Submitochondrial Localization of Rat Liver β -Alanine-Oxoglutarate Aminotransferase

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Summary β -Alanine-oxoglutarate aminotransferase from rat liver was mainly distributed in the mitochondrial fraction, while β -ureidopropionase, the last one of uracil-metabolizing enzymes to β -alanine, was predominantly distributed in the cytosolic fraction. When rat liver mitochondria were separated into submitochondrial fractions, β -alanine-oxoglutarate aminotransferase was localized in the mitochondrial matrix. β -Alanine was transported into mitochondria with time and the influx of β -alanine into the matrix was 0.47 nmol/mg of mitochondrial protein at 2 min after incubation.

Key Words mitochondria, mitochondrial matrix, β -alanine, β -alanine-oxoglutarate aminotransferase, β -ureidopropionase

It is well known that β -alanine-oxoglutarate aminotransferase (4-aminobutyrate aminotransferase [EC 2.6.1.19]) has been shown to play an important role in GABA shunt in brain (1, 2). The enzyme is also present in non-neuronal tissues of mammalian species (3–5) and purified from liver (6–8). The immunological and kinetic properties of the enzyme from rat liver were similar to those of the enzyme from the brain (8). β -Alanine-oxoglutarate aminotransferase catalyzes the transamination of a wide variety of ω -amino acids such as 4-aminobutyrate, β -alanine and β -aminoisobutyrate which are present in mammals, when 2-oxoglutarate is used as an amino acceptor (6–8).

β -Alanine is formed by degradation of uracil (9, 10). The rate-limiting step in uracil to β -alanine is NADPH-dependent dihydrouracil dehydrogenase [EC 1.3.1.2] (11, 12). The enzyme activity was located predominantly in the 100,000 $\times g$ supernatant fractions (13, 14). Uracil was rapidly catabolized to β -alanine with little accumulation of the two intermediate compounds, dihydrouracil and β -ureidopropionate, *in vitro* (15). However, β -alanine is not present in serum and urine of human (16) or rabbit (17). Therefore, β -alanine formed in liver may be further

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degraded *in vivo*.

β -Alanine-oxoglutarate aminotransferase in brain is localized in the mitochondrial matrix (18) or in the inner mitochondrial membrane (19). However, the localization of β -alanine-oxoglutarate aminotransferase in liver is not clear. This paper described that β -alanine-oxoglutarate aminotransferase was localized in the mitochondrial matrix in rat liver and we discussed the relationship between uracil catabolism and β -alanine-oxoglutarate aminotransferase.

MATERIALS AND METHODS

Chemicals. All chemicals used were of analytical grade and were purchased from Nakarai Chemicals Ltd., Kyoto unless otherwise stated. β -[2- ^{14}C]Alanine and [^{14}C (U)]sucrose were purchased from New England Nuclear, U.S.A.

Animals. Male Wistar rats, weighing 150–200 g, were housed in screen-bottomed cages in a room maintained at $23 \pm 1^\circ\text{C}$ with 50% humidity under controlled lighting conditions (12 to 12 light-dark cycle). The animals were fed on a commercial stock diet (Oriental Yeast Ltd., Tokyo) and water *ad libitum* for 1 week before the experiment to acclimate them to the new environment. Rats were decapitated and livers were rapidly removed to be provided as a sample preparation.

Tissue fractionation. Rat liver was cut into small pieces, and homogenized in 5 volumes (v/w) ice-cold medium (5 mM Tris-HCl buffer, pH 7.3, containing 0.25 M sucrose) in a Potter-Elvehjem homogenizer. The homogenate was filtered through two layers of cheese-cloth and then centrifuged at $480 \times g$ for 10 min. The pellets contained nuclei and cell debris. The supernatant was removed and the pellet was homogenized in a further 3 volumes (v/w) of the above medium and then centrifuged. The supernatant was combined with the above supernatant and further fractionations were performed as described by de Duve *et al.* (20): a mitochondrial fraction was obtained as a pellet after 7 min centrifugation of the supernatant at $4,200 \times g$, a lysosomal fraction after 5 min centrifugation of the post-mitochondrial fraction at $19,000 \times g$, a microsomal fraction after 60 min at $105,000 \times g$ and a cytosolic fraction of the final supernatant. The pellets were suspended in 5 mM Tris-HCl buffer, pH 7.3, containing 1% Triton X-100 and assayed immediately.

Submitochondrial fractionation. The crude mitochondrial preparation was suspended in 20 mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, pH 7.4, containing 70 mM sucrose and 220 mM mannitol (buffer A), and then centrifuged at $8,500 \times g$ for 8 min. In order to remove lysosomes, this process was repeated 3 times. Submitochondrial fractionation was carried out from this purified mitochondria by the method of Greenawalt (21).

Density-gradient fractionation. Sucrose density gradients were used to separate mitochondria from peroxisomes. To facilitate the separation of lysosomes, Triton WR-1339 (85 mg/100 g body weight in 0.9% (w/v) NaCl) was injected intraperi-

toneally 4 days before the rats were killed (22, 23). The crude mitochondrial fraction was obtained as described above, and resuspended in 5 mM Tris-HCl buffer, pH 7.3, containing 0.21 M mannitol, 0.07 M sucrose and 0.1 mM EDTA. A 0.1 ml of the mitochondrial suspension was put on 4.5 ml linear sucrose 24–54% gradient in 20 mM glycylglycine buffer, pH 7.5, and centrifuged at $58,000 \times g$ for 2.5 h in a RPS swing-out bucket rotor in a Hitachi 65P ultracentrifuge at 3–4°C.

Measurement of β -alanine uptake. β -Alanine uptake by mitochondria was determined by the method of Palmieri and Klingenberg (24). Mitochondria (4–5 mg/ml) were incubated for 0–4 min at 28°C in the standard assay mixture (20 mM Tris-HCl buffer, pH 7.0, containing 10 mM KH_2PO_4 , 10 mM KCl, 10 mM succinate, 1 mM MgCl_2 and 1 mM β -[2- ^{14}C]alanine (0.5 $\mu\text{Ci}/\mu\text{mol}$)). The mitochondrial suspension was then sedimented by centrifugation for 2 min at $6,500 \times g$ in an Eppendorf model 3200 microcentrifuge. The supernatant was removed as completely as possible, part of it was immediately acidified with HClO_4 (final concentration, 5% (w/v)) and the radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer (460 CD type). The walls of the centrifuge tubes were wiped clean and the drop of fluid on the pellet was absorbed by filter papers. The pellet was resuspended with 0.1 ml H_2O , and extracted with 0.4 ml of 1.8 M HClO_4 . The suspension was then centrifuged and 0.3 ml of the supernatant were assayed for radioactivity.

Sucrose is transported into mitochondrial intermembrane components (sucrose-permeable space) but not into the matrix. Therefore, the volume of intermembrane components of mitochondria could be determined by uptake of [$^{14}\text{C}(U)$]sucrose into mitochondria. Mitochondria (4–5 mg/ml) were incubated for 10 min at 28°C in the mixture of 20 mM Tris-HCl buffer, pH 7.0, containing 10 mM KH_2PO_4 , 10 mM KCl, 10 mM succinate, 1 mM MgCl_2 , 1 mM β -alanine and 2 μCi [$^{14}\text{C}(U)$]sucrose (1–5 mCi/mmol). The mitochondrial suspension was sedimented by centrifugation. By counting of the radioactivity of the pellet and the supernatant, the sucrose space was calculated. The amount of β -alanine taken up by the mitochondrial matrix was calculated by using the following equation (23):

$$\beta\text{-Ala}_{\text{matrix}} = \left(\beta\text{-Ala}_p - \frac{\text{Suc}_p}{\text{Suc}_{\text{sup}}} \times \beta\text{-Ala}_{\text{sup}} \right) \frac{1}{\text{mg protein}}$$

where $\beta\text{-Ala}_{\text{matrix}}$ is the amount of β -alanine in the mitochondrial matrix; $\beta\text{-Ala}_p$, the amount in the whole pellet; $\beta\text{-Ala}_{\text{sup}}$, the concentration in the supernatant; Suc_p , the amount in the whole pellet; Suc_{sup} , the concentration of sucrose in the supernatant. The mean of the respiratory control ratios, when measured using the standard incubation mixture and 5 mM succinate as substrate, was 5.5.

Enzyme assays. β -Alanine aminotransferase activity was determined by measuring the rate of formation of malonic semialdehyde as previously described (7). β -Ureidopropionase [EC 3.5.1.6] was determined by measuring the rate of formation of ammonia using glutamate dehydrogenase. The standard assay mixture contained 0.1 M sodium phosphate buffer, pH 7.0, including 10 mM β -

ureidopropionate in a total volume of 0.9 ml, and was incubated for 1 h. The reaction was terminated by the addition of 0.1 ml 50% (w/v) trichloroacetic acid. The solution was mixed, and then kept for 30 min at room temperature. The precipitate was removed by centrifugation. Ammonium ions in the supernatant were assayed by the addition of 0.5 ml of the supernatant to 0.52 ml of the reaction solution, containing 0.38 M KHCO_3 , 0.19 M Tris-HCl, pH 8.0, 19 mM 2-oxoglutarate and 0.46 mM NADH. After reading of the extinction at 365 nm, 0.01 ml of glutamate dehydrogenase (12 units) in 50% (v/v) glycerol was added. The reaction mixture was kept for 60 min at room temperature, and then the concentration of the ammonium ions was calculated from the difference of the extinction at 365 nm. The enzyme activity was revealed as the amount of enzyme which catalyzed the formation of the ammonium ions per min under the given conditions.

The following enzyme markers were assayed to assess the purity of the various subcellular fractionations: lactate dehydrogenase [EC 1.1.1.27] for cytosol; glutamate dehydrogenase [EC 1.4.1.2] for mitochondrial matrix; glucose 6-phosphatase [EC 3.1.3.9] for microsomes; monoamine oxidase [EC 1.4.3.4] for mitochondrial outer membrane; adenylate kinase [EC 2.7.4.3] for mitochondrial intermembrane components; cytochrome *c* oxidase [EC 1.9.3.1] for mitochondrial inner membrane; catalase [EC 1.11.1.6] for peroxisomes (30); acid phosphatase [EC 3.1.3.2] for lysosomes. Lactate dehydrogenase was assayed in the direction of pyruvate reduction essentially as described by Stolzenbach (25). Glutamate dehydrogenase was assayed in the direction of glutamate synthesis essentially by the method of Schmidt (26). Glucose 6-phosphatase activity was assayed by measuring the inorganic phosphate liberated (27). Monoamine oxidase was assayed in the direction of benzylamine to benzaldehyde at 250 nm as described by Tabor *et al.* (28). Adenylate kinase was assayed in the direction of ADP synthesis essentially by the method of Bergmeyer *et al.* (29). Cytochrome *c* oxidase was assayed by observing the reoxidation of reduced cytochrome *c* as described by Orii and Okunuki (30). Catalase was measured by the spectrophotometric method of Aebi (31). Acid phosphatase was assayed with *p*-nitrophenyl phosphate as substrate (32). β -Alanine aminotransferase, β -ureidopropionase, and glucose 6-phosphatase were assayed at 37°C, and all other enzymes at 25°C. Protein concentration was measured by the Lowry method (33) or the biuret method (34) in the case of sub-mitochondrial fractionation, with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Subcellular distribution of β -alanine-oxoglutarate aminotransferase and β -ureidopropionase from rat liver

The purity and yield of the isolated subcellular fractions were evaluated by measurement of the glutamate dehydrogenase, glucose 6-phosphatase and lactate dehydrogenase activities of both the total homogenate and the various fractions. To facilitate comparison between the activities of different enzymes, the results of the

Table 1. Subcellular distribution of β -alanine aminotransferase and β -ureidopropionase in rat liver.

Protein (mg)	β -Alanine aminotransferase		β -Ureido- propionase		Glutamate dehydrogenase		Glucose 6-phosphatase		Lactate dehydrogenase	
	T.A. ^a (nmol/ min)	S.A. ^b (nmol/ min/mg)	T.A. (nmol/ min)	S.A. (nmol/ min/mg)	T.A. (μ mol/ min)	S.A. (μ mol/ min/mg)	T.A. (μ mol/ min)	S.A. (μ mol/ min/mg)	T.A. (μ mol/ min)	S.A. (μ mol/ min/mg)
Homogenate	843	1,114	1.32	1.10	929	1.10	1,163	1.38	40.6	0.048
Nuclei and cell debris	196	382	1.95	0.14	28	0.14	251	1.28	9.0	0.046
Mitochondria	210	651	3.10	0.40	85	0.40	445	2.12	10.6	0.050
Lysosome	62.4	30	0.48	0.61	38	0.61	28	0.45	3.9	0.063
Microsome	80.3	1	0.01	0.51	41	0.51	16	0.20	13.4	0.167
Cytosol	244	38	0.16	2.57	626	2.57	2	0.01	0.8	0.003
									1,920	2.28
									240	1.22
									212	1.01
									96	1.54
									130	1.62
									1,400	5.74

Rat liver (4 g) was homogenized and fractionated. The enzyme activities of each fraction were determined as described in MATERIALS AND METHODS. The specific activity was calculated per mg protein. ^a T.A., total activity; ^b S.A., specific activity.

Fig. 1. Density-gradient fractionation of crude mitochondrial fraction. Crude mitochondrial fraction from rat liver was placed over a linear sucrose gradient and centrifuged at $58,000 \times g$ for 2.5 h. After centrifugation, fractions of 3 drops were collected from the upper of the tube and then enzyme activities were immediately assayed. ●, β -alanine-oxoglutarate aminotransferase; ○, glutamate dehydrogenase; △, catalase. The peaks of glutamate dehydrogenase and catalase were found at density of about 1.19 and 1.24 g/ml, respectively.

mean amount of activity and specific activity were shown in Table 1. β -Alanine-oxoglutarate aminotransferase was mainly located in the mitochondrial fraction (Table 1). About 34% β -alanine-oxoglutarate aminotransferase activity was recovered in the nuclei and cell debris fractions. As glutamate dehydrogenase, which is a mitochondrial marker enzyme, was also recovered from this fraction, the nuclei and cell debris fraction might be contaminated by mitochondria.

In order to separate mitochondria from peroxisomes, the crude mitochondrial fraction prepared by centrifugation was subjected to sucrose gradient centrifugation (Fig. 1). β -Alanine-oxoglutarate aminotransferase was cosedimented with the mitochondrial marker enzyme, glutamate dehydrogenase. These enzymes were separated from catalase, which is a peroxisomal marker enzyme. A considerable amount of catalase was found at the top of the gradient and this may be due to the broken peroxisomes, as shown by Noguchi *et al.* (35). When the homogenate was prepared from livers pretreated with Triton WR-1339, β -alanine aminotransferase could separate from acid phosphatase as the lysosomal marker by sucrose density-gradient centrifugation (not shown). From these results, it was concluded that β -alanine-oxoglutarate aminotransferase from rat liver was predominantly distributed in the mitochondrial fraction but not in the peroxisomal fraction.

As shown in Table 1, β -ureidopropionase activity was mainly located in the cytosolic fraction. Therefore, β -alanine produced by β -ureidopropionase in the cytosolic fraction may transport into mitochondria and then may be catabolized to malonic semialdehyde by β -alanine-oxoglutarate aminotransferase.

Table 2. Intramitochondrial distribution of β -alanine aminotransferase in rat liver.

Protein (mg)	β -Alanine aminotransferase		Monoamine oxidase		Adenylate kinase		Cytochrome <i>c</i> oxidase		Glutamate dehydrogenase	
	T.A. ^a (nmol/ min)	S.A. ^b (nmol/ min/mg)	T.A. (nmol/ min)	S.A. (nmol/ min/mg)	T.A. (μ mol/ min)	S.A. (μ mol/ min/mg)	T.A. (min ⁻¹)	S.A. (min ⁻¹ / mg)	T.A. (μ mol/ min)	S.A. (μ mol/ min/mg)
Mitochondria ^c	169.6	1.17	1,231	7.3	434	2.6	16,900	99.6	833	4.91
Outer membrane	11.2	1.25	423	37.9	8	0.7	446	39.8	8	0.71
Inner membrane components	9.1	0.99	409	44.8	269	29.6	30	3.3	38	4.18
Mitoplast	121.1	1.21	372	3.1	46	0.4	13,200	109.0	859	7.09
Mitoplast ^d	241.4	0.84	—	—	—	—	6,500	27.0	1,320	5.47
Inner membrane	66.2	0.35	—	—	—	—	4,750	71.8	90	1.36
Matrix	115.5	1.57	—	—	—	—	40	0.35	1,030	8.92

Enzyme activities of each fraction were determined as described in MATERIALS AND METHODS. ^aT.A., total activity; ^bS.A., specific activity. ^cA volume of mitochondrial suspension (100 mg protein/ml) was treated with 0.5 volumes of 2% digitonin solution for 8 min to break the outer mitochondrial membrane. The suspension was then centrifuged and fractionated into outer membrane, intermembrane components, and mitoplast fractions. ^dA volume of mitochondrial suspension (100 mg protein/ml) was treated with 0.5 volumes of 2% digitonin solution for 15 min. The mitoplast fraction prepared from it was mixed with Lubrol solution (19 mg/ml) to give a final concentration of 0.16 mg Lubrol per mg mitoplast protein, and treated for 25 min. The suspension was then fractionated into inner membrane and matrix fractions.

Intramitochondrial distribution of β -alanine-oxoglutarate aminotransferase

β -Alanine-oxoglutarate aminotransferase functions on catabolism of γ -aminobutyrate in brain and β -alanine in liver. The intramitochondrial localization of β -alanine-oxoglutarate aminotransferase is not clear. Therefore, intramitochondrial distribution of the enzyme from rat liver is described here. Mitochondria were separated into outermembrane, intermembrane soluble and mitoplast fractions. β -Alanine-oxoglutarate aminotransferase activity (73% of the activity in mitochondria) was recovered in the mitoplast fraction (Table 2a). After Lubrol treatment, the mitoplast fraction could be separated into innermembrane and matrix fractions from the distribution of cytochrome *c* oxidase and glutamate dehydrogenase (Table 2b). β -Alanine-oxoglutarate aminotransferase activity (90% of the activity in mitoplast) was recovered in the matrix fraction (Table 2b). These results show that β -alanine-oxoglutarate aminotransferase is localized in the mitochondrial matrix of rat liver.

Uptake of β -alanine into hepatic mitochondrial matrix

Preliminary evidence of high affinity sodium-dependent β -alanine transport system in liver was reported by Hardison and Weiner (36). Recently, the detailed studies of the sodium-dependent β -alanine transport systems in membrane vesicles derived from dog renal brush-border (37) and from rat brain synaptosomes (38) were published. However, it is not clear whether β -alanine could be transported into mitochondrial matrix from cytosol. As shown in Fig. 2, we measured time-dependent uptake of β -[2- 14 C]alanine by mitochondria at 28°C. After 2 min incubation the amount of β -alanine uptake into mitochondria was 2.54 nmol/mg of protein (Fig. 2) and the concentration of β -alanine in the supernatant was 0.92 nmol/ μ l. The sucrose space ($\text{Suc}_p/\text{Suc}_{\text{sup}}$) was 2.25 μ l/mg of mitochondrial

Fig. 2. Uptake of β -[2- 14 C]alanine by isolated rat liver mitochondria as a function of time. Values are means of three separate samples. Conditions of incubation were described in MATERIALS AND METHODS.

protein. Therefore, at 2 min of incubation time the amount of β -alanine uptake into mitochondrial matrix, calculated to be 0.47 nmol/mg of mitochondrial protein from MATERIALS AND METHODS and the β -alanine contents in the matrix, was 18% of that in whole mitochondria. The concentration of β -alanine was varied from 0.18 and 10.95 μ mol/g wet tissue in livers of control and 6-azauracil treated rats, respectively (39). Therefore, the concentration of 1mM β -alanine in the incubation medium is the range of physiological concentrations. The mechanism of β -alanine uptake into mitochondria and its effectors are now being investigated.

When [6- 3 H]uracil was incubated with the rat liver cytosolic fraction, the accumulation of β -[3- 3 H]alanine with time was found and so β -alanine was proposed to be the final end product of uracil (15). However, as described here, mitochondria is important to metabolize β -alanine, which is the catabolic product of uracil, because uracil is degraded to β -alanine in cytosolic fraction and then transported into mitochondria matrix. Therefore, we would like to propose that mitochondria has an important role in overall pyrimidine catabolism.

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