Methods for Purification of Each Subunit of the Mitochondrial Oligomycin-Insensitive Adenosine Triphosphatase†

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ABSTRACT: Methods are described for the purification of each of the five different subunit species of the beef heart mitochondrial oligomycin-insensitive ATPase enzyme. The enzyme complex was first depolymerized in 8 M guanidine hydrochloride, then the subunits were separated and purified by ion-exchange chromatography in buffers containing urea. Each purified subunit was shown to be homogeneous in size by the technique of electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate, and homogeneous in charge by electrophoresis in polyacrylamide gels containing urea at pH 5.2 and 8.5. The amino acid composition of each purified subunit is presented.

In previous work, we described a procedure for the purification of the beef heart mitochondrial oligomycin-insensitive ATPase enzyme and examined its behavior in depolymerizing solvents (Senior and Brooks, 1970). We found that the enzyme contained four components of different molecular weight upon electrophoresis in 5% polyacrylamide gels containing sodium dodecyl sulfate. In later studies, one band was further resolved into two bands in 10% polyacrylamide gels run under similar conditions (Senior and Brooks, 1971) and the approximate molecular weights of the five different components were found to be 53,000, 50,000, 25,000, 12,500, and 7500. We found this same pattern of components, in the same relative proportions, in enzyme prepared from different sources and by different techniques. One preparation contained, in addition, a protein with a molecular weight of 10,500 that was identified as the specific mitochondrial ATPase inhibitor protein (Brooks and Senior, 1971). Therefore, it seemed that, depending on the method of preparation, the ATPase always contained five tightly bound components and could contain an additional sixth component, the inhibitor protein.

In order to achieve a more complete understanding of the role of this enzyme in oxidative phosphorylation, it will be necessary to have available a detailed chemical analysis of each subunit. Our first task in this direction therefore was to separate and purify the subunits.

In this paper we describe methods devised to allow purification of each subunit, together with electrophoretic evidence establishing the homogeneity of each purified subunit. The amino acid composition of each individual subunit is presented.

Materials and Methods

Preparation and Assay of ATPase. The enzyme was prepared from beef heart mitochondria as described previously (Senior and Brooks, 1970). At step 4 of that procedure, the enzyme was eluted from the DEAE-Sephadex with buffer containing 0.5 M KCl (replacing buffer containing 0.2 M KCl). This modification gave a higher yield, and a homogeneous preparation (judged by gel electrophoresis and ultracentrifugation) which was used directly in this work. The specific activity of the enzyme, assayed as described (Senior and Brooks, 1970) was 80–120.

Polyacrylamide Gel Electrophoresis. Electrophoresis and staining in 10% polyacrylamide gels containing sodium dodecyl sulfate were carried out as we have described previously (Brooks and Senior, 1971). We also used two electrophoretic systems containing buffered urea solutions at pH 5.2 and 8.5. The gels for both systems were 5 cm long. Gels for the acid system were 7.5% in acrylamide and contained 7 M urea in 0.1 M acetic acid. These gels were polymerized at 50° for 15 min. The electrode buffer was 0.1 N acetic acid. The gels at pH 8.5 were 5% in polyacrylamide and contained 7 M urea in 30 mM Tris–25 mM glycine (pH 8.5). The electrode reservoir buffer was 30 mM Tris–25 mM glycine (pH 8.5). Preelectrophoresis was carried out for 3 hr at 2 mA/tube for both systems. At the end of this time both reservoir buffers were replaced, samples were applied, and electrophoresis was carried out for 30 min and 3 hr at 3 mA/tube. Samples were run toward both the anode and cathode. The gels were stained for 8 hr with 0.2% Coomassie Blue in a solution of methanol–acetic acid–water (5:1:5, v/v) and destained by soaking in the solvent. Destained gels were stored in 7% acetic acid. Samples were prepared by dissolving lyophilized protein in the appropriate buffer containing 1% (v/v) 2-mercaptoethanol and 7 M urea. They were allowed to stand overnight at room temperature before use. Usually 10–30 μg of protein was applied to the gel.

Amino Acid Analysis. Protein samples were dialyzed for several days against distilled water at 4° and were lyophilized. The samples were then hydrolyzed with 6 N HCl at 110° for 24 hr in sealed, evacuated ampoules. Hydrolysates were analyzed by the procedure of Spackman *et al.* (1958) in a Beckman amino acid analyzer, Model MS.

Routine Procedures and Chemicals. Protein was determined by the biuret method of Gornall *et al.* (1949). Dialysis tubing used for all the work reported here was first acetylated for 16–24 hr at room temperature as described by Craig (1967). This procedure was necessary to prevent loss of the smallest subunit of the ATPase during dialysis.

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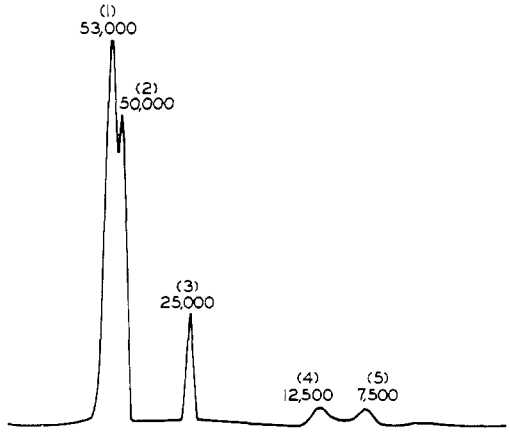

FIGURE 1: Electrophoresis of mitochondrial ATPase in sodium dodecyl sulfate gels. This is a densitometric tracing of a 10% sodium dodecyl sulfate gel. The enzyme (40 μg) was applied. The origin is at the left and the estimated molecular weight of each component (1–5) is given above the peaks in the diagram.

Urea buffers were made from freshly deionized urea solutions. Urea was dissolved to 10 M in water and deionized with the mixed-bed ion-exchange resin AG 501-X8(D). Buffer components were added and the solution was diluted to the extent necessary to make the final concentration of urea 8 M.

Ion-exchange resins were purchased from the following sources: Reeve-Angel, Whatman CM-52; Pharmacia, DEAE-Sephadex A-50; Bio-Rad, AG 501-X8(D). TPCK-treated trypsin was purchased from Worthington Biochemicals.

Results

Subunit Nomenclature. A densitometric tracing of a sodium dodecyl sulfate gel of the beef heart mitochondrial ATPase is shown in Figure 1. We have assigned a number to each subunit and these numbers will be used throughout for clarity. This figure is reproduced from Brooks and Senior (1971).

Procedure for Purification of Subunits 1, 2, and 3. Our earlier work (Senior and Brooks, 1970) had shown us that the enzyme is not completely dissociated in 8 M urea, but is dissociated completely in 8 M guanidine hydrochloride. Preliminary work convinced us that ion-exchange chromatography was the most promising technique for purification of the subunits. Thus we decided as a general procedure first to depolymerize the enzyme in 8 M guanidine hydrochloride, then to transfer it to 8 M urea solutions by gel filtration in order to allow chromatographic separation on ion-exchange resins. The success of this procedure showed that reaggregation of the enzyme in the 8 M urea, if it occurred at all, did not interfere with clean separation of the individual subunits.

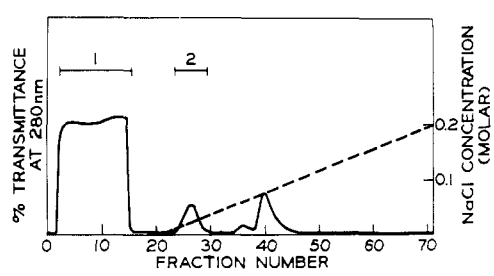

FIGURE 2: Elution profile for the chromatography of ATPase on Whatman CM-52 at pH 8.0 in a buffer containing urea; see the text for details. The second peak (pool 2) contains pure subunit 3.

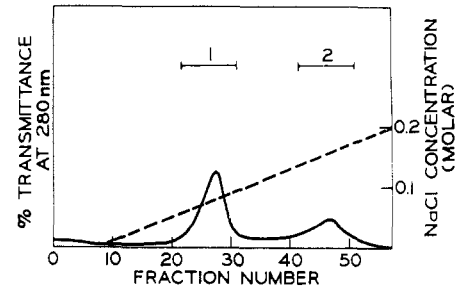

FIGURE 3: Elution profile for the chromatography of components 1 and 2 on DEAE-Sephadex A-50 at pH 8.0 in a buffer containing urea; see the text for details. The peaks containing subunits 1 or 2 are labeled 1 and 2, respectively, in the diagram.

The enzyme (325 mg of precipitate in 55% saturation $(NH_4)_2SO_4$ solution) was centrifuged and dissolved in 5 ml of a solution 8 M in guanidine hydrochloride and 10 mM in dithiothreitol. The pH was adjusted to 8.5 with KOH and the sample sat overnight at room temperature. All subsequent operations were also done at room temperature, except where stated otherwise. The protein was equilibrated with a solution ("urea buffer") containing 8 M urea, 20 mM Na_2HPO_4, 1 mM dithiothreitol, and 0.1 mM EDTA at pH 8.0 (adjusted with 2 M H_3PO_4) by passage down a 40 × 2 cm column of Sephadex G-25 equilibrated with this solution. The sample (35 ml) was applied to a 15 × 1.5 cm column of Whatman CM-52 equilibrated with the buffered urea solution. After the sample had flowed into the resin, the column was washed with urea buffer until the effluent showed no absorbance at 280 nm. This first eluate (pool 1) contained subunits 1 and 2. The column was developed with a linear gradient between 100 ml of urea buffer and 100 ml of urea buffer containing 0.2 M NaCl (pH 8.0). The elution profile is shown in Figure 2. Fractions were pooled as indicated in the figure, dialyzed against large volumes of distilled water at 4° and lyophilized. The material from each pool was examined by electrophoresis in 10% sodium dodecyl sulfate gels. The first pool contained material not adsorbed by the resin (subunits 1 and 2). The second pool contained subunit 3 in pure form. A sodium dodecyl sulfate gel of this material is shown in Figure 5c. The protein also migrated as a single species (data not shown) in urea gels at pH 5.2; it did not enter the gels at pH 8.5 for migration toward either electrode.

A portion of the excluded material (150 mg of pool 1, Figure 2) from the CM-52 chromatography was dissolved in 8 M guanidine hydrochloride–10 mM dithiothreitol solution and equilibrated with a solution containing 8 M urea, 20 mM Tris, 1 mM dithiothreitol, and 0.1 mM EDTA at pH 8.0 (adjusted with 6 N HCl) on Sephadex G-25 (technique as above). The sample was applied to a 15 × 1.5 cm column of DEAE-Sephadex A-50, previously equilibrated with the same buffer. The column was developed with a linear salt gradient between 150 ml of urea buffer and 150 ml of urea buffer containing 0.2 M NaCl (pH 8.0). An elution profile for this column is shown in Figure 3. The fractions were pooled, dialyzed at 4° and lyophilized as described above. The first peak (pool 1) contained subunit 1 and the second peak (pool 2) contained subunit 2, each in pure form. Densitometric tracings of sodium dodecyl sulfate gels of these proteins are shown in Figure 5A,B, respectively. Subunit 2 migrated as a single species in urea gels at pH 5.2 and 8.5 (not shown); at pH 8.5 subunit

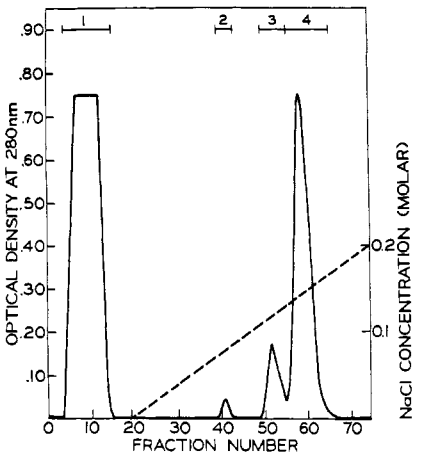

FIGURE 4: Elution profile for the chromatography of components 4 and 5 on CM-52 at pH 7.0 in a buffer containing urea; see the text for details. The first peak contains subunit 4 and the fourth contains subunit 5.

1 would not enter the gel. Subunits 1 and 2 migrate very close together in gels at pH 5.2.

In some instances it was necessary to rechromatograph the material in the second pool in order to eliminate some contamination by subunit 1. When this procedure was repeated, the conditions were the same as those used for the original chromatography.

Procedure for Purification of Subunits 4 and 5. These two subunits are present in the enzyme to the extent of no more than 5% of the total protein, estimated from the relative staining intensity of the bands in sodium dodecyl sulfate gels. For this reason subunits 4 and 5 were first separated together from the other components of the enzyme, and were then separated from each other chromatographically on Whatman CM-52.

An $(NH_4)_2SO_4$ precipitate of ATPase (100 mg) was dissolved in 10 ml of 8 M guanidine-HCl containing 10 mM dithiothreitol and the pH was adjusted to 8.5 with KOH. The sample sat for 4 hr at room temperature and was then dialyzed at 4° for 3 days against 6 l. of water with changes twice daily. The dialyzed material was centrifuged at 20,000*g* for 10 min. The supernatant was carefully decanted and lyophilized. (The precipitate could be used for the preparation of subunits 1 and 2, as above.) The lyophilized material from the supernatant contained subunits 4 and 5 plus a small amount of a contaminant that was not one of the components of the enzyme. About 1 g of enzyme was treated in this fashion and the material from the ten lyophilized supernatants was combined.

The mixture of subunits 4 and 5 was dissolved in 20 ml of a solution containing 8 M urea, 20 mM Na_2HPO_4, 1 mM dithiothreitol, and 0.1 mM EDTA at pH 7.0 (adjusted with 2 M H_3PO_4) and kept at room temperature for at least 3 hr. The sample was applied to a 15 × 1.5 cm column of Whatman CM-52 previously equilibrated with the same buffer. Following sample application, urea buffer was applied until the effluent absorbance at 280 nm dropped to zero. The column was developed with a linear salt gradient between 75 ml of urea buffer and 75 ml of urea buffer containing 0.2 M NaCl (pH 7.0), this chromatography was done at room temperature. The elution profile for this column is shown in Figure 4. Material from each peak was examined by electrophoresis in sodium dodecyl sulfate gels. The first peak (excluded material) contained pure subunit 4 and the fourth peak (pool 4)

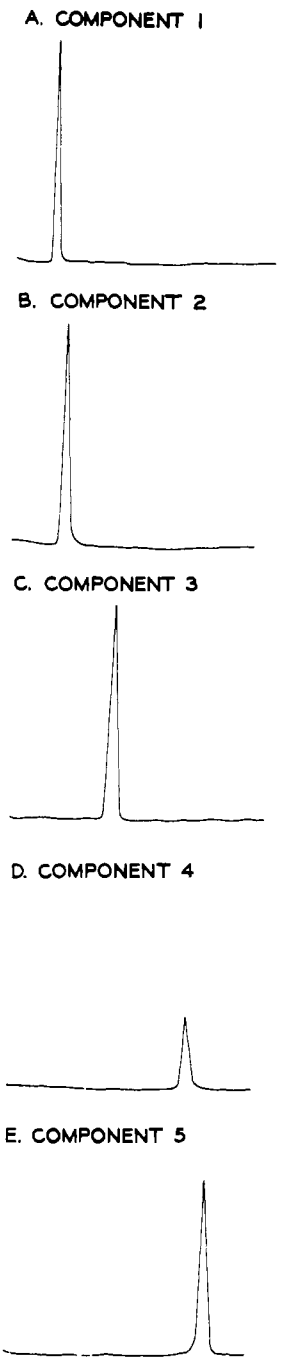

FIGURE 5: Polyacrylamide gel electrophoresis of mitochondrial ATPase subunits. These are densitometric tracings of 10% sodium dodecyl sulfate gels of the component proteins of mitochondrial ATPase. The origin is at the left. The amount of sample applied to the gels was 20–30 μg. Each component was positively identified by coelectrophoresis with a sample of the unfractionated ATPase.

contained pure subunit 5. Densitometric tracings of sodium dodecyl sulfate gels of these purified proteins are shown in Figure 5D,E, respectively. Both proteins migrated as single species in urea gels at pH 5.2 and 8.5 (data not shown). Subunit 5 migrates well ahead of cytochrome *c* (toward the cathode) in urea gels at pH 5.2 and is therefore a very basic protein. In this gel system electrophoresis for 3 hr allows the protein to migrate off the gel into the lower buffer chamber, so an electrophoresis time of 30 min was used.

The pool containing component 5 was sometimes contaminated slightly with a protein of higher molecular weight. This

TABLE I: Amino Acid Composition of the Subunits of Mitochondrial ATPase.[a]

Amino Acid	Subunit 1	2	3	4	5	Whole ATPase
Lysine	6.29	5.40	9.63	3.28	12.75	6.12
Histidine	1.03	2.03	1.76	1.52	0.99	1.56
Arginine	6.74	4.96	6.69	2.91	4.66	5.41
Aspartic acid	8.38	7.38	8.72	8.03	6.31	8.34
Threonine	5.31	6.03	6.73	5.24	4.73	5.49
Serine	6.14	4.89	9.72	6.87	10.51	5.52
Glutamic acid	12.04	13.93	10.08	14.65	11.04	12.06
Proline	3.47	4.92	0.0	4.56	1.64	4.09
Glycine	9.62	9.61	5.90	6.76	12.02	9.39
Alanine	9.75	10.19	11.08	18.33	10.97	10.50
Valine	7.31	8.14	4.67	9.90	5.71	8.01
Methionine	1.96	2.03	2.33	1.40	1.31	1.63
Isoleucine	6.92	6.59	8.13	3.11	5.45	6.80
Leucine	9.28	8.71	7.85	8.64	7.23	9.25
Tyrosine	2.96	2.30	3.41	0.86	2.23	2.78
Phenylalanine	2.76	2.89	3.30	3.90	2.43	2.98

[a] Cysteine and tryptophan were not estimated. Each value given is the average of two or more analyses. ATPase was prepared by the method of Senior and Brooks (1970) and was free of inhibitor protein. Values are moles found per 100 moles of total amino acids.

protein was removed by chromatography of the pooled material on a 60 × 0.9 cm column of Sephadex G-200 in 0.1 M NH_4HCO_3 containing 1% sodium dodecyl sulfate. The pool was first dialyzed and lyophilized as before. The lyophilized protein was then dissolved at 10 mg/ml in a solution containing 3% sodium dodecyl sulfate, 10 mM dithiothreitol, 0.1 M NH_4HCO_3, and 10% sucrose and allowed to sit overnight at room temperature. A 0.1-ml sample was applied to the column. Fractions of 0.3 ml were collected, diluted to 1.0 ml with water and the absorbancy was measured at 225 nm. Pooled fractions were dialyzed at room temperature against 200 volumes of 80% acetone with one change after 3 hr in order to remove sodium dodecyl sulfate.

Amino Acid Composition. The amino acid composition of each of the purified components is given in Table I. Each component shows a different amino acid composition, although components 1 and 2 appear to be generally similar in composition.

A molecular weight greater than 10,000 was calculated for component 5 from the amino acid analysis, assuming a single histidine residue. This value is higher than the value of 7500 which we have obtained using the technique of electrophoresis in sodium dodecyl sulfate gels and may reflect known inaccuracy in calculation of molecular weight of small proteins in sodium dodecyl sulfate gels.

Discussion

In previous work we have shown that mitochondrial ATPase contains several proteins with different molecular weights when examined in various depolymerizing solutions (Senior and Brooks, 1970). Our more recent studies have demonstrated that there are five subunits of different molecular weight present in the enzyme (Senior and Brooks, 1971). In this paper we have gone further and described procedures which allow purification of each subunit to homogeneity.

As the first step toward chemical characterization of each subunit we have also presented the amino acid composition of each subunit. Similar data have been previously presented for the specific inhibitor protein (Brooks and Senior, 1971). We hope that with the ability to obtain each subunit in pure form, and with this limited chemical knowledge, detailed investigations of the mechanism of ATP hydrolysis and ATP synthesis at the active site of this enzyme will now be both facilitated and encouraged. We may also envisage that in time comparison of the amino acid sequences of each subunit from several different ATPase enzymes of mitochondrial, chloroplast, or bacterial origin, which are known to be similar in molecular weight and subunit structure (Catterall and Pederson, 1971; Schnebli *et al.*, 1970; Farron, 1970; Tzagoloff and Meagher, 1971; R. J. Birzborn and E. Racker, personal communication; Lambeth and Lardy, 1971) will throw light on the evolution and interrelation of these various bioenergetic systems.

From the behavior of the subunits of the enzyme on ion-exchange resins it is apparent that the ATPase subunits dissolved in 8 M urea vary widely in their isoelectric points. Components 1, 2, and 4 appear to be acidic in nature, while components 3 and 5 are basic, the latter to an unusual degree.

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