tion was not realized. It is not clear at present whether the complex kinetics arise from heretofore unrecognized substrate self-association at concentrations below the cmc or from problems arising from the apparent multisubunit structure of the enzyme and its apparent instability in aqueous solutions lacking polyhydric alcohols. These problems are currently under investigation.

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Isolation and Characterization of Argininosuccinate Synthetase from Human Liver†

William E. O'Brien

ABSTRACT: This communication describes the purification and characterization of argininosuccinate synthetase from human liver. By numerous criteria including electrophoresis in sodium dodecyl sulfate containing gels, electrophoresis in nondissociating gels, and analytical ultracentrifugation, the protein is homogeneous at a specific activity of 4.2 μmol/(min mg) assayed at 37 °C in the direction of argininosuccinate synthesis. The enzyme has a molecular weight of 183 000, as determined by gel filtration. Electrophoresis in the presence of sodium dodecyl sulfate yielded a single band migrating with an R_f corresponding to 43 000 daltons. Thus, the enzyme is considered to contain four subunits of identical molecular weight. The $s_{20,w}$ of the enzyme is 8.2 S. Antibodies were prepared in rabbits directed against the purified protein. These antibodies react specifically with argininosuccinate synthetase, as determined by electrophoretic analysis of the immunoadsorbed product from crude extracts of human liver. The human enzyme has very similar properties to those published for the beef and rat liver enzymes.

Argininosuccinate synthetase (EC 6.3.4.5) catalyzes reaction 1. The enzyme was described first by Ratner and her colleagues in beef liver (Ratner & Petrack, 1951, 1653) and since has been studied extensively (Rochovansky et al., 1977; Rochovansky & Ratner, 1961, 1967). The enzyme also has been isolated from hog kidney (Schuegraf et al., 1960) and rat liver (Saheki et al., 1975) and has been found in numerous human cultured cell lines (Lockridge et al., 1977; Schimke, 1964; Irr & Jacoby, 1978).

$$\text{citrulline} + \text{aspartate} + \text{ATP} \rightleftharpoons \text{AMP} + \text{argininosuccinate} + \text{PP}_i \quad (1)$$

In ureatelic organisms this enzyme plays a vital role in the disposal of ammonia via the Krebs–Henseleit urea cycle. This metabolic process occurs predominately in the liver, and this organ has many-fold higher levels of enzyme than other tissues. In tissues not capable of ureagenesis, the enzyme presumably serves to synthesize arginine from citrulline. Due to the bifunctional role of the enzyme, it might be expected that different tissues would have evolved different mechanisms to control the levels of the enzyme. In cultured lymphocytes and in Hela and KB cells argininosuccinate synthetase levels are influenced by the availability of arginine and citrulline in the culture fluid (Schimke, 1964; Irr & Jacoby, 1978). In rat and monkey liver the levels of the urea cycle enzymes including argininosuccinate synthetase are influenced by the amount of protein in the diet (Nuzum & Snodgrass, 1971; Schimke, 1962). Nothing is known about the mechanism by which these control processes occur. Our interest in inborn errors of metabolism involving urea cycle enzymes in humans and the control of these enzymes necessitated that more information be made available concerning these proteins. Carbamylphosphate synthetase (Pierson & Gilbert, 1978), ornithine transcarbamylase (Kalausek et al., 1978), and arginase (Beruter et al., 1978) have been isolated from human liver and their properties described. This report describes the isolation and characterization of argininosuccinate synthetase from human liver.

† From the Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030. *Received June 21, 1979; revised manuscript received September 5, 1979.* This work was supported by a Basil O'Connor research grant from the National Foundation–March of Dimes and National Institutes of Health Research Grant HD12284.

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Materials and Methods

Materials. Enzyme-grade ammonium sulfate was purchased from Schwarz/Mann. NADH, ATP, FMN, fructose 6-phosphate, trizma base, citrulline, and aspartic acid were obtained from Sigma Chemical Corp. [U-^{14}C]Aspartic acid was supplied by Amersham. Pyrophosphatase, aldolase, bovine serum albumin, triosephosphate isomerase, glycerol-3-phosphate dehydrogenase, ovalbumin, chymotrypsinogen, and catalase were supplied by Boehringer. Bio-Gel A1.5, CM-Bio-Gel, and all reagents for electrophoresis were obtained from Bio-Rad Laboratories. Formalin-killed *Staphylococcus aureus* (IgGsorb) was purchased from the Enzyme Center. All other chemicals were from commercial sources and of the best grade available. Human liver was obtained at autopsy within 8 h following death and stored at −80 °C until use.

Analytical Methods. The enzyme activity during purification and other routine studies was determined as described by O'Brien (1977). For the immunological studies a more sensitive enzyme assay procedure was developed based on similar assays described previously (Lockridge et al., 1977; Kato et al., 1976). This assay determines the amount of [^{14}C]argininosuccinate formed from [^{14}C]aspartate. Each reaction contained citrulline (5 mM), Tris-HCl (pH 7.5, 10 mM), ATP (0.1 mM), $MgCl_2$ (6 mM), KCl (20 mM), phosphoenolpyruvate (1.5 mM), [U-^{14}C]aspartate (1 mM, 900 cpm/nmol), pyruvate kinase (4.5 units), myokinase (4 units), and pyrophosphatase (0.2 unit) in a final volume of 0.30 mL. Reactions were run in 12 × 75 mm glass tubes at 37 °C for 90 min. At the end of the incubation period, 0.05 mL of 1 M acetic acid was added and the tubes were heated at 90 °C for 30 min. Following this procedure, 0.65 mL of H_2O was added and the contents of the tube were applied to 0.5 × 4 cm columns of Dowex 1-X8 in 0.05 M acetic acid. The tubes were rinsed with 1 mL of 0.05 M acetic acid, which was applied to the column, followed by a final 1 mL of 0.05 M acetic acid. The entire 3 mL was collected and radioactivity determined by liquid scintillation counting in Aquasol (New England Nuclear). Enzyme activity was linear under these conditions if no more than 50 nmol of asparate was utilized. Protein was determined by the biuret method (Gornall et al., 1949) and by the method of Warburg & Christian (1941). Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) was conducted essentially as described by Laemmli (1970). Sucrose density centrifugation was conducted according to the methodology of Martin & Ames (1961).

Purification of Argininosuccinate Synthetase. Step 1: Preparation of Crude Extract. All procedures were conducted at 4 °C. Human liver, 127 g, was chopped, suspended in 260 mL of CAP buffer (1 mM citrulline, 1 mM asparate, and 20 mM potassium phosphate, pH 6.3), and homogenized with a Polytron (Brinkmann Instruments) homogenizer. The resulting fluid was centrifuged at 30000*g* for 30 min. The supernatant fluid was removed and diluted with CAP buffer to 360 mL.

Step 2: Ammonium Sulfate Precipitation. The crude extract from step 1 was brought to 60% saturation by the addition of crystalline ammonium sulfate with stirring. The resulting precipitate was collected by centrifugation at 30000*g* for 20 min, and the supernatant fluid was discarded. The precipitate was dissolved in a minimal volume of CAP buffer and then dialyzed for 18 h against the same CAP buffer.

Step 3: CM-Bio-Gel Chromatography. A 2.6 × 30 cm column of CM-Bio-Gel A was packed and equilibrated in CAP buffer. The dialyzed protein from step 2 was applied to this column, and the nonbound protein was eluted with CAP buffer until all nonbound protein was removed. The enzyme was eluted with CAP buffer containing 15 mM KCl. All tubes containing enzyme with a specific activity greater than 1 μmol/(min mg) were pooled. The enzyme was concentrated by precipitation in 60% saturated ammonium sulfate. Care must be taken to maintain a neutral pH during precipitation to prevent inactivation of the enzyme.

Step 4: Bio-Gel A1.5 Chromatography. The enzyme obtained from step 3 was applied to a 2.6 × 95 cm column containing Bio-Gel A1.5 previously equilibrated in CAT buffer (1 mM citrulline, 1 mM aspartate, and 100 mM Tris-HCl, pH 7.5). All tubes containing enzyme with a specific activity of 4 μmol/(min mg) or greater were pooled. The protein was concentrated by precipitation in 60% saturated ammonium sulfate.

Preparation of Rabbit Antibodies to Human Liver Argininosuccinate Synthetase. The homogeneous argininosuccinate synthetase [4.2 μmol/(min mg)] was emulsified with an equal volume of Freund's complete adjuvant, and 2 mL of this mixture containing 200 μg of protein was injected subcutaneously at four sites into New Zealand white rabbits. The animals were then boosted every 2 weeks with the identical material. Antibodies were detected by double immunodiffusion 4 weeks after the initial immunization and reached a peak 8 weeks after the initial immunization. Animals were bled every 2 weeks from the marginal ear vein. Sera were separated from the clot and stored at −20 °C until their use. Immunoglobulin was purified by column chromatography. A 1.5 × 21 cm column was packed with Blue Sepharose (Pharmacia) and connected in series to 0.9 × 24 cm DEAE-Bio-Gel column. The columns were equilibrated with 0.02 M potassium phosphate, pH 8.0, containing 0.02% sodium azide. Four-milliliter aliquots of sera were dialyzed for 18 h against three 500-mL changes of the same buffer. The serum was applied to the column and washed with the equilibrating buffer. Under these conditions immunoglobulins are not retained by the column. The nonbound protein was pooled and precipitated by the addition of solid ammonium sulfate to 40% saturation. The immunoglobulins were resuspended in phosphate-buffered saline and stored at −20 °C.

Analysis of the Antigen–Antibody Complex by Electrophoresis in $NaDodSO_4$-Containing Polyacrylamide Gels. For ascertainment of the specificity of the antibodies produced by the rabbits, the antigen–antibody complexes were analyzed as described by Kessler (1975). Crude liver extract, 10 μL containing 0.0065 unit, was mixed with 50 μL of antiserum (or preimmune serum) and allowed to incubate for 30 min at room temperature. To this was added 50 μL of a 10% suspension of *S. aureus* (IgGsorb) cells, and the mixture was allowed to incubate at 4 °C for 10 min. The cells, containing the bound IgG and IgG–antigen complex, were removed by centrifugation, and the supernatant fluid was discarded. The cell pellet was washed 3 times in NET buffer (0.05% Noniodet P40, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 0.02% NaN_3, pH 7.5). The resulting pellet was suspended in 60–120 μL of 6 M urea containing 4% $NaDodSO_4$ and placed in a boiling water bath for 3 min. After centrifugation to remove the cells, the supernatant fluid was applied to a standard $NaDodSO_4$–polyacrylamide gel (Leammli, 1970).

Results

Enzyme Purification and Criteria of Purity. Table I summarizes the purification procedure for argininosuccinate synthetase. The enzyme was purified ~400-fold from crude extracts of liver with 45% recovery. On the basis of a specific

Table I: Purification of Argininosuccinate Synthetase

step	procedure	total enzyme (μmol/min)	sp act. [μmol/(min mg)]	recovery (%)
1	crude extract	115	0.01	100
2	0–60% ammonium sulfate	130	0.02	110
3	CM-Bio-Gel	68	1.4	54
4	Bio-Gel A1.5	52	4.2	45

FIGURE 1: Polyacrylamide gel electrophoresis of purified argininosuccinate synthetase in the presence of sodium dodecyl sulfate. The gel was loaded with ~7 μg of the enzyme at a specific activity of 4.2 μmol/(min mg). Electrophoresis was from top to bottom.

activity of 4.2 μmol/(min mg) for the purified enzyme, there is 0.2 mg of argininosuccinate synthetase per g of original tissue.

The enzyme from step 4 of Table I yielded a single band on electrophoresis in sodium dodecyl sulfate containing gels. A representative gel is shown in Figure 1. Electrophoresis under nondissociating conditions also revealed only one band at pH 8.9 in the gel system of Brewer & Ashworth (1969). Analytical ultracentrifugation revealed only a single symmetrical moving boundary. On the basis of these observations, we believe the enzyme to be essentially homogeneous at a specific activity of 4.2 μmol/(min mg).

Argininosuccinate synthetase is unstable in crude extracts as well as in the purified state unless citrulline is present. The purified protein is quite stable frozen or as an ammonium sulfate precipitate. We have noted irreversible precipitation upon repeated freezing and thawing, however.

Physical and Chemical Properties. The molecular weight of the native protein was determined by gel filtration through Bio-Gel A1.5. A molecular weight of 183 000 was calculated. The molecular weight of the subunit was determined by sodium dodecyl sulfate electrophoresis. A minimum molecular weight of 42 800 was determined. These observations indicate that the enzyme is composed of four subunits of equal molecular weight and the subunits are presumably identical. Centrifugation of the native enzyme in sucrose density gradients yielded a sedimentation coefficient of 8.2 S.

The apparent K_m values were determined to be 0.017 mM for asparate, 0.016 mM for citrulline, and 0.041 mM for ATP. We did not observe the non-Michaelis–Menton kinetics described by others for the beef liver enzyme (Rochovansky et al., 1977), but we did not investigate the kinetics of this enzyme in great detail.

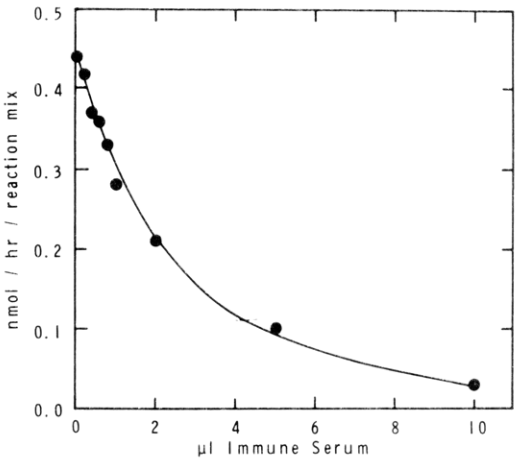

FIGURE 2: Immunoadsorption of argininosuccinate synthetase from crude extracts of human liver. A crude extract was prepared as described under Materials and Methods and diluted to give ~0.5 nmol/(h mg) in the final reaction mixture. The indicated volumes of immune serum were added to the mixture and allowed to react for 10 min (total volume 40 μL). Then 20 μL of a 10% suspension of formalin-treated *S. aureus* was added. After a 5-min incubation, the mixture was centrifuged and an aliquot of the supernatant removed for subsequent activity determination.

Immunochemical Studies. Detectable antibodies were observed 4 weeks after the initial injection and reached a maximum titer in 8 weeks. Serum obtained at 8 weeks was used to perform the immunotitration experiment shown in Figure 2, which demonstrates that the antibodies are directed against the enzyme. The antibody does not inactiviate the enzyme upon binding; hence, the antibody–antigen complex must be removed prior to performing activity determinations. This was accomplished by employing formalin-fixed *S. aureus* cells which bind the F_c region of IgG molecules by virture of the protein A content of the cell walls of these bacteria (Goding, 1978; Kessler, 1975). The data in Figure 2 were derived by treating a constant amount of crude extract of human liver with an increasing quantity of antiserum. Identical plots were obtained when the purified enzyme was used. (Data are not shown.)

We examined the specificity of the antibodies produced against human liver argininosuccinate synthetase by using formalin-fixed *S. aureus* cells as described by Kessler (1975) to remove the antigen–antibody complexes from crude liver extracts. Subsequent washing to remove nonspecifically bound protein and treatment with $NaDodSO_4$–urea solutions to denature the antibody–antigen complex produced a sample which could be subjected to electrophoresis in $NaDodSO_4$–polyacrylamide gels. The results are presented in Figure 3. Comparison of the protein bands adsorbed by immune serum vs. nonimmume serum revealed the presence of only one major protein band in the lane containing immune serum corresponding in mobility to purified argininosuccinate synthetase. The large protein band in Figure 3 above the band labeled AS is the heavy chain of the rabbit IgG molecule, and the faint bands below AS correspond to the light chains of rabbit IgG. In addition, two faint bands migrating above the heavy-chain IgG band may be seen also in lane C. These bands appear to be due to nonspecific precipitation since they are not seen reproducibly. In addition to being an index concerning the purity of the antibody, the comigration of the purified argininosuccinate synthetase with the immunoadsorbed material

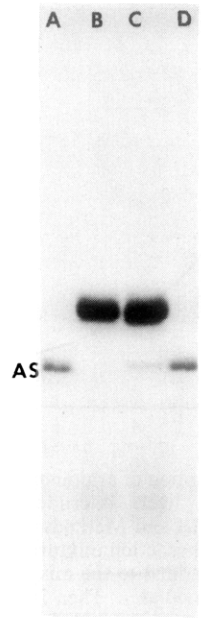

FIGURE 3: Identification of the immunoadsorbed product from crude liver as argininosuccinate synthethase. Procedures for the preparation of the samples are described under Materials and Methods. Lanes A and D contained purified human liver argininosuccinate synthetase. Lanes B and C resulted from the immunoadsorption of crude liver extract with preimmune and immune serum, respectively. AS denotes the position of the purified human liver enzyme.

Table II: Comparison of Properties of Argininosuccinate Synthetase

parameter	enzyme source		
	human[a]	beef[b]	rat[c]
M_r	183000	185000	192000
$s_{20,w}$ (S)	8.2	7.9	
M_r subunit	42800	46500	48000
V_m [μmol/(min mg)]	4.2	3.8	2.1

[a] Data are from this communication. [b] Data are from Rochovansky et al. (1977). [c] Data are from Saheki et al. (1975).

from crude liver extracts as shown in Figure 3 is strong evidence that proteolysis of the enzyme has not occurred during our purification procedure.

Discussion

The properties of the human liver enzyme are quite similar to the properties of argininosuccinate synthetase from other sources. Table II shows a comparison of some parameters for human, beef, and rat liver. No significant differences are noted in physical or chemical parameters among the three enzymes. Antibodies prepared against the human liver enzyme cross-react with the enzyme from both beef and rat liver with similar affinity (unpublished experiments).

Argininosuccinate synthetase provides an interesting example of an enzyme which catalyzes a reaction that is involved in two metabolic roles. In liver its primary role undoubtedly is to dispose of the toxic metabolite ammonia. The observations of many workers that protein intake regulates the level of argininosuccinate synthetase (Nuzum & Snodgrass, 1971; Schimke, 1962) in mammalian liver support the role of the enzyme in this capacity. In peripheral tissues, however, argininosuccinate synthetase does not play such a metabolic role since very few tissues other than liver are capable of urea synthesis. In these tissues argininosuccinate synthetase serves to synthesize arginine from citrulline. Data concerning the control of the enzyme in cultured human cell lines (Schimke, 1964; Irr & Jacoby, 1978) certainly support this concept.

Since argininosuccinate synthetase has tissue-specific functions, it is possible that isoenzymes of this enzyme might exist. Patients with citrullinemia have a deficiency of argininosuccinate synthetase in liver (Okken et al., 1973; Kennaway et al., 1975). There have been reports suggesting that some of these patients have a deficiency of the enzyme in liver but not other tissues (Okken et al., 1973). To date there has been no mechanism by which such observations could be further studied. The availability of purified human liver enzyme and antibodies to that protein now will allow for further probes into such interesting questions.

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