Comparison of Argininosuccinate Synthetase from Young and Old Rat Livers

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Properties of argininosuccinate synthetases (ASS), including immunological properties and heat stability, from young and old rats were quite similar. However the degradation rates of ASS from young and old rats were found to be different as shown in the experiment using the double labeling technique. Three forms of this enzyme from young and old rat livers were separated by DEAE-Sephadex A-50 column chromatography, and they were called ASS 1, 2 and 3 in order of elution. The degradation rate of ASS 1 from young and old rat livers was very similar, but the rate of degradation of ASS 2 form old rat livers was greater than that from young rat livers.

(Key Words: Argininosuccinate Synthetase, Aging, Heat Stability, Immunotitration, Turnover)

INTRODUCTION

There is evidence showing that enzymes are altered in aged organisms(13). The qualitative alteration of enzymes related to aging may be caused by a post-synthetic modification or by a sequence change. Some data suggest the presence of post-synthetic modification of enzymes, but there is no evidence indicating amino acid substitution(4, 16). On the other hand, the quantitative alteration of the enzyme may be due to the alteration of the rate of synthesis or degradation of the enzyme in old animals.

Argininosuccinate synthetase (ASS) (EC 6.3.4.5.) is a rate limiting enzyme of the urea cycle and may be a regulatory enzyme. Recently, ASS was purified from rat liver and was separated into three forms by DEAE-Sephadex A-50 column chromatography (14). The physicochemical characteristics of each form of ASS have been extensively studied in our laboratory (15). Takada et al. reported that ASS 2 is the most stable against heat inactivation and digestion by chymotrypsin and trypsin (17).

In this paper, we discuss whether ASS 1, 2 and 3 from young and old rat livers are immunochemically identical, and whether there is any difference in in vitro and in vivo stability of the enzymes from young and old rats.

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MATERIALS AND METHODS

Materials
Phosphoenolpyruvate was purchased from Boehringer Mannheim GmbH (Mannheim, Germany); NADH from the Oriental Yeast Co. (Tokyo, Japan); ATP, citrulline and aspartate, from the Kyowa Hakko Co. (Tokyo, Japan); and Freund’s adjuvant from Difco Laboratories (Detroit, U.S.A.). L-(U-14C) leucine (348 mCi/m mol), L-(4, 5-H3) leucine (50 Ci/m mol), and NCS solubilizer were purchased from The Radiochemical Centre, Amersham, U.K. Argininosuccinate was synthesized enzymatically from arginine and fumarate according to the method of Ratner (9). Lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), adenylate kinase (EC 2.7.4.3) and inorganic pyrophosphatase (EC 3.6.1.1) were purified according to the methods of Racker (8), Tiez and Ochoa (18), Kress et al. (6), and Cooperman et al. (2), respectively. All other chemicals were of analytical grade and they were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

Enzyme assay
Argininosuccinate synthetase activity was measured spectrophotometrically according the method of Rochovansky and Ratner (12), except that the reaction was carried out at 25°C. One unit was defined as the amount of enzyme producing 1 µmol of AMP per min at 25°C.

Animals
Male Wistar rats 6 and 25 months old were used for heat stability and immunotitration studies, and HOS-Donryu rats 2 and 26 months old for the studies of turnover and immunodiffusion, respectively. All animals were given Laboratory Chow diet made by Clea Japan, Inc. (Tokyo, Japan) and water ad libitum at 22°C.

Immunochromical analysis
The preparation of specific antisera was described previously (15). Ouchterlony double diffusion analysis was performed by the method of Clausen (1) in 2% agarose containing 0.1% NaN3 and 0.05 M Tris-HCl, pH 7.5.

Double isotope experiments
A rat was given an intraperitoneal injection of 50 µCi of L-(U-14C) leucine/100 g of body weight. Five days after the initial injection, the animal was injected with 150 µCi of L-(4, 5-H3) leucine/100 g of body weight. The animal was killed 4 hours after the final injection of isotope. The liver was homogenized in four volumes of 0.25 M sucrose containing 0.05 M Tris-HCl buffer, pH 7.5, with a teflon homogenizer. After centrifugation at 105,000 × g for 60 min, the supernatant fraction was obtained. The supernatant solution was chromatographed through a Sephadex G-50 column (5.6 × 25 cm) equilibrated with 0.007 M Tris-HCl buffer, pH 8.5. The protein fraction was applied to a DEAE-Sephadex A-50 column (2.5 × 25 cm) equilibrated with the same buffer. The column was washed with 160 ml of the same buffer, and then argininosuccinate synthetase was eluted from the column with a linear gradient of Tris-HCl buffer (0.005 to 0.05 M), pH 8.0. The enzyme activity was present in three peaks called ASS 1, 2 and
3 as shown in Fig. 1. The amount of ASS 3 was so small that the double isotope experiment could not be performed. The fractions of ASS 1 and 2 were collected and concentrated by Centriflo (Amicon Co.). To 1 ml of the concentrated enzyme solution were added 0.2 ml of 0.25 M argininosuccinate dissolved in distilled water and 0.2 ml of 1.0 M potassium phosphate buffer, pH 7.4, in an Eppendorf microtube. Then the mixture was heated at 56°C for 7 min, cooled in an ice bath and centrifuged at 8,000 x g for 2 min. The specific antiserum was added to one portion of this supernatant solution and the same volume of control serum was added to the other. The mixtures were incubated at 37°C for 60 min. The resulting precipitates were collected by centrifugation and washed five times with cold 0.9% NaCl. The precipitates were dissolved in 0.6 ml of NCS solubilizer and counted in 8 ml of a toluene-based scintillation mixture. The soluble liver protein was precipitated with the same volume of 10% trichloroacetic acid, boiled at 95°C for 10 min and centrifuged at 8,000 x g for 2 min. The resulting precipitate was washed five times with 5% trichloroacetic acid and with ethanol/hydrochloric acid (98/2, v/v) and ethanol. The protein was dissolved in NCS solubilizer. Counting was performed in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Enzyme activity

The specific activities of the enzyme in crude preparations of young and old rats were indistinguishable (Table 1). Mean specific enzyme activities of young and old Wistar rats were 0.033 and 0.031 units/mg, respectively. Those of HOS-Donryu rats were 0.029 units/mg for young and 0.022 units/mg for old rats.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Units/mg of protein</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ASS 1</td>
</tr>
<tr>
<td>Wistar</td>
<td>0.039</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.026</td>
<td>—</td>
</tr>
<tr>
<td>Young</td>
<td>0.037</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>0.028</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>0.022</td>
<td>65</td>
</tr>
<tr>
<td>HOS-Donryu</td>
<td>0.033</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.028</td>
<td>—</td>
</tr>
<tr>
<td>Old</td>
<td>0.021</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>0.017</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.028</td>
<td>—</td>
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</table>
Fig. 1  DEAE-Sephadex A-50 column chromatography of young rat liver homogenate.

Elution was performed with a linear gradient of 0.005 to 0.05 M Tris-HCl buffer, pH 8.0, and fractions of 10 ml were collected. The tubes pooled to obtain ASS 1, 2 and 3 are indicated by arrows. The pattern of DEAE-Sephadex column chromatography of old rat liver was essentially the same as that of young rat liver.

Relative enzyme activities of ASS 1, 2 and 3 after DEAE-Sephadex A-50 column chromatography were 47 to 65%, 25 to 41% and 10 to 12% for young rats, and 44%, 40% and 16% for old rats, respectively.

**Heat stability**

The heat inactivation curve of crude enzyme from young and old rat livers is shown in Fig. 2. The young and old enzymes lost their activity at the same constant rate. Young and old enzymes without 10mM argininosuccinate lost their activity more rapidly than enzymes with 10mM argininosuccinate.

**Ouchterlony double diffusion analysis**

In the Ouchterlony double diffusion analysis, antisera to ASS was prepared from rabbits by injecting purified ASS 1 or 2 obtained from young rat livers. Fig. 3 shows that all forms of ASS from young and old rat livers reacted to both the antisera and showed clear precipitin lines which completely fused with each other. This result indicates that all forms of ASS from young and old rat livers were immunochemically identical.

**Immunotitration**

Fig. 4 shows the effect of addition of various amounts of ASS from young or old rats to a constant amount of antiserum. The equivalence points for ASS in homoginate from young and old rat livers were identical.
This result also shows that ASS from young and old rats was indistinguishable immunochemically.

**Fig. 2**  Heat inactivation of crude ASS from young and old rats.

The temperature was 45°C. The initial enzyme activity was 0.1 units in 0.2 ml of 0.05 M Tris-HCl buffer, pH 7.5, and amounts of protein (1.9 mg/0.2 ml) in both preparations were adjusted to the same level by adding bovine serum albumin. The reaction mixtures were centrifuged at 8,000 × g for 2 min and the supernatant was assayed for ASS catalytic activity.

○: young and △: old enzymes with 10 mM argininosuccinate.

●: young and ▲: old enzymes without 10 mM argininosuccinate.

**Fig. 3**  Ouchterlony double diffusion of crude ASS preparations from young and old rat livers.

The center wells contained antisera against purified ASS 1 (P 1) or ASS 2 (P 2) from young rats. Other wells contained the following antigens: Y1, Y2 and Y3: ASS 1, 2 and 3 of a young rat and O1, O2 and O3: ASS 1, 2 and 3 of an old rat, respectively.
Fig. 4 Immunotitration of ASS activity in young and old rat livers.

Extracts containing 10 mM argininosuccinate to protect against enzyme inactivation were used. Varying amounts of ASS were added to the rabbit antiserum for ASS. After incubation at 37°C for 60 min, the mixtures were centrifuged at 8,000 x g for 2 min and the supernatants were assayed for ASS catalytic activity.

O: young and △: old enzyme.

Double labeling studies

ASS in crude extract was separated into three forms by DEAE-Sephadex A-50 column chromatography (Fig. 1), and each form of ASS had different stabilities to heat and protease in vitro (17).

To examine the stabilities of each form of ASS in vivo, and to compare them with those of young and old rat ASS, we used the double labeling technique. The incorporation of H3 and C14 into ASS 1 and 2 and soluble liver protein in young and old rats is shown in Table 2. In a young rat, the H3/C14 ratio for ASS 1, 4.39, was greater than for ASS 2, 3.10. In an old rat, the H3/C14 ratios for ASS 1 and 2 were 3.22 and 2.75, respectively. Since it has been demonstrated that the ratio of final leucine incorporation (H3 leucine) to initial leucine incorporation (C14) after a decay period (five days in this case) can be used to compare the degradation rate constants (3), our results appear to show that ASS 1 is more rapidly degraded than ASS 2 from both young and old rats. To compare the H3/C14 ratio of ASS from young and old rats, the results are expressed as the A/B value which is the ratio of H3/C14 of ASS (A) to that of total soluble protein (B). The normalized A/B value of ASS 1 of a young rat, 0.52, was about the same as that of a old rat, 0.51. However a difference was observed in ASS 2, for which the values of young and old rats were 0.37 and 0.43, respectively.
Table 2: Ratios of H³ to C¹⁴ dpm in ASS 1, 2 and 3 and soluble protein of young and old rats.

Young (2 months old) and old (26 months old) rats were injected with 50μCi of L-(U-C¹⁴) leucine per 100 g of body weight. After 5 days, they were given 150μCi of L-(4, 5-H³) leucine per 100 g of body weight. The rats were killed 4 hours after the final injection of isotope. After DEAE-Sephadex A-50 column chromatography, ASS 1 and 2 were isolated from liver extracts, and the radioactivity was measured. See text for more details.

<table>
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<tr>
<th></th>
<th>Total activities</th>
<th>H³ (dpm/mg)</th>
<th>C¹⁴ (dpm/mg)</th>
<th>H³/C¹⁴</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(units)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASS 1</td>
<td>9.3</td>
<td>5440</td>
<td>1240</td>
<td>A</td>
<td>4.39</td>
</tr>
<tr>
<td>ASS 2</td>
<td>8.1</td>
<td>4150</td>
<td>1340</td>
<td>A</td>
<td>3.10</td>
</tr>
<tr>
<td>Soluble protein</td>
<td>—</td>
<td>10340</td>
<td>1220</td>
<td>B</td>
<td>8.48</td>
</tr>
<tr>
<td>Old</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASS 1</td>
<td>10.3</td>
<td>8990</td>
<td>2790</td>
<td>A</td>
<td>3.22</td>
</tr>
<tr>
<td>ASS 2</td>
<td>9.4</td>
<td>7290</td>
<td>2650</td>
<td>A</td>
<td>2.75</td>
</tr>
<tr>
<td>Soluble protein</td>
<td>—</td>
<td>25390</td>
<td>4010</td>
<td>B</td>
<td>6.33</td>
</tr>
</tbody>
</table>

DISCUSSION

Senescence may start from the progressive accumulation of faulty enzyme molecules which is indicated by changes in heat stability (5, 10, 11, 16) or immunochemical properties (7, 10, 11), but some data showed contradictory results due to differences in the animals used or the assay conditions (13). For example, fructose-1, 6-diphosphate aldolase shows a difference in heat sensitivity between young and old nematodes only for purified enzyme preparations (11) but not for crude homogenates (20). Our studies on ASS in crude homogenates from young and old rats showed no apparent differences in specific and relative activities of ASS 1, 2 and 3; heat stability; immunotitration and immunodiffusion, but these results seem to leave some room for consideration because of the presence of some enzyme factors in the homogenates.

In addition to physicochemical and immunological properties, we examined age-related changes in the metabolism of ASS in rat livers by double isotope experiments (Table 2). It was shown that the rate of degradation of ASS 1 from young and old rat livers are very similar, but the rate of degradation of ASS 2 from an old rat liver was greater than that from a young rat liver. Takada et al. reported that ASS 1, 2 and 3 were not isozymes, but were post-translational modification products (17). Their data indicated that: a) the molecular weights of ASS 1, 2 and 3 were identical; b) optimum pH and Km values for substrates were similar in all ASS; c) all ASS purified from young rats had similar specific activity; d) the amino acid compositions of ASS 1 and 2 were about the same; and e) ASS 1 and 2 contained four and eight moles of argininosuccinate per mol of the enzyme, respectively, and ASS 3 contained no argininosuccinate. Based on such results, our data presented two possibilities: 1) one is that the rates of synthesis and degradation of ASS 1 are greater than those of ASS 2 if there is no conversion of ASS 1 to ASS 2; and 2) the other is that ASS 1 predominates in the synthesis process of the enzyme, and a part of ASS 1 is converted to
ASS 2 during the 5 days after the synthesis, so that ASS 1 and 2 are degraded at the same rate if the conversion occurs. (The latter possibility is coincident with the previous result that the degradation of all ASS appears to follow first order kinetics) (19). Although we can not explain the reason why the A/B value of ASS 2 in young rats was less than that of old rats, we wish to stress that there are some differences in the metabolism of ASS, including degradation and/or interconversion, between young and old rats, in spite of the physicochemical and immunochemical similarity of ASS in young and old rat livers.

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