Argininosuccinate Lyase: Purification and Characterization from Human Liver†

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ABSTRACT: Argininosuccinate lyase has been purified to near homogeneity and partially characterized from extracts of human liver. The purified enzyme had a specific activity of 10.3 μmol min^{-1} mg^{-1} in the forward, argininosuccinate cleaving, reaction and 8.0 μmol min^{-1} mg^{-1} in the reverse reaction. On the basis of electrophoretic mobility in sodium dodecyl sulfate containing polyacrylamide gels, the protein had a minimum molecular weight of 49 000. Sedimentation equilibrium centrifugation revealed a molecular weight of 187 000. On the basis of these data, the enzyme appears to be a tetramer composed of subunits of identical molecular weight. The K_m values were 0.20 mM for argininosuccinate, 5.3 mM for fumarate, and 3.0 mM for arginine. The enzyme exhibited normal Michaelis–Menten kinetics, and guanosine 5′-triphosphate (GTP) had no affect on the activity of the enzyme. With the exception of its kinetic properties, the enzyme is very similar to the beef liver enzyme. Antibodies were prepared in rabbits and were specific for the human protein, reacting only slightly with the beef liver enzyme and not at all with the rat liver enzyme. The antibodies reacted identically with purified enzyme and enzyme in extracts of human skin fibroblasts. Immunoadsorption of crude human liver extracts followed by analysis of the immunoprecipitates by sodium dodecyl sulfate gel electrophoresis showed only one protein band which corresponded in mobility to purified argininosuccinate lyase, demonstrating that the antibodies were specific for argininosuccinate lyase.

Argininosuccinate lyase (EC 4.3.2.1) catalyzes the following reaction: argininosuccinate ⇌ arginine + fumarate. The enzyme has been studied from bovine liver (Havir et al., 1965), bovine kidney (Bray & Ratner, 1971), algae (Matagne & Schlosser, 1977), and other sources (Ratner, 1972). The bovine liver enzyme has been extensively studied (Ratner, 1972; Lusty & Ratner, 1972; Rochovansky, 1975; Schulze et al., 1970). Argininosuccinate lyase activity had been found in all human tissues examined (Ratner, 1973), and in cultured human skin fibroblasts the gene was mapped on human chromosome 7 (Naylor et al., 1978). The enzyme is found in greatest concentrations in the livers of urea-forming animals, where it catalyzes the fourth reaction of the Krebs–Henseleit ornithine cycle. In other tissues, the primary role of the enzyme is presumably in the biosynthesis of arginine from ornithine or citrulline.

In humans, deficiency of argininosuccinate lyase results in the clinical condition argininosuccinic aciduria (Hsia, 1974; Shih, 1978). There is one description of a patient with a documented deficiency of the enzyme in the liver with normal enzyme levels in the kidney and brain (Glick et al., 1976) and a recent report of a patient with normal activity in the brain but deficient activity in the liver and kidney (Perry et al., 1980). Such observations could be explained if more than one gene controls the expression of argininosuccinate lyase. There is some evidence that argininosuccinate synthetase is under the control of different genes in different tissues (Roerdink et al., 1973; Saheki et al., 1979). The lack of availability of purified argininosuccinate lyase from human tissues has hampered the study of this interesting human genetic disease. Our interest in urea cycle enzyme deficiencies in humans and the control of these enzymes in various human tissues prompted this investigation.

Experimental Procedures

Materials. Enzyme-grade ammonium sulfate was obtained from Schwarz/Mann. Argininosuccinate, fumarate, and arginine were obtained from Sigma. All electrophoretic supplies and Bio-Gel A-0.5 were from Bio-Rad Laboratories. Columns and CM-Sephadex were purchased from Pharmacia. Proteins for molecular weight markers were obtained from Boehringer-Mannheim. Formalin-killed *Staphylococcus aureus* cells (IgGsorb) were purchased from the Enzyme Center. All other chemicals were purchased from commercial sources and were of the best grade available. Human liver was obtained at autopsy within 12 h postmortum and stored at −80 °C until use. Tissue was used within 4 weeks.

Analytical Methods. Enzyme activity was assayed by following the rate of urea formation in crude extracts (Nuzum & Snodgrass, 1976) and by monitoring fumarate production at 37 °C in a thermostatically controlled spectrophotometer when the purified enzyme was employed (Havir et al., 1965). In this forward reaction, 1 unit of enzyme catalyzed the formation of 1 μmol of fumarate in 1 min. For immunoadsorption and kinetic experiments, a more sensitive assay was required; hence, we utilized a method which follows the incorporation of [^{14}C]fumarate into argininosuccinate and was a modification of the procedure of Kato et al. (1976). Reactions contained 100 mM potassium phosphate at pH 6.8, 12 mM [2,3-$^{14}C_2$]fumarate (500 dpm/nmol), 50 mM arginine, and enzyme, in a total volume of 0.1 mL. Reactions were incubated at 37 °C, terminated by the addition of 0.05 mL of 1 M HOAc, and then heated for 30 min at 90 °C to convert argininosuccinic acid to its anhydrides. After the mixture was heated, 0.85 mL of H_2O was added, and the entire 1 mL was applied to a 0.5 × 4.0 cm column of Dowex-1-8X acetate previously equilibrated in 50 mM HOAc. Two washes of 1 mL of 50 mM HOAc were used to rinse the tube and were applied to the column. The anhydrides of argininosuccinic acid were totally eluted in the 3-mL volume, and the radioactivity in an aliquot was measured to quantify enzyme activity. Protein was determined by either the biuret reaction (Gornall et al., 1949) or the method of Warburg & Christian (1941). Polyacrylamide gel electrophoresis was conducted as described by Laemmli (1970) with 15% polyacrylamide gels. Molecular weight determinations in NaDodSO_4[1]-containing gels were

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made according to Weber & Osborn (1969). Analytical ultracentrifugation was conducted in a Beckman Model E centrifuge. Centrifugation was conducted at 10000 rpm and 278 K until equilibrium was obtained.

Purification of Argininosuccinate Lyase. (Step 1) Extract Preparation. A 230-g sample of liver was homogenized in 920 mL of 0.02 M potassium phosphate, pH 6.5, containing 0.02% NaN_3 and 0.10 mM phenylmethanesulfonyl fluoride (PMSF). The pH of the resulting homogenate was lowered to 5.1 by addition of 5 N acetic acid, and 1 h later the homogenate was centrifuged for 30 min at 17000 rpm in a Sorvall SS-34 rotor. The resulting supernatant fluid (805 mL) was referred to as crude extract. These procedures were conducted at 4 °C.

(Step 2) $(NH_4)_2SO_4$ Precipitation. The crude extract was made 1.1 M in $(NH_4)_2SO_4$ by addition of solid $(NH_4)_2SO_4$ (165 g). The pH was maintained at 5.1 by the addition of 1 M Tris base. After a 1-h incubation a 4 °C, the precipitate was collected by centrifugation for 30 min at 10000 rpm in a Sorvall GSA rotor. The precipitate from this procedure was dissolved in 29 mL of 0.01 M potassium phosphate, pH 6.1, containing 0.1 mM PMSF and 0.02% NaN_3 and dialyzed for 18 h against four 1-L changes of the same buffer at 4 °C.

(Step 3) CM-Sephadex Chromatography. A 2.6 × 15 cm column containing CM-Sephadex was equilibrated at room temperature with 0.01 M potassium phosphate, pH 6.1, containing 0.1 mM PMSF and 0.02% NaN_3. The enzyme from the previous step was applied to the column, and nonbound protein was removed by continued washing with the same buffer. Argininosuccinate lyase was eluted with 0.08 M potassium phosphate, pH 6.1, containing 0.1 mM PMSF and 0.02% NaN_3. All tubes containing enzyme with a specific activity of 10.3 units/mg were pooled and precipitated by addition of solid $(NH_4)_2SO_4$ to 1.1 M. The enzyme was dissolved in 0.1 M potassium phosphate, pH 7.0, dialyzed overnight against two 500-mL changes of the same buffer, and stored at −20 °C.

Immunological Procedures. For preparation of antibodies, 50 μg of enzyme from step 3 (above) in 1 mL of phosphate-buffered saline was emulsified with 1 mL of Freund's complete adjuvant and injected subcutaneously at four sites in New Zealand white rabbits. The animal received an additional 50 μg emulsified in Freund's incomplete adjuvant 3 weeks later. Five weeks after the initial inoculation, the animals were bled from the marginal ear vein. Sera were removed from the clotted blood and stored frozen until use. Subsequently, animals were given 50 μg of protein in incomplete Freund's adjuvant every 4 weeks for a period of 12 weeks. Seven days after the final injection, 20 μg of enzyme was administered intravenously, and 5 days later the animals were sacrificed.

For the immunoadsorption and immunodiffusion experiments, extracts of liver were prepared by homogenizing 1 part tissue to 2 parts (w/v) 0.10 M potassium phosphate, pH 7.0. Homogenates were centrifuged at 30000*g* for 30 min, and the supernatant fluid was used without further fractionation.

Double-diffusion plates were purchased from Cordis Laboratories. After the plates were loaded, they were incubated at room temperature for 24–48 h. Plates were washed extensively with phosphate-buffered saline containing 1% Triton X-100, and the immunoprecipitates were stained with Coomassie brilliant blue.

Electrophoretic analysis of the antigen–antibody complexes was performed as described previously (O'Brien, 1979; Kessler, 1975). A 80-μL aliquot of crude extract prepared as described above was mixed with 20 μL of either immune or nonimmune serum and incubated for 30 min at room temperature. After incubation, 100 μL of a 10% suspension of IgGsorb was added, and a second 30-min incubation was allowed. The cells were collected by centrifugation and washed 3 times with buffer (0.05% Noniodet P-40, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3, and 50 mM Tris-HCl, pH 7.5) and once with phosphate-buffered saline. The final pellet was suspended in 65 μL of $NaDodSO_4$ containing 0.7 M 2-mercaptoethanol and 30% glycerol and heated for 3 min at 100 °C. Cells were removed by centrifugation, and 40 μL of the supernatant fluid was applied to a 15% polyacrylamide gel. As a control, 16 μg of purified argininosuccinate lyase was taken through the identical protocol in place of the human liver extract.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

Table I: Purification of Argininosuccinate Lyase

procedure	total units	total protein (mg)	sp act. (μmol min^{-1} mg^{-1})	purification	% yield
crude extract	390	19883	0.02	1	100
$(NH_4)_2SO_4$ precipitation	395	648	0.61	31	100
CM-Sephadex	225	22	10.30	515	58

Cell Culture. Human skin fibroblasts were grown in Eagle's minimum essential media (Gibco) supplemented with 10% fetal calf serum in an atmosphere containing 10% carbon dioxide. Cells were harvested at confluency by scraping into 0.6 mL of 0.1 M potassium phosphate, pH 7.0. Cells were freeze-thawed 3 times and centrifuged at 30000*g* for 30 min. The supernatant fluid was used for all assays.

Results

Purification of Argininosuccinate Lyase. Table I summarizes the purification procedure for the enzyme. The enzyme was purified approximately 500-fold to a final specific activity of 10.3 μmol min^{-1} mg^{-1} at 37 °C. On the basis of this specific activity, the original tissue contained 0.16 mg of lyase per g of tissue (wet weight). The purified protein was stable when stored frozen in 0.1 M potassium phosphate, pH 7.0. Repeated freezing and thawing did not appreciably alter the activity of the enzyme. $NaDodSO_4$ gel analyses and sedimentation equilibrium centrifugation indicated that the enzyme was in excess of 95% homogeneous.

Chemical and Physical Characterization. The K_m for argininosuccinate was 0.20 mM. The human liver enzyme exhibited normal Michaelis–Menton kinetics, and no evidence of negative cooperativity was noted under the conditions of the experiment. This was in contrast to the beef liver enzyme, which showed marked negative cooperativity (Rochovansky, 1975) under similar conditions. Addition of GTP at 0.2 mM had no effect on the K_m for argininosuccinate or the V_m of the reaction when tested under identical conditions. The apparent K_m for arginine was 3.0 mM when a constant fumarate concentration of 12 mM was employed, and the apparent K_m for fumarate was 5.3 mM at a constant concentration of 50 mM arginine. All double-reciprocal plots were linear. A pH optimum of 7.5 was determined for the forward, argininosuccinate-forming, reaction, and a broad optimum pH of 6.8–7.2 was determined for the reverse reaction. These pH optima were determined in 0.1 M potassium phosphate buffers. The V_m was 10.3 and 8.0 μmol min^{-1} mg^{-1} in the forward and reverse reactions, respectively, under the assay conditions described under Experimental Procedures.

The minimum molecular weight of the enzyme was determined by electrophoresis in $NaDodSO_4$-containing poly-

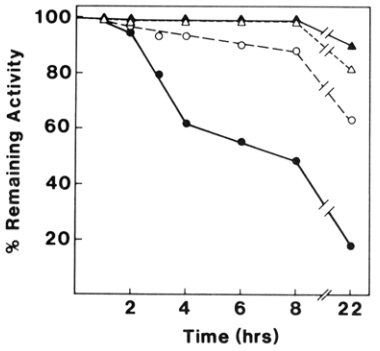

FIGURE 1: Effect of pH and temperature on argininosuccinate lyase. Enzyme of specific activity 10.3 units/mg was transferred from 0.1 M potassium phosphate, pH 7.0, into 0.01 M imidazole-HCl, pH 7.0, by passage over a 0.9 × 17 cm column of Sephadex G-25. The enzyme was immediately placed into the buffers noted below. Each reaction contained 0.33 unit of enzyme in a final volume of 0.4 mL. At the indicated times, 0.02 mL was removed and assayed by monitoring fumarate production as described under Experimental Procedures. Conditions tested were the following: (▲) 0.08 M imidazole-HCl, pH 7.0, at 4 °C; (△) 0.08 M imidazole-HCl, pH 7.0, and 25 °C; (○) 0.0 M imidazole-HCl, pH 8.2, at 25 °C; (●) 0.08 M imidazole-HCl, pH 8.2, at 4 °C.

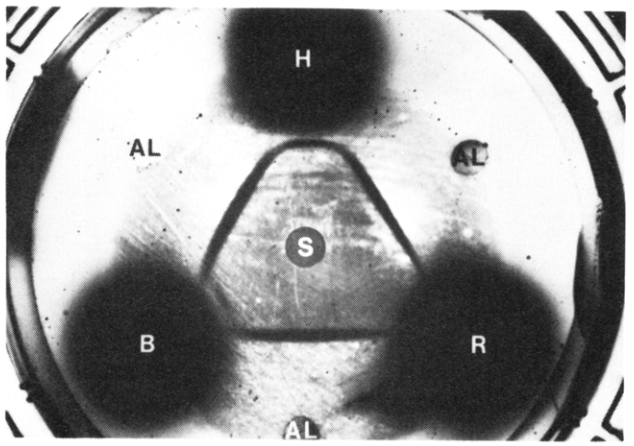

FIGURE 2: Double-diffusion analysis of antihuman liver argininosuccinate lyase. The center well (S) contained 40 μL of a 1:2 dilution of immune serum. Wells designated AL contained 23 μg of purified argininosuccinate lyase in 40 μL. Wells designated H, R, and B contained 40 μL of extract from human, rat, and beef liver, respectively. The precipitated protein was stained with Coomassie blue.

acrylamide gels. Comparison of the R_f of the enzyme with proteins of known molecular weight indicated a molecular weight of 49 000 for argininosuccinate lyase. Sedimentation equilibrium centrifugation revealed a weight-average molecular weight of 187 400 for the native enzyme. On the basis of these data, the enzyme is a tetramer composed of subunits of identical molecular weight.

The effect of buffer, pH, and temperature on the stability of the enzyme is demonstrated in Figure 1. In imidazole buffer, the enzyme was stable at pH 7.0 under both temperature conditions, but lost activity rapidly at pH 8.2 and 4 °C. The enzyme was considerably more stable at pH 8.2 and 25 °C. Tris buffers gave the same results as imidazole buffer (data not shown). Under identical conditions, phosphate buffers stabilize the enzyme at both pH and temperature conditions investigated (data not shown).

Immunochemical Studies. Rabbit antihuman argininosuccinate lyase was produced as described under Experimental Procedures. The specificities of the antibodies were determined by double-diffusion analysis as shown in Figure 2. A single line of identity was observed when immune serum was reacted

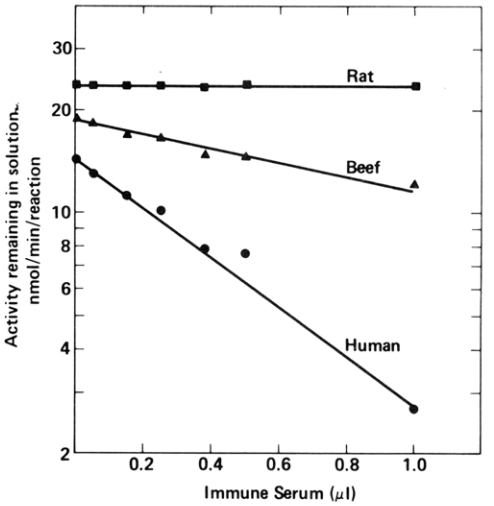

FIGURE 3: Immunoadsorption of argininosuccinate lyase from human, beef, and rat liver extracts. Reactions contained 0.017 unit of beef enzyme, 0.014 unit of human enzyme, 0.024 unit of rat enzyme, 7 mg/mL bovine serum albumin, and the indicated volumes of antiserum in a total volume of 70 μL. After a 15-min incubation at room temperature, 10 μL of a 10% suspension of IgGsorb was added, and an additional 15-min incubation was allowed. The reactions were centrifuged, and a 20-μL aliquot of the supernatant fluid was removed for activity determination. Normal rabbit serum was utilized as a control and had no affect on any extract. (■) Rat liver; (▲) beef liver; (●) human liver.

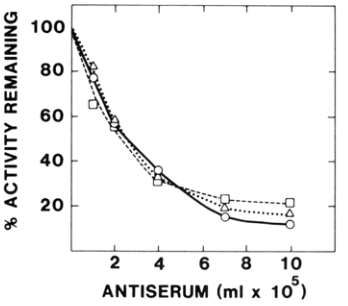

FIGURE 4: Immunoprecipitation of argininosuccinate lyase from extracts of cultured human skin fibroblasts. All reactions contained 0.2 mg of bovine serum albumin, extract, and phosphate-buffered saline in a total volume of 80 μL. (□) Purified enzyme, 0.19 milliunit; (△) extract of fibroblast line C.C., 0.26 milliunit; (○) extract of fibroblast line R.N., 0.19 milliunit. The indicated amounts of antiserum were added, and a 15-min incubation at 25 °C was carried out. A 10-μL aliquot of a 10% suspension of IgGsorb was added, and an additional 15-min incubation at 25 °C was allowed. IgGsorb was removed by centrifugation, and the enzyme activity remaining in the supernatant fluid was determined. Activity was determined by using the argininosuccinate-forming reaction as described under Experimental Procedures.

with extracts of human liver. Figure 2 also demonstrates the reaction of the antibodies with crude extracts of rat and beef liver. There was no visible precipitation line between immune serum and rat liver, but a line of nonidentity with spurring was observed with beef liver.

Proof that the antibodies were directed against argininosuccinate lyase was obtained by monitoring the removal of enzyme activity from solution upon incubation of extracts of liver with immune serum and removal of the antigen–antibody complex with IgGsorb. The data are presented in Figure 3. The antiserum clearly binds argininosuccinate lyase. Antiserum did not react with the enzyme from rat liver and reacted to a greatly reduced extent with the enzyme from beef liver. These data were in agreement with the observations from the double-diffusion plate (Figure 2). The observed specificity of the antibodies was not expected, and it was considered that

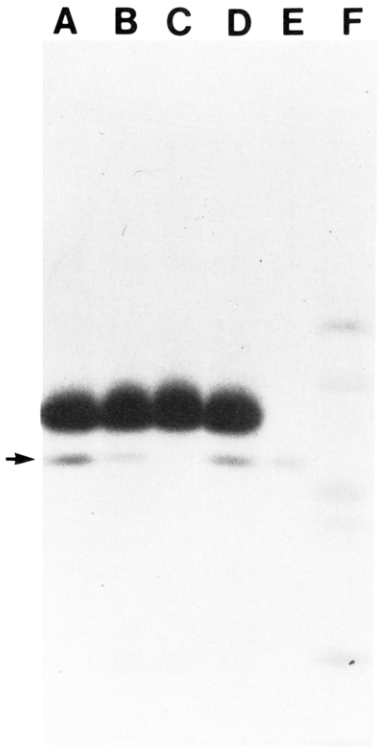

FIGURE 5: Analysis of antibody–antigen complexes. Antibody–antigen complexes were prepared as described under Experimental Procedures and electrophoresed in 15% polyacrylamide gels containing $NaDodSO_4$. Lanes A and D contained 10 μL of antiserum and 10 μg of purified argininosuccinate lyase. Lane B contained 80 μL of extract of human liver (0.06 unit) and 20 μL of antiserum. Lane C contained 80 μL of extract and 20 μL of normal rabbit serum. Lane E contained 4 μg of purified enzyme. Lane F contained the molecular weight markers bovine serum albumin (68 000), catalase (60 000), ovalbumin (45 000), aldolase (39 500), and chymotrypsinogen (25 000). The arrow marks the location of argininosuccinate lyase.

the results were related to some alteration of the enzyme prior to or during purification since the human tissue was obtained at autopsy. However, Figure 4 demonstrates that the antibodies react identically with purified enzyme and enzyme in extracts of cultured human skin fibroblasts. Thus, it is unlikely that the observed specificity was related to alterations in antigenicity that might have occurred postmortum or during enzyme purification.

Further proof of the specificity of the antibodies for argininosuccinate lyase and no other protein was obtained by analyzing the antibody–antigen complex on $NaDodSO_4$-containing polyacrylamide gels. The data in Figure 5 demonstrate that only one protein species was precipitated upon incubation of the antiserum with extracts of human liver. The comigration of the immunoadsorbed protein from extracts of human liver (lane B) with purified argininosuccinate lyase (lane A) clearly identifies the immunoadsorbed protein as argininosuccinate lyase. The data in Figure 5 also demonstrate that the purification procedures employed have not resulted in any major proteolysis of the enzyme. The large protein band above argininosuccinate lyase and the fainter staining bands below are due to the heavy and light chains of the rabbit immunoglobin molecules. It should be noted that the heavy chains of rabbit IgG molecules migrate more slowly on $NaDodSO_4$ gels than expected from their molecular weight of 50 000. This was presumably due to the glycosylation of these proteins.

Discussion

We have presented a rapid procedure for obtaining homogeneous argininosuccinate lyase fom extracts of human liver. The enzyme resembles the enzyme from beef liver with regard to its physical properties. The beef liver enzyme has a molecular weight of 202 000, and the enzyme from human liver has a molecular weight of 187 000. Both enzymes are composed of four identical subunits, and respond similarly to changes in pH and temperature. The only significant difference observed was in the kinetics of the reaction. The enzyme from human liver did not show the negative cooperativity or GTP effect that was observed for the enzyme from beef liver. The GTP effect and the native cooperativity have been postulated to serve a role in the regulation of argininosuccinate lyase in beef liver and thereby have an affect on urea synthesis (Rochovansky, 1975). The absence of these kinetic activity modulators in the enzyme from human liver suggests that the control of the enzyme in the human is at another level, perhaps in the rate of synthesis and degradation of the protein. The level of this enzyme has been shown to be altered by the protein content of the diet of rats (Schimke, 1962) and primates (Nuzum & Snodgrass, 1971).

Antibodies prepared in rabbits against the purified enzyme showed marked species specificity as exemplified by the lack of recognition of enzyme in rat liver and only partial recognition of enzyme from beef liver. Antibodies reacted identically with purified lyase, enzyme in extracts of human liver, and enzyme from extracts of human skin fibroblasts. Antibodies were demonstrated to be specific for argininosuccinate lyase by analysis of antibody–antigen complexes in $NaDodSO_4$-containing polyacrylamide gels. These analyses demonstrated that no significant proteolysis of the enzyme had occurred during the purification procedure.

Whenever enzymes are prepared from tissues obtained postmortum, the question arises concerning the possible alterations of these enzymes during tissue autolysis prior to purification. We have several pieces of data to argue that this was not a problem in our work: (1) six independent preparations of enzyme from different sources have yielded enzyme of identical molecular weight, kinetic parameters, and specific activity; (2) antibodies prepared against a single preparation react identically with all subsequent preparations as well as against the enzyme in liver extracts and human skin fibroblast extracts. We have observed no indications that would suggest that this protein has undergone any changes prior to or during the purification procedure.

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Phosphorus-31 Nuclear Magnetic Resonance Studies of the Adenosine 5′-Triphosphate–Calcium–G-actin Complex†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance spectra of the adenosine 5′-triphosphate–calcium–G-actin complex were obtained, and the resonances of the three phosphates of the protein-bound ATP were detected. The exchange of the ATP between its protein-bound and free states was found to be slow on the NMR time scale, with an exchange rate of less than 480 s^{-1} at pH* 7.8, 4 °C. The line width of the protein-bound γ-phosphate resonance (corrected for spin–spin splitting by the β phosphate) was used to calculate a rotational correlation time for the G-actin-bound ATP. With the assumption that chemical shift anisotropy is the dominant relaxation mechanism at 109.29 MHz and that the chemical shielding tensor for pyrophosphate serves as a good model for the γ phosphate of the bound ATP, a correlation time of 60 ns was estimated. Since the theoretical correlation time of a globular protein the size of G-actin is 36 ns, the line width of the bound γ-phosphate resonance is consistent with that expected for ATP bound to G-actin without large-scale rapid internal mobility. The addition of 1.5 M urea to the ATP–Ca–G-actin complex caused exchange broadening of the γ and β phosphates, but no effect on the α phosphate. This indicates an increase in the rate of exchange for the β and γ phosphates between the protein-buried and solvent-exposed environments at 1.5 M urea. At 6 M urea, the intensities of the protein-bound ATP resonances were greatly reduced, and the intensities of the free ATP resonances were greatly increased, indicative of complete protein unfolding and liberation of protein-bound ATP.

The necessity of a bound nucleotide and divalent cation in the maintenance of the native conformation of G-actin has been previously demonstrated (Laki et al., 1950; Straub & Feuer, 1950; Oosawa & Kasai, 1971). However, the roles of the bound nucleotide in the physiological functions of actin, such as cross-bridge formation with myosin heads (Huxley, 1969; Mannherz & Goody, 1970), polymerization to form thin filaments (Oosawa & Kasai, 1971; Engel et al., 1977), and binding with tropomyosin and troponin (Mannherz & Goody, 1970; Ebashi & Ebashi, 1965), are not clearly understood. ^{31}P NMR[1] spectroscopy offers a noninvasive probe to monitor the microenvironments of each of the three phosphates of ATP (Cohn & Hughes, 1960, 1962). The utility of ^{31}P NMR in studying ligand binding to various proteins, such as alkaline phosphatase (Hull et al., 1976), glycogen phosphorylase (Withers et al., 1979), and pyruvate, phosphoglycerate, and arginine kinases (Nageswara Rao et al., 1978, 1979; Nageswara Rao & Cohn, 1977), has already been well shown. Herein, we report the first ^{31}P NMR spectra of G-actin-bound ATP, with an interpretation of the spectral features in terms of protein structure and ATP binding.

Materials and Methods

Actin was prepared from rabbit skeletal muscle by the method of Spudich & Watt (1971). The protein migrated in one band on sodium dodecyl sulfate gel electrophoresis (Weber & Osborn, 1969). G-actin, after dialysis against 2 mM Tris, 0.2 mM $CaCl_2$, 0.5 mM dithiothreitol (DTT), and 0.2 mM ATP at pH 7.8, 4 °C, was diluted with an equal volume of D_2O and concentrated to 0.3–0.4 mM by using a Minicon B15

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; D_2O, deuterium oxide.

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