ASPARTATE TRANSCARBAMOYLASE: DISTRIBUTION IN VARIOUS TISSUES AND IDENTIFICATION OF THREE MOLECULAR FORMS[1]

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Summary. Aspartate transcarbamoylase, obtained from a variety of tissues, was found to exist in two macromolecular forms in crude extracts with approximate molecular weight of 900,000 and 600,000. A constant sedimentation profile was obtained with a given tissue in the same physiological stage, but the ratios of the two peaks for the enzyme varied considerably in different tissues. The peaks were not affected by agents known to disrupt secondary bonding. Guanidine treatment or preincubation of the crude liver extract at 37° resulted in the appearance of polydisperse heavier complexes. When the two peaks were treated with proteolytic enzymes all the activity of ATC was recovered in a third peak (MW 80,000), which had a Km for carbamoyl phosphate 10-50 times greater than those obtained with ATC in the other peaks. Evidence for feedback inhibition of ATC by pyrimidine nucleotides was not obtained.

In many organisms, the second step in the de novo pyrimidine biosynthetic pathway is catalyzed by aspartate transcarbamoylase (ATC: EC 2.1.3.2). In E. coli, control of pyrimidine biosynthesis is mediated by allosteric inhibition of ATC by the end product, CTP (1,2). ATC purified from E. coli has a molecular weight (MW) of 310,000 which can be dissociated into two types of subunit, one of which contains a catalytic site and the other a regulatory site (3).

In yeast, the activities of the first and second enzymes of the de novo pyrimidine biosynthetic pathway, glutamine-dependent carbamoyl phosphate synthetase (CPS) and ATC, are associated within a single complex with a MW of 600,000. This complex can be dissociated into units of MW 300,000 and 140,000 (4). Both of the larger complexes (MW 600,000 and 300,000) have CPS and ATC activities sensitive to inhibition by UTP.

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We previously reported that when extracts of rat liver were subjected to zone-sedimentation analysis, ATC was separated into macromolecular components of MW 900,000 and 600,000 (5). In this report these two forms of ATC have been investigated as to their biological distribution, structure, and catalytic properties.

METHODS

Tissue extracts were obtained by homogenizing (teflon-glass) 1 part of fresh tissue with 3 to 4 volumes of 0.25 M sucrose-TKM (0.05 M Tris-HCl, pH 7.5; 0.025 M KCl; and 0.005 M $MgCl_2$) and centrifuging at 75,000 x g for 1 hour at 4°. Zonal centrifugation in linear 20%-30% sucrose-gradients was carried out in a SW65 rotor using the Spinco L2-65 ultracentrifuge. The gradients contained 0.5% bovine serum albumin (BSA) throughout, unless stated otherwise. Activity of ATC was determined with a modification (5) of the method of Porter et al (6).

Molecular size was estimated with a Sephadex G-100 column (100 x 2.6 cm) (7). The enzyme-sample was prepared as described in the text and applied to the column with the following reference proteins: lactic dehydrogenase (LDH, 2 units), BSA (25 mg), ovalbumin (20 mg), and α-chymotrypsinogen (7 mg). The sample of trypsin-treated ATC was applied to the bottom of the column in a total volume of 1.6 ml and eluted by reverse flow at a constant rate of 12-14 ml per hour at 4°. The eluate from the column was collected in 3.0 ml fractions and immediately assayed for activity of LDH (8) and ATC. The protein content of each fraction was estimated from absorbance at 280 mμ in a 1 cm light path.

RESULTS

Extracts of several tissues from the rat, mouse, chicken, and from tissue culture cells were subjected to zone-sedimentation analysis. In all the preparations, activity of ATC was associated with two macromolecular components with sedimentation patterns similar to those observed with ATC derived from neonatal rat liver (5). The approximate molecular weight of these components were 900,000 (Peak I) and 600,000 (Peak II). The sedimentation profile was constant for each particular tissue from animals of similar age and physiological status. However, the ratio of activities between Peak I and Peak II differed characteristically from tissue to tissue as shown in Table I. Peak I was the predominant form in rat liver, spleen, and heart but constituted the minor component of growing uterus and crypts of intestine. Analysis of liver 5-58 hours post-hepatectomy showed no marked differences in the ratio of Peak I to Peak II. Selected sedimentation patterns of ATC are depicted in Fig. 1 showing differences between: a) tissues of the same species at the same developmental stage (liver of newborn mouse vs. spleen of newborn mouse), b) same tissue of different species at the same developmental stage (spleen of new-

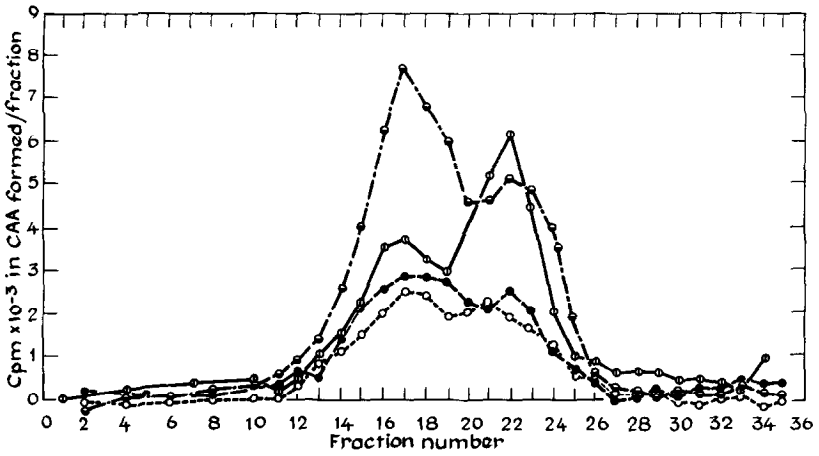

Figure 1. Sedimentation patterns of ATC obtained from extracts of various tissues. Fractions were derived from 20-30% sucrose density gradients containing 0.5% BSA and centrifuged at 300,000 x g for four hours. Aliquots of each fraction were assayed at 37° for 15 minutes at pH 9.2: (●---●) spleen from newborn rat; (◐---◐) spleen from newborn mouse; (○---○) spleen from adult rat; and (◉---◉) liver from newborn mouse.

born rat vs. spleen of newborn mouse), and c) same tissue of the same species at different developmental stages (spleen of newborn rat vs. spleen of adult rat).

The ultracentrifugal characteristics of ATC were not affected either by incubation of extract from rat liver with various detergents and ribonuclease (5) or by preparation of the extract at 4° in the presence of 0.5 M KCl and either p-hydroxymercuribenzoate (1.6 mM) or urea (1.0M). Incubation of the extract for 15 min. at 37° did not reduce the total enzymatic activity, but heavier, polydisperse particles appeared which could be obtained as a pellet by centrifugation at 160,000 x g for 2 hours. About 30% of the enzymatic activity was lost following pretreatment with 0.2 M guanidine-HCl and 0.01 M dithiothreitol at 4° for 15 hours. The enzyme was rapidly inactivated by concentrations of guanidine greater than 0.2 M. Incubation of liver extract with concentrations greater than 0.0005% trypsin or 0.03% papain for 15 min. at 37° resulted in minimal loss of enzymatic activity. Zonal sedimentation patterns of liver extracts which had been incubated in the presence of trypsin (>0.0005%) revealed complete disappearance of the Peak I and Peak II forms of the enzyme and appearance of a single third peak (III) of considerably lesser density (Fig. 2). No intermediate peaks were obtained with this treatment. Attempts to obtain similar conversions employing 0.05% carboxypeptidase A were unsuccessful.

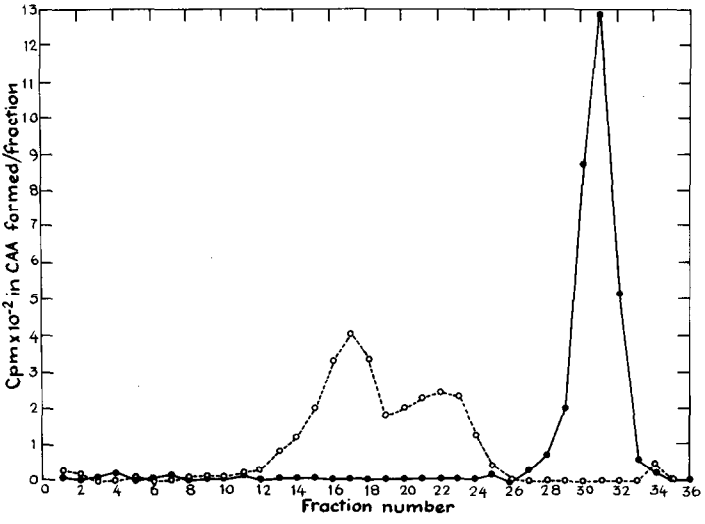

Figure 2. Changes in sedimentation patterns of ATC from newborn rat liver produced by trypsin. Sedimentation patterns include: Extract of rat liver, control (0---0), and incubated with trypsin (0.03%) for 15 minutes at 37° (●---●). Centrifugation was as described for Fig. 1. Aliquots of each fraction were assayed for ATC at 37° for 15 minutes.

In order to estimate the molecular size of Peak III an extract of liver from newborn rat was pre-incubated at 37° for 30 min., then centrifugated at 160,000 x g for 2 hours. The resulting pellet was resuspended and, following incubation with trypsin, centrifuged at 160,000 x g for 30 min. The trypsin-released enzyme was in the supernatant fluid and was applied with reference proteins to a column of Sephadex G-100. The component demonstrating ATC activity was eluted as a single peak with a molecular weight of approximately 80,000 (Fig. 3).

ATC in Peaks I, II, and III each exhibited a maximum reaction velocity at approximately pH 9.2. The effect of elevated temperatures on ATC stability was studied by heating at 50° and 60°. Each of the molecular forms displayed similar decreases of activity as a function of heating so that only 35-50% of the activity remained after 15 minutes at 60°. Preliminary kinetic measurements were carried out on the crude fractions; the Km for aspartate was similar for each of the peaks but the Km for carbamoyl phosphate was 52 μM for Peak I, 82 μM for Peak II and 1400 μM for Peak III.

When UTP or CTP was employed prior to initiation of the reaction in a concentration ranging from 10^{-5} to 7.5 x 10^{-3} M, or when nucleotide mixtures (CMP, CTP, UMP, UTP, TMP, dCMP, dCTP, dUTP) were added at increasing concentrations to 10^{-3} M for each of the compounds there was no inhibition or activation of enzymatic activity observed with any of the molecular forms of the enzyme.

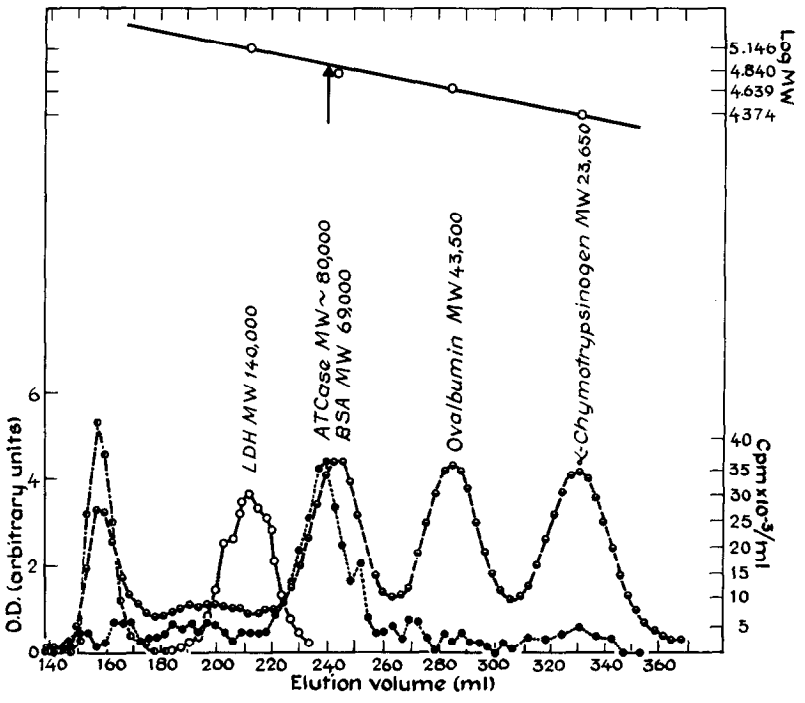

Figure 3. Estimation of molecular size using a Sephadex G-100 column (100 x 2.6 cm) with standard markers and ATC from trypsin-treated extract of newborn rat liver. The equilibration and elution buffer was Tris-HCl, 0.05M, pH 7.5, containing 0.05 M KCl and 0.001 M EDTA. The left ordinate represents absorbancy: $O.D._{600} \times 10^2$ for Blue Dextran (●···●), $O.D._{280} \times 10$ for proteins (●---●), and $O.D._{340} \times 20$ for the activity of LDH (○——○). The upper ordinate on the right represents the log of molecular weights of marker proteins. The lower ordinate on the right represents counts per minute x 10^{-3} of ^{14}C-carbamoyl aspartate formed by ATC in each fraction (●·····●).

DISCUSSION

Previously, we reported that activity of ATC was associated with two macromolecular peaks (MW 900,000 and 600,000) separable by zonal centrifugation of extracts from newborn and adult rat liver (5). Using the same technique, we have now observed these same peaks in a number of mammalian and avian tissues as well as cultured cells. The ratio of activity of ATC between these peaks was found to vary considerably from tissue to tissue (Table I). However, the pattern of distribution of the multiple forms was always the same for a particular tissue of a given age, indicating that they were not artifacts. Recently, Inagaki and Tatibana (12) also reported that the ATC from the soluble fraction of hematopoietic mouse spleen could be separated into two forms.

Bottomley and Lovig (9), in the study of subcellular distribution of ATC, concluded that the enzyme probably is associated with lipid portions of the

microsomal membrane. However, previous studies (5) and our present findings that Peaks I and II are susceptible to proteolytic digestion, suggest that the main components associated with the peaks of ATC activity are not RNA, DNA or lipid in nature, but rather predominately protein. The use of agents capable of disrupting hydrogen or ionic bonds (e.g., guanidine, $(NH_4)_2SO_4$, mercurials, or high concentrations of salts) resulted in either considerable loss of

TABLE I

The Ratio of Macromolecular Forms of ATCase in Different Tissues and Cultured Cells[1]

Enzyme Source	Age	Peak I/Peak II
Rat liver	17 day fetus	2.6
liver	newborn	2.6
liver	adult	1.5
liver	adult, 5 hrs.[2]	1.4
liver	adult, 7 hrs.[2]	1.1
liver	adult, 10 hrs.[2]	1.0
liver	adult, 18 hrs.[2]	1.6
liver	adult, 32 hrs.[2]	1.4
liver	adult, 58 hrs.[2]	1.2
spleen	newborn	1.4
spleen	adult	1.5
heart	17 day fetus	3.0
uterus	3 weeks	0.4
intestinal crypt	adult	0.3
Mouse liver	newborn	2.6
liver	adult	1.7
spleen	newborn	0.5
spleen[3]	adult	0.5
spleen[3]	adult	0.4
spleen[4]	adult	0.6
Ehrlich's ascites cells	-	0.8
Chick embryo	4-5 days	5.9
intestine	18 day embryo	5.1
liver	19 day embryo	2.7
Cell cultures		
Hela, stationary phase		2.4
mouse lymphoma, growth phase		0.5
mouse lymphoma, stationary phase		0.7

[1] The total activity in Peak I (MW 900,000) was divided by that in Peak II (MW 600,000).

[2] Liver taken post-hepatectomy at the noted times.

[3] Spleen from mouse treated with 1 mg phenylhydrazine, pH 7.3 for 2 days.

[4] Spleen from mouse treated with phenylhydrazine for 4 days.

enzyme activity or a polydisperse pattern of activity of ATC on sedimentation analysis.

The complexes containing ATC can be split into a smaller unit of MW 80,000 without loss of enzymatic activity. If Peaks I and II are composed of ATC alone, combinations of 8 and 12 times this small unit would result in MW of 640,000 and 960,000. Alternatively ATC might be complexed with: a) other proteins of a regulatory nature similar to the regulatory subunit of bacterial ATC, b) one or more enzymes in the same pathway, e.g. an ATC-CPS complex (10,11) or c) some non-enzymatic components. We found no evidence for a "regulatory subunit" in ATC. The enzyme might be attached to two different sized particles, or different numbers of enzyme molecules can be attached to a single particle, leading to similar heterogeneity.

The heat stability characteristics and kinetic properties are similar for the various molecular forms of the enzyme. Although widely differing K_m and V_{max} values for carbamoyl phosphate were obtained between the trypsin-derived enzyme product and the original macromolecular forms of the enzyme, the significance of these differences remains to be assessed.

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