver and kidney were about a half of that in intestine, but still higher than those of other tissues. In rat, the liver and kidney have the highest OAT activities (Herzfeld and Knox, 1968). It has been reported that almost the entire OAT activity from the

Table 1. Distribution of OAT in Mouse Tissues. Homogenate of each tissue was prepared in 9 volumes of 0.25 M sucrose solution containing 0.2 mM pyridoxal phosphate using a Teflon pestle homogenizer. OAT activity of each homogenate was assayed by the ninhydrin method described in the text.

Tissues	OAT Activity (units/g tissue)
Small Intestine	105.6 ± 1.4
Liver	62.1 ± 6.1
Kidney	48.3 ± 5.8
Lung	23.4 ± 1.3
Spleen	22.5 ± 1.3
Brain	21.0 ± 2.2
Heart	17.8 ± 1.1
Muscle	6.2 ± 0.3

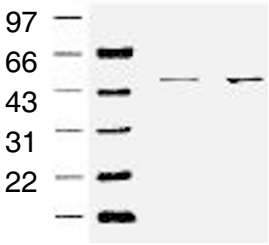

Figure 1. SDS-polyacrylamide gel electrophoresis of purified OAT. The lane 1 is molecular weight markers, lane 2, the intestinal OAT from mouse small intestine and lane 3, the hepatic OAT from mouse liver.

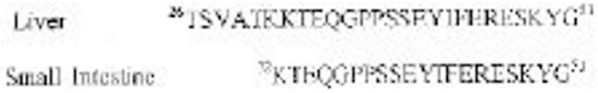

Figure 2. N-terminal amino acid sequences of purified intestinal and hepatic OATs from mouse.

liver and kidney could be recovered in the mitochondrial fraction (Peraino and Pitot, 1963; Strecker, 1965). The OAT from mouse small intestine was also mainly distributed in the mitochon-drial fraction (data not shown). OAT is known to play different roles in arginine, ornithine and proline metabolism depending on the tissues and their physiological needs (Mestichelli *et al*., 1979; Merril and Pitot, 1983). Highest enzyme activity in mouse small intestine suggests the possibility of different metabolic needs between the species.

The OAT was purified from mouse small intestine as summarized in Table 2. The purified OAT from mouse small intestine had a specific activity of 1,145 units per mg protein. This represents 89-fold purification with 51% recovery from the mitochondrial fraction of mouse small intestine.

The OAT was also purified from mouse liver by the same procedures, and the purified hepatic OAT had a specific activity of 1,485 units per mg protein.

Figure 1 shows the SDS-polyacrylamide gel electrophoresis pattern of the OATs purified from mouse small intestine and liver. Intestinal OAT migrated as a single band (lane 2) with the estimated molecular weight of 46 kDa. It was hard to identify any molecular size difference with hepatic OAT (lane 3) on the electrophoregram. The molecular weight of the hepatic OAT was reported as 43-46 kDa by SDS-gel electrophoresis (Kalita *et al*., 1976; Lyons and Pitot, 1976; Sanada *et al*., 1976), and confirmed by the available primary structure in rat, to be 45,749 (Simmaco *et al*., 1986).

N-terminal heterogeneity

It has been known that the OAT from mouse liver was synthesized as a 49-kDa precursor in the cytoplasm and the leader peptide was cleaved between Ala-25 and Thr-26 and to leave an N-terminal of Thr-26 in the mature mitochondrial OAT (Giometti *et al*., 1992). The N-terminal amino acid sequences up to 20 residues of purified mouse hepatic OAT was TSVATKKTEQGPPSSEYIFE (Figure 2), consistent with the previous report (Giometti *et al*., 1992). Simmaco *et al*. (1986) also reported that Thr-26 is the amino terminus of the rat liver OAT.

The N-terminal amino acid sequence of the OAT purified from small intestine was found to be KTEQGPPSS EYIFERESKYG (Figure 2). Compared with the sequence of hepatic OAT, six N-terminal amino acid residues were missing, and it started with Lys-32. The subsequent sequences of intestinal OAT were identical with those of hepatic OAT reported by Giometti *et al* (1992). We also purified intestinal OAT in a buffer containing protease

Table 2. Purification of OAT from Mouse Small Intestine.

Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Recovery (%)	Purification (folds)
Crude Extract	354.0	4,536	12.8	100	1.0
Heat Treatment (65˚C, 5 min)	226.0	4,450	19.7	98	1.5
$(NH_4)_2SO_4$ Fractionation	32.1	4,172	130.0	92	10.2
Octyl-Sepharose Chromatography	4.1	3,394	827.8	75	64.7
Sephadex G-150 Chromatography	2.0	2,324	1,145.0	51	89.4

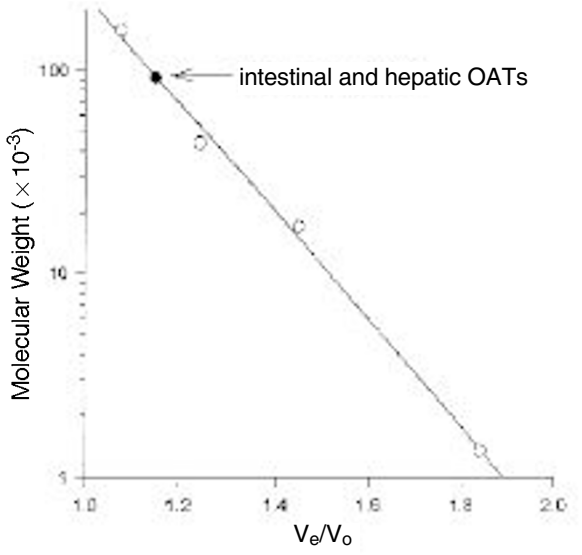

Figure 3. Determination of the molecular weight of mouse intestinal OAT by gel filtration on Superose 6. The standards used were gamma-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa). The arrow indicates the position of intestinal OAT. V_e, volume of the elution buffer; V_o, void volume of the column.

inhibitors including aprotinin, trypsin inhibitor and phenylmethylsulfonylfluoride (PMSF). The N-terminal amino acid of intestinal OAT purified in the presence of protease inhibitors was also Lys-32, suggesting that there six N-terminal amino acid residues absent in the hepatic OAT is not due to degradation by intracellular protease during purification procedure. In addition 20 N-terminal amino acids of the intestinal OAT purified from rat were identical with there from mouse (data not shown).

Biochemical Properties

The molecular weights of native OATs from small intestine and liver were also similar to each other and estimated as about 92 kDa by gel filtration on Superose 6 column (Figure 3). This result indicates that both OATs are composed of two identical subunits. There is considerable disagreement in the reported value for the molecular weight of native OAT; they range from 105 to 180 kDa (Peraino *et al*., 1969; Sanada *et al*., 1976). Morris *et al*. (1974) reported that the quaternary structure of purified OAT was concentration dependent. In dilute solutions (0.1 mg/ml), enzyme usually existed as a dimer, but at the higher protein concentrations, higher multimeric species are formed.

The K_m values of intestinal OAT for L-ornithine and α-ketoglutarate were 2.9 mM and 3.6 mM, respectively, and intestinal OAT showed its maximal activity around pH 8.0 (data not shown). No difference in K_m values or optimal pH was observed between intestinal and hepatic OATs.

The enzyme activity of the purified intestinal OAT was lost progressively by trypsin treatment (Figure 4). Loss of enzyme activity was accompanied by the appearance of a new protein band of 43 kDa on SDS-polyacrylamide gel electrophoresis (Figure 5). Simmaco *et al*. (1989) reported that the loss of OAT activity by trypsin was caused by hydrolysis of the peptide bond between Lys-24 and Tyr-25 of mature hepatic OAT. However, purified hepatic OAT was much more resistant to trypsin treatment under the same conditions. The absence of six N-terminal amino acids in intestinal OAT seems to increase the lability of the enzyme to tryptic digestion. However, the difference in molecular weight between intestinal and hepatic OATs was not detected by SDS-gel electrophoresis and gel filtration.

In α_1-antitrypsin (Hercz, 1985) and α_1-antichymotrypsin (Lindmark *et al*., 1989), amino-terminal heterogeneity occurs as a result of posttranslational proteolytic

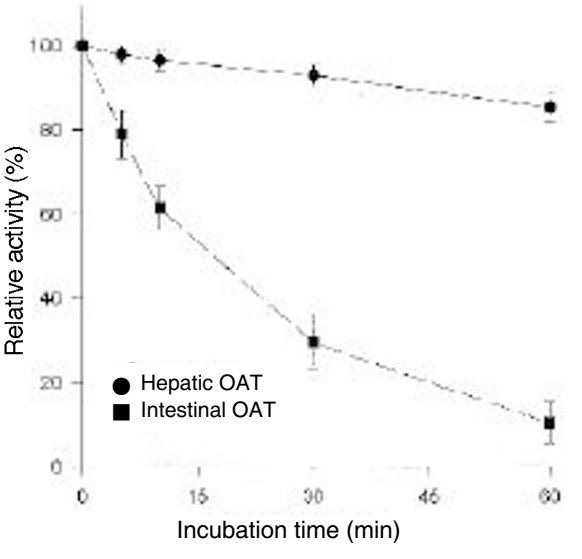

Figure 4. Loss of OAT activity by tryptic digestion. Intestinal and hepatic OATs (0.1 mg dissolved in 0.1 ml of 10 mM Tris, pH 8.0) were treated with 10 mg of trypsin at 37°C. At the indicated time, 20 ml of this solution was added to 20 ml of aprotinin. Each sample was used for determination of enzyme activity.

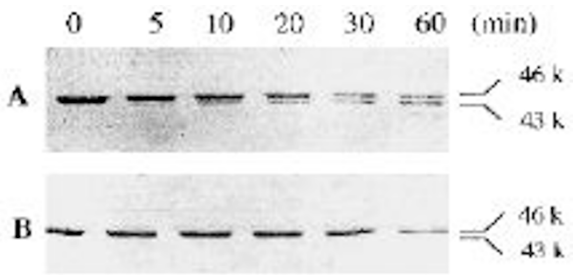

Figure 5. Polyacrylamide gel electrophoresis of tryptic digestion of OAT from mouse small intestine (A) and liver (B). Each sample was taken from the hydrolysate illustrated in Figure 4.

cleavage. The cleavage of one N-terminal amino acid of angiotensin II by aminopeptidase causes the production of angiotensin III. The activity of angiotensin III is generally less than that of angiotensin II (Reid, 1985). For OAT, this is the first report of N-terminal heterogeneity. However, at present no difference of biochemical properties other than the resistance to tryptic digestion was observed between two isoforms.

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