CRYSTALLIZATION AND SOME PROPERTIES OF ARGININOSUCCINATE SYNTHASE FROM RAT LIVER

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1. Introduction

In the urea cycle argininosuccinate synthase activity is rate-limiting [1,2] and may be a regulatory enzyme. While the other urea cycle enzymes have been purified to homogeneity [3,4,5,6], this enzyme has been purified from steer liver and hog kidney to only 50% purity [7,8]. Recently Ratner described in Advances in Enzymology [1] briefly that they succeeded in the crystallization of the enzyme from steer liver. In the present paper, the isolation, the crystallization and some properties of argininosuccinate synthase from the rat liver are described. The enzyme consisted of four subunits with identical mol. wt of 48 000 ± 1000. Its catalytic properties such as optimal pH and K_m values were similar to those of the enzyme from steer liver and hog kidney [7,9].

2. Materials and methods

High protein diet (70%) was supplied from Clea Japan, Inc. Argininosuccinate synthase was assayed with coupling enzymes and NADH by the method of Rochovansky and Ratner [7]. One enzyme unit was defined as the amount of enzyme that produces 1 μmole of AMP per min at 25°C. DE-52 used on the step-3 in the purification procedure was equilibrated with 0.2 M potassium phosphate, pH 7.5, and then washed extensively with 0.005 M potassium phosphate, pH 7.5. Analytical disc-gel electrophoresis was carried out according to the method of Davis [10] and sodium dodecylsulfate gels were prepared by the method of Weber and Osborn [11]. Molecular weight of the subunit was determined by the method of Weber and Osborn [11] using bovine serum albumin, lactate dehydrogenase, pyruvate kinase, γ-globulin, chymotrypsin and cytochrome C as standards. Cross-linking of the subunits was performed by the method of Penninckx et al. [12].

3. Results and discussion

3.1. *Purification of the enzyme from rat liver*

(Step 1) Livers were collected from 20 male Wistar strain rats fed 70% protein diet for 10–15 days, and homogenized in 4 vol of 0.25 M sucrose with homogenizer. After centrifugation at 27 000 *g* for 20 min, the supernatant fraction was obtained.

(Step 2) The precipitate formed between 0.45 and 0.6 saturation of ammonium sulfate was collected and dissolved in 0.05 M potassium phosphate, pH 7.5.

(Step 3) Ammonium sulfate was removed by gel filtration on Sephadex G-50 equilibrated with 0.005 M potassium phosphate, pH 7.5. About 100 g wet weight of the washed DE-52 was added to the above eluate (about 3 g protein). After stirring for 30 min, the suspension was filtered through a Buchner funnel.

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A colorless solution was obtained. The DE-52 left on the funnel was washed once with an equal volume of 0.005 M potassium phosphate, pH 7.5, and the eluate was combined to the above filtrate.

(Step 4) To the filtrate 50% polyethylene glycol 6000 solution was added and the precipitate formed between 6 and 12% was collected by centrifugation and dissolved in a buffer of pH 7.5 containing 0.1 M citrulline, 0.1 M aspartate and 0.1 M potassium phosphate.

(Step 5) The solution from step 4 was diluted to about 10 mg protein/ml with the above buffer. The diluted solution was heated to 65°C for 1 min, then cooled to 5°C in an ice bath, and centrifuged. The supernatant fluid was removed and the enzyme was precipitated with polyethylene glycol (final concentration of 15%). The precipitate was dissolved in 0.05 M Tris–HCl, pH 7.5.

(Step 6) The solution from step 5 was chromatographed through Sephadex G-50 equilibrated with 0.01 M Tris–HCl, pH 8.5. The protein fraction was placed on a column of DEAE-Sephadex A-50 equilibrated with the Tris–HCl buffer. The column was first washed with 0.01 M Tris–HCl, pH 8.5, until unabsorbed protein was removed. Then the absorbed proteins were eluted with a linear gradient made with 500 ml of 0.01 M Tris–HCl, pH 8.0, in the mixing chamber and 500 ml of 0.1 M Tris–HCl, pH 8.0, in the reservoir. The enzymatic activities were present in three peaks named Peak 1, 2, and 3 according to the order of elution. Specific activities of these three fractions were almost the same.

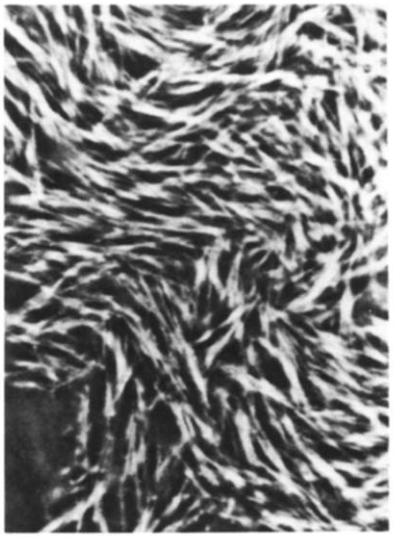

Fig.1. Photomicrograph of the crystals of argininosuccinate synthase (× 4000)

(Step 7) The main peak (Peak 2) fractions were made 0.6 saturation with ammonium sulfate. The precipitate was dissolved in 0.05 M potassium phosphate and applied on a column of Sephadex

Table 1
Purification of argininosuccinate synthase from rat liver

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
1 supernatant	1100	1400	21 000	0.067	100
2 ammonium sulfate fractionation	194	800	4150	0.19	62
3 DE-52	345	620	2100	0.30	48
4 polyethylene glycol fractionation	55	572	930	0.62	44
5 heat treatment	75	569	833	0.68	44
6 DEAE-Sephadex A-50					
peak 1	59	146	66	2.2	11
peak 2	50	270	115	2.3	21
Further purification of peak 2					
7 Sephadex G-200	80	250	120	2.1	18
8 crystallization	2.4	149	70.6	2.1	11

G-200 which had been equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.05 M citrulline and 0.05 M aspartate. The activity emerged in a small first and a large second symmetrical peak coinciding with protein peaks.

(Step 8) The enzyme preparation from step 6 of 7 was precipitated with 0.6 saturation of ammonium sulfate and dissolved in a small volume of 0.05 M Tris–HCl, pH 7.5, containing 0.05 M citrulline and 0.05 M aspartate under the conditions that the preparation contained about 30 mg protein per ml in 0.5 saturation of ammonium sulfate. After centrifugation the supernatant was stored in a refrigerator at 5°C. In two or three days needle-shaped crystals appeared, as shown in fig.1. Representative results of purification of argininosuccinate synthase from rat liver are shown in table 1.

3.2. *Homogeneity*

The acrylamide gel electrophoresis with dodecylsulfate of the crystalline enzyme gave a single band (fig.2b). The gel electrophoresis without dodecylsulfate usually revealed a single band (fig.2a), but occasionally it was accompanied by minor four or five components even with the enzyme preparations of the highest specific activity. However, these minor components disappeared when electrophoresed in the presence of 5 mM argininosuccinate. Homogeneity of the enzyme was also proven by the sedimentation pattern. S_{20} value was calculated to be 8.0 at 11.8 mg protein/ml in 0.05 M potassium phosphate, pH 7.5, containing 0.05 M citrulline and 0.05 M aspartate.

3.3. *Molecular weight of subunit and oligomeric structure as determined by cross-linking of the subunits*

The molecular weight of the subunits was determined by electrophoresis in 7.5% acrylamide gel with dodecylsulfate using the molecular weight markers [11]. The mol. wt of the subunits was calculated to be 48 000 ± 1000 (fig.3). The oligomeric structure was studied by cross-linking of the subunits with glutaraldehyde followed by the gel electrophoresis with dodecylsulfate [12]. Four bands were seen when the enzyme was cross-linked in 0.05 M potassium phosphate, pH 7.5, containing 0.02% glutaraldehyde with the mobilities expected for the monomer, dimer, trimer and tetramer (fig.2, c-2). At

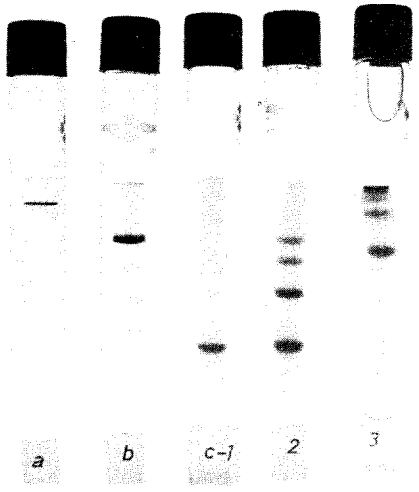

Fig.2. Acrylamide gel electrophoresis of rat liver argininosuccinate synthase. (a) Migration band for native enzyme at pH 8.9 in normal 7.5% acrylamide gel; (b) migration pattern of the enzyme in dodecylsulfate gel (7.5% acrylamide); (c-1, -2 and -3) dodecylsulfate gel electrophoresis of the enzyme cross-linked with 0%, 0.02% and 0.05% glutaraldehyde, respectively, at 1.0 mg protein/ml (4% acrylamide gel).

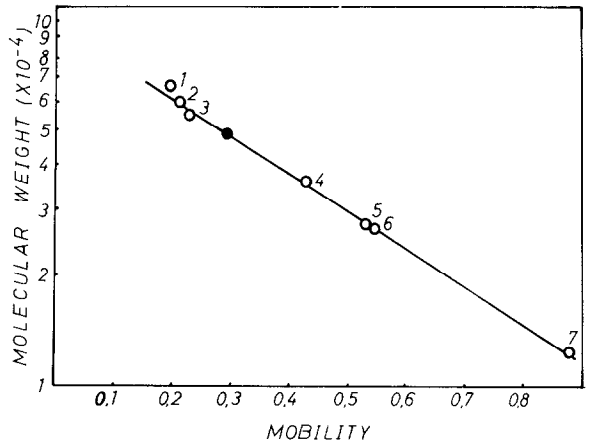

Fig.3. Determination of the mol. wt of the subunit of argininosuccinate synthase by gel electrophoresis with sodium dodecylsulfate. Electrophoresis was performed under the condition as described in the legend of Fig.2b. (1) Bovine serum albumin; (2) pyruvate kinase; (3) H-chain of bovine γ-globulin; (4) lactate dehydrogenase; (5) chymotrypsin; (6) L-chain of bovine γ-globulin; (7) cytochrome C; • argininosuccinate synthase.

0.05% glutaraldehyde, more slowly moving bands were seen, probably tetramer, octamer and so on as expected (fig.2, c-3). From the mol. wt of the subunits and the cross-linking behavior of the subunits, the native enzyme appeared to have a mol. wt of 192 000 which consisted of four subunits with identical molecular weight of 48 000.

3.4. *Optimal pH and K_m values*

The enzyme is active at broad range of pH with the optimum at pH 8.4–8.8. Rochovansky and Ratner reported that the optimal pH of steer liver enzyme was 8.7 [9]. The K_m values were determined at pH 7.5. The apparent K_m values of the crystalline rat liver enzyme for citrulline (4.4×10^{-5} M), aspartate (2.0×10^{-5} M) and ATP (1.5×10^{-4} M) were quite similar to those reported by Rochovansky and Ratner [7], 4.6×10^{-5} M, 3.5×10^{-5} M (data on steer liver enzyme) and 3.2×10^{-5} M (data on hog kidney enzyme), respectively.

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