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ATPase of *Escherichia coli:* Purification, Dissociation, and Reconstitution of the Active Complex from the Isolated Subunits†

Günter Vogel* and Roswithe Steinhart

ABSTRACT: A simple procedure for the purification of Mg^{2+}-stimulated ATPase of *Escherichia coli* by fractionation with poly(ethylene glycols) and gel filtration is described. The enzyme restores ATPase-linked reactions to membrane preparations lacking these activities. Five different polypeptides (α, β, γ, δ, ϵ) are observed in sodium dodecyl sulfate electrophoresis. Freezing in salt solutions splits the enzyme complex into subunits which do not possess any catalytic activity. The presence of different subunits is confirmed by electrophoretic and immunological methods. The active enzyme complex can be reconstituted by decreasing the ionic strength in the dissociated sample. Temperature, pH, protein concentration, and the presence of substrate are each important determinants of the rate and extent of reconstitution. The dissociated enzyme has been separated by ion-exchange chromatography into two major fragments. Fragment I_A has a molecular weight of about 100000 and contains the α, γ, and ϵ polypeptides. The minor fragment, I_B, has about the same molecular weight but contains, besides α, γ, and ϵ, the δ polypeptide. Fragment II, with a molecular weight of about 52000, appears to be identical with the β polypeptide. ATPase activity can be reconstituted from fragments I_A and II, whereas the capacity of the ATPase to drive energy-dependent processes in depleted membrane vesicles is only restored after incubation of these two fractions with fraction I_B, which contains the δ subunit.

The membrane-associated Mg^{2+}-stimulated adenosinetriphosphatases from mitochondria, chloroplasts, and bacteria appear to function in energy transduction reactions. Several extensive reviews have appeared (see, e.g., Senior, 1973; Beechey and Cattell, 1973; Baltscheffsky and Baltscheffsky, 1974; Racker, 1970; Abrams and Smith, 1974). The available information about these enzymes shows a remarkable similarity with respect to catalytic mechanism, molecular weight, and overall subunit composition. The enzyme complexes exist as multimeric proteins with molecular weights of 300000–400000 and contain five different polypeptides.

The ATPase (EC 3.6.1.3) of *Escherichia coli* has been purified by several workers. Two preparations yielded an enzyme which appeared to contain five polypeptide chains (α–ϵ) by sodium dodecyl sulfate electrophoresis, ranging in molecular weight from about 60000 to about 10000 (Bragg and Hou, 1972; Futai et al., 1974). These enzyme preparations were active as "coupling factor" in reconstituting energy-linked reactions on incubation with ATPase-depleted membranes. Several other purification procedures yielded an enzyme containing only four polypeptides, the δ subunit being the missing component. These preparations were not capable of restoring energy coupling (Evans, 1970; Kobayashi and Anraku, 1972, 1974; Hanson and Kennedy, 1973; Nelson et al., 1974). Direct evidence for the involvement of the δ subunit in the attachment of the ATPase to the membrane was obtained recently (Smith and Sternweis, 1975). These authors showed that the coupling factor capacity of the four-subunit enzyme could be restored by addition of a fraction containing mainly the δ and ϵ polypeptide chains.

The stoichiometry of the polypeptide chains in the ATPases from beef heart (Senior and Brooks, 1971) and rat liver (Catterall et al., 1973) was suggested to be $\alpha_3\beta_3\gamma\delta\epsilon$, based on staining intensities after dodecyl sulfate electro-

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phoresis. The same stoichiometry was proposed for the ATPases from *Escherichia coli* and *Salmonella typhimurium*, as determined with the ^{14}C-labeled enzymes based on the estimation of the radioactivity distribution between the peptide chains (Bragg and Hou, 1975). However, new evidence suggests that this proposal for the overall subunit structure may be incorrect (Senior, 1975). The data for the location of sulfhydryl groups and the position of disulfide bridges in the various subunits indicate that only two copies of the α, γ, and ϵ chains are present in the complex. The stoichiometry of subunits β and δ could not be calculated by the technique used since they were found to contain no cysteine.

In the present studies reconstitutively active ATPase from *Escherichia coli* was purified to homogeneity. Procedures were designed so that large quantities of enzyme could be quickly purified in high yield. A simple method for reversible dissociation of the enzyme complex was worked out. In order to understand the structural organization of the complex and to relate the functional properties of the subunits to their quaternary interactions, the different reconstitutively active fragments were isolated and partially characterized. The effects of various conditions upon the reconstitution of active enzyme were investigated.

Materials and Methods

ATPase Assay. The ATPase activity was measured with an ATP-regenerating system at 25°C. The standard ATPase reaction mixture contained 50 m*M* Tris-HCl (pH 8), 2.5 m*M* $MgCl_2$, 5 m*M* ATP, 1.5 m*M* phosphoenolpyruvate, 0.2 m*M* NADH, 50 m*M* KCl, 6 units of pyruvate kinase (EC 2.7.1.40) and 6 units of lactate dehydrogenase (EC 1.1.1.27), and enzyme in a volume of 1.0 ml. ATPase activity was calculated from the rate of NADH oxidation followed at 334 nm.

One unit of ATPase is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmol of ATP per min at 25°C at pH 8.

Fluorescence Assay. The standard reaction mixture contained 50 m*M* Mops[1] (pH 7.0), 50 m*M* KCl, 10 m*M* $MgCl_2$, 2 μM ACMA, and membrane vesicles (0.1–0.3 mg of protein). Fluorescence was measured in an Aminco Bowman spectrofluorometer using an excitation wavelength of 410 nm and an emission wavelength of 490 nm. The reaction was started by addition of 2 m*M* ATP and an appropriate amount of ATPase if required.

Purification of Reconstitutively Active ATPase. All operations were carried out at 2–5°C.

Step 1: Membrane Preparation and Extraction of the Enzyme. Frozen cells of *E. coli* K 12 (λ) grown in peptone medium and harvested in late exponential phase were purchased from Merck and suspended in 600 ml of 50 m*M* Mops (pH 7)–10 m*M* $MgCl_2$ containing 10 μg/ml of DNase. Cells were disrupted by one passage through a Ribi press at 25000 lb/in.^{2}. Unbroken cells and debris were removed by centrifugation at 8000*g* for 10 min. The supernatant fraction was centrifuged at 120000*g* for 90 min. The resulting pellet was washed once by resuspending in 300 ml of 1 m*M* Tris-HCl (pH 8.0) containing 0.5 m*M* EDTA, 2.5 m*M* 2-mercaptoethanol, and 10% glycerol, and centrifuging at 120000*g* for 90 min. To release the ATPase, the resulting pellet was homogenized in 600 ml of the washing buffer described above, incubated for at least 1 hr (usually overnight), and centrifuged again for 90 min at 120000*g*. The washed membranes were suspended at the highest possible concentration in 50 m*M* Mops (pH 7)–10 m*M* $MgCl_2$ and stored in liquid nitrogen.

Step 2: Poly(ethylene glycol) Fractionations. The supernatant fraction, which contained most of the original ATPase activity, was mixed with methanol (4:1) and then brought to 50 m*M* $MgCl_2$ by addition of 1 *M* $MgCl_2$. The resulting precipitate was removed by centrifugation at 10000*g* for 20 min. To each 100 ml of the supernatant 25 ml of 50% (w/w) aqueous poly(ethylene glycol) (average molecular weight 6000) was added slowly while the mixture was stirred. The solution was stirred for a further 20 min and centrifuged for 30 min at 10000*g*.

The supernatant fraction was discarded and the precipitate was resuspended in 50 ml of 50 m*M* Mops (pH 7) containing 20% methanol and 2.5 m*M* 2-mercaptoethanol. $MgCl_2$ was added to 50 m*M* and the resulting precipitate was removed by centrifugation at 40000*g* for 10 min. A 2.5-ml portion of 50% (w/w) aqueous poly(ethylene glycol) (average molecular weight 1550) was added to each 10 ml of the supernatant fraction. The precipitated protein was collected by centrifugation at 40000*g* for 15 min and dissolved in about 5 ml of 50 m*M* Mops (pH 7) containing 20% methanol and 2.5 m*M* 2-mercaptoethanol. Any insoluble material was removed by centrifugation at 10000*g* for 10 min.

Step 3: Gel Filtration on Sepharose 6B. The clear, slightly yellow enzyme solution was applied to a column of Sepharose 6B (2.6 × 100 cm), previously equilibrated with the above buffer. The protein was eluted at a flow rate of approximately 20 ml/hr, and 5-ml fractions were collected. Enzyme activity appeared after an elution volume of about 270 ml, in the second of the two major protein peaks. Active fractions were pooled and precipitated by addition of $MgCl_2$ to 50 m*M*, and 2.5 ml of aqueous poly(ethylene glycol) (MW 6000) (50% w/w) per 10 ml of enzyme solution.

The precipitated enzyme was collected by centrifugation at 40000*g* for 15 min and dissolved in about 4 ml of 50 m*M* Mops (pH 7) containing 20% methanol and 2.5 m*M* 2-mercaptoethanol.

Dissociation and Reactivation of Purified Enzyme. The enzyme was precipitated by addition of $MgCl_2$ to 50 m*M* and 0.25 ml of poly(ethylene glycol) (MW 1550) (50% w/w) per ml of enzyme solution. The precipitate was collected by centrifugation at 15000*g* for 15 min and dissolved in 50 m*M* Mes (pH 6.1) containing 5 m*M* ATP and 1 *M* LiCl, at a protein concentration of 10–15 mg/ml; 0.5–1-ml portions of the enzyme solution in small test tubes were frozen in an alcohol bath at approximately −70°C. After thawing at room temperature the process was repeated. Enzyme solutions inactivated by the above treatment were reactivated by at least tenfold dilution with 50 m*M* Mes (pH 6.1) containing 10% glycerol, 2.5 m*M* $MgCl_2$, and 2.5 m*M* ATP, followed by incubation at 25°C until maximal reactivation was obtained. Alternatively, the salt concentration was decreased by passage of the dissociated sample through a small Sephadex G-25 column equilibrated with the same buffer.

Separation of Reconstitutively Active Subunits. About 10–15 mg of pure enzyme was dissociated as described above. Thereafter, all buffers used contained 2.5 m*M* ATP and 2.5 m*M* 2-mercaptoethanol. The dissociated sample was desalted by passage through a Sephadex G-25 column

[1] Abbreviations used were: ACMA, 9-amino-6-chloro-2-methoxyacridine; EDTA, sodium ethylenediaminetetraacetate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 2-(*N*-morpholino)propanesulfonic acid; Tris, *N*-tris(hydroxymethyl)aminomethane.

Table I: Purification of Reconstitutively Active ATPase from *E. coli*.

Step	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)
1. Crude solubilized ATPase	600	3100	4.7
2. Poly(ethylene glycol) fractionations	71	2100	29.5
3. Sepharose 6B eluate	43	1700	39.5

(0.5 × 4 cm) equilibrated with 20 m*M* potassium phosphate (pH 6.5) and applied to a DEAE-cellulose column (1 × 5 cm) previously equilibrated with 20 m*M* potassium phosphate (pH 6.5). The column was washed with a small amount of starting buffer and subsequently eluted with a 50-ml linear gradient of 50–300 m*M* potassium phosphate (pH 6.5). Thirty-four fractions of equal volume were collected.

Electrophoretic Procedures. Gelatinized cellulose acetate strips (Chemetron, Milano) were immersed 30 min before use in the electrophoresis buffer (usually 50 m*M* Tris-glycine (pH 8.3) containing 10% glycerol). After blotting between two filter paper sheets, the strips were placed on the cooled surface of the electrophoretic tank, the sample was applied, and electrophoresis was carried out for 30–45 min at 500 V. Protein bands were then visualized by staining with Coomassie Blue and destaining in 10% acetic acid–20% methanol.

Acrylamide gel electrophoresis was performed in either 5 or 7.5% polyacrylamide gels prepared and run in 50 m*M* Tris-glycine (pH 8.3).

Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Weber et al. (1972). Protein samples were dissociated by heating at 100°C for 1 min in a solution containing 1% dodecyl sulfate and 1% 2-mercaptoethanol.

Gradient slab gel electrophoresis was performed in exponential gradient gels (Pharmacia) in 90 m*M* Tris-borate buffer (pH 8.35). The gradient ranged from 4 to 30% acrylamide. Gels were stained for protein with Coomassie Blue and for ATPase activity by the method of Abrams and Baron (1967). Densitometric traces were made in a Joyce-Loebl-Microdensitometer Mk III B. For molecular weight estimations of ATPase and its isolated subunits electrophoresis was carried out for 12 hr at 120 V. Reference proteins were: apoferritin (460000), catalase (240000), lactate dehydrogenase (140000), and bovine serum albumin (67000).

Immunological Studies. Antibodies against ATPase were prepared by intramuscular injection of 600 μg of purified enzyme in 2 ml of 0.1 *M* potassium phosphate (pH 7) mixed with an equal volume of Freund's complete adjuvant into each of two rabbits. Intramuscular booster injections were performed at 4 and 6 weeks using 2 mg of antigen each time. Seven days after the last injection, about 60 ml of blood was collected from the ear vein of each rabbit. The bleeding was repeated 4 days later. After clotting, the blood serum was collected by centrifugation. Serum was fractionated by ammonium sulfate precipitation. The γ-globulins (precipitating between 30 and 50% saturated ammonium sulfate) were further purified by ultrafiltration in a Minicon A75 cell (Amicon).

Ouchterlony double diffusion experiments (Ouchterlony and Nilson, 1973) were carried out on immunodiffusion plates (10 mm radius, 1 mm thickness) containing 1% agarose in a phosphate-buffered saline (pH 7.2). Unprecipitated material was eluted by washing the plates in physiological saline and precipitin lines were stained with Coomassie Blue and destained by washing with 10% acetic acid–1% glycerol.

Immunoelectrophoresis was done on gelatinized cellulose acetate sheets. Samples (3 μl) were applied as round spots and electrophoresis was carried out as described before. After electrophoresis, antiserum was applied as a central line and the immunodiffusion pattern was allowed to develop for 15 hr at room temperature in a moist chamber. After extensive washing of the strips in 0.9% NaCl the precipitin lines were visualized by staining with Coomassie Blue.

Chemicals and Routine Procedures. Protein was determined by the procedure of Lowry et al. (1951) using 10% trichloroacetic acid to precipitate the samples, when necessary, and using bovine serum albumin as a standard.

ACMA was a gift from Dr. P. Overath. Coomassie Brillant Blue G-250, sodium dodecyl sulfate (>99%), poly(ethylene glycol) (MW 1550), and poly(ethylene glycol) (MW 6000) were obtained from Serva; auxiliary enzymes and standard proteins were from Boehringer. Sephadex G-25 and Sepharose 6B were purchased from Pharmacia and DEAE-cellulose (DE52) was from Whatman. All other chemicals were of reagent grade purity.

Results

Purification and Purity of E. coli *ATPase.* The membrane-associated ATPase of *E. coli* was released in a soluble form by washing membrane vesicles with buffers of low ionic strength. The soluble enzyme was purified by fractionation with poly(ethylene glycols) and gel filtration on Sepharose 6B as described above. The results of a typical purification procedure using 200 g (wet weight) of cells are summarized in Table I. A specific activity of 30–40 units/mg was generally obtained. This corresponds to a specific activity of about 100 units/mg at 37°C and is thus comparable with the highest specific activities previously reported (Futai et al., 1974; Nelson et al., 1974). The purified enzyme recombines with ATPase-deficient membranes to reconstitute ATP synthesis in oxidative phosphorylation, ATP-driven transhydrogenase (Table II), and ATP-driven quenching of ACMA fluorescence (Kraayenhof and Fiolet, 1974; Nieuwenhuis et al., 1973) (Figure 3). The final preparation migrated as a single band upon electrophoresis on cellulose acetate strips and in polyacrylamide gels (Figure 2a). It formed a single precipitin line in Ouchterlony double diffusion plates (Figure 1b) and on immunoelectrophoresis (Figure 1), using antisera prepared against the purified enzyme. As judged by dodecyl sulfate polyacrylamide electrophoresis, the enzyme consisted of five different polypeptides with molecular weights of 56000, 52000, 32000, 21000, and 11500 (Figure 11a). These values are in agreement with those reported previously for the *E. coli* ATPase active as coupling factor (Futai et al., 1974; Bragg and Hou, 1972).

Reversible Dissociation of E. coli ATPase. Dissociation Procedure. Cold lability is a common feature of soluble ATPase enzymes from different sources. In previous reports it was shown that cold inactivation of mitochondrial ATPase was accompanied by dissociation of the enzyme (Penefsky and Warner, 1965; Forrest and Edelstein, 1970; Rosing et al., 1975). Irreversible inactivation of the *E. coli* ATPase in the cold was shown to be accompanied by the appearance of

Table II: Reversible Inactivation of *E. coli* ATPase by Freezing and Thawing.

Sample[a]	ATPase Activity (units/mg)	ATP[b] Driven Transhydrogenase (nmol of NADH Formed per min per mg of Membrane Protein)	[c] Uncoupler Sensitive Phosphorylation of ADP (nmol of ATP Formed in 10 min)
Untreated enzyme	34	24	56
Dissociated enzyme	0.2	0	1.3
Reconstituted enzyme	28	16	38

[a] ATPase (4 mg) was inactivated by freezing and thawing twice as described under Materials and Methods. An aliquot of the dissociated sample was diluted 1:10 in Tris-HCl (pH 8), and kept at 0°C. No reactivation took place under these conditions. Another aliquot was diluted 1:10 in 50 m*M* Mes (pH 6.1) containing 20% glycerol, 2.5 m*M* $MgCl_2$ and 2.5 m*M* ATP. After incubation for 1 hr at 25°C maximal reconstitution was observed. [b] Depleted membrane vesicles (0.5 mg of protein) were incubated at 25°C for 10 min with either native ATPase (30 μg of protein) or the same amounts of the dissociated and reconstituted sample. ATP-driven transhydrogenase was assayed as described by Futai et al. (1974). [c] Incubation conditions were those described above and uncoupler sensitive phosphorylation of ADP with NADH as substrate was assayed by the method of Hertzberg and Hinkle (1974).

faster migrating components on acrylamide electrophoresis (Hanson and Kennedy, 1973; Carreira et al., 1973). Freezing and thawing in salt solutions was suggested as a useful method to dissociate a number of multimetric proteins into subunits (Chilson et al., 1965).

ATPase is stable at room temperature in a 50 m*M* Mes buffer (pH 6.7) containing 1 *M* LiCl. Rapid freezing to −70°C and thawing at room temperature cause complete loss of enzymatic activity. The presence of 20% methanol in the incubation mixture effectively protects the enzyme against inactivation by this treatment.

Evidence for the dissociation of the enzyme into subunits was obtained from electrophoresis on cellulose acetate strips. The complex was split into two subunits which differed from each other and from the native enzyme in their electrophoretic mobilities. Both of these subunits gave a precipitin line against anti-ATPase γ-globulin in immunoelectrophoresis (Figure 1). The presence of two different subunits was further confirmed by polyacrylamide electrophoresis in a gradient slab gel (Figure 2b), indicating a different molecular weight for the two fragments formed. Figure 2c shows that the ATPase can be reconstituted from the dissociated components by decreasing the ionic strength and incubating at room temperature.

Enzyme reassociated in this way regained ATPase activity and restored ATP-synthesis capacity, ATP-driven transhydrogenase activity, and ATP-driven quenching of ACMA. These activities are summarized in Table II and Figure 3.

Effect of pH. The effect of pH on the rate and extent of reactivation of the split enzyme is demonstrated in Figure 4. The reactivation process is favored by low pH values, with a maximum at pH 5. Plots of either maximal yield or initial rate of reconstitution against pH give sigmoid curves with inflection points between pH 6 and 6.5 (Figure 4b and c).

Other experiments showed that a dissociated sample

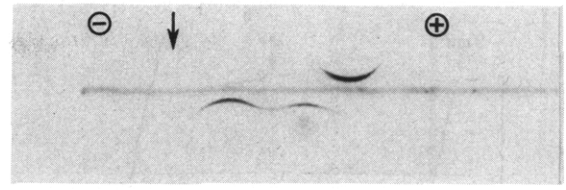

FIGURE 1: Immunoelectrophoresis on cellulose acetate sheet of native ATPase (top) and the same preparation after it was frozen and thawed twice (bottom). The diffusion pattern was developed and visualized as described under Materials and Methods.

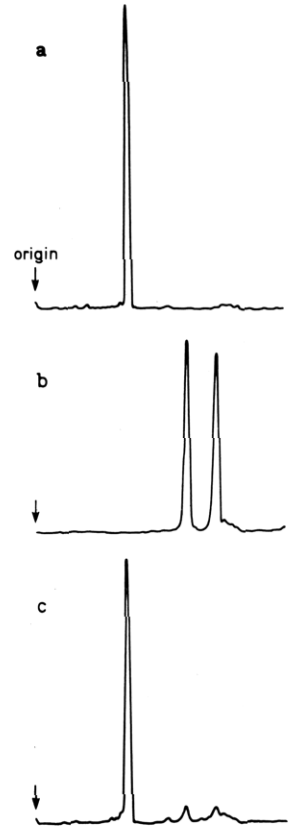

FIGURE 2: Densitometric trace after slab-gel electrophoresis (4–30% polyacrylamide) of: (a) native ATPase; (b) same preparation after two freezings and thawings; (c) same as (b) after reactivation (see Materials and Methods); 30 μg of each enzyme preparation was applied to the gel and electrophoresis was performed for 6 hr at 120 V in 90 m*M* Tris-borate (pH 8.35).

brought to alkaline pH using Sephadex G-25 filtration at 0°C could be completely reactivated by rewarming at low pH immediately after the filtration procedure. After aging for a few hours at alkaline pH in the cold, however, irreversible denaturation of one or both of the subunits occurred and no reactivation was obtained by incubation of the sample at acid pH at room temperature.

Effect of Temperature. Since the ATPase has been shown by many workers to be cold labile and to be protected against this effect by glycerol or methanol, it was of interest to investigate the effect of these parameters on the reconstitution process. Results are summarized in Figure 5. No significant reconstitution occurred at temperatures below 18°C. Between 20 and 35°C the initial rate of reconstitution increased. The maximal yield of enzyme activity was not significantly increased at temperatures above 22°C. The sharp reduction in reconstitution below 18°C was not due to an irreversible inactivation of subunits since a shift from lower to higher temperature allowed reconstitution of

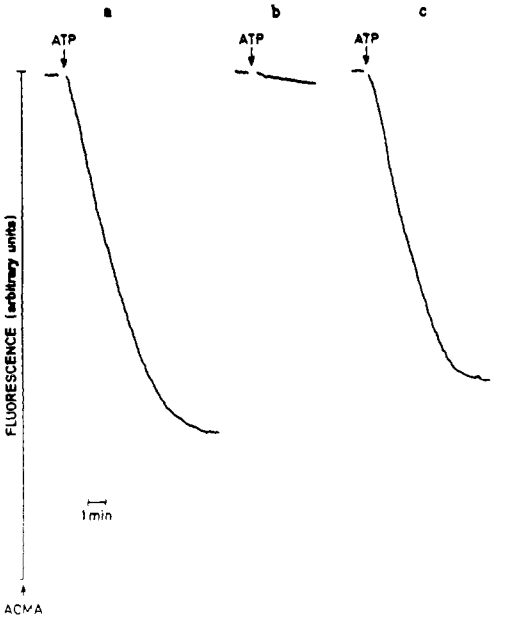

FIGURE 3: Quenching of ACMA fluorescence by ATPase-depleted vesicles (0.2 mg) after preincubation for 10 min with: (a) native ATPase (50 μg); (b) dissociated ATPase (50 μg); (c) reactivated ATPase (50 μg). For details of fluorescence quenching and reversible dissociation procedure see Materials and Methods.

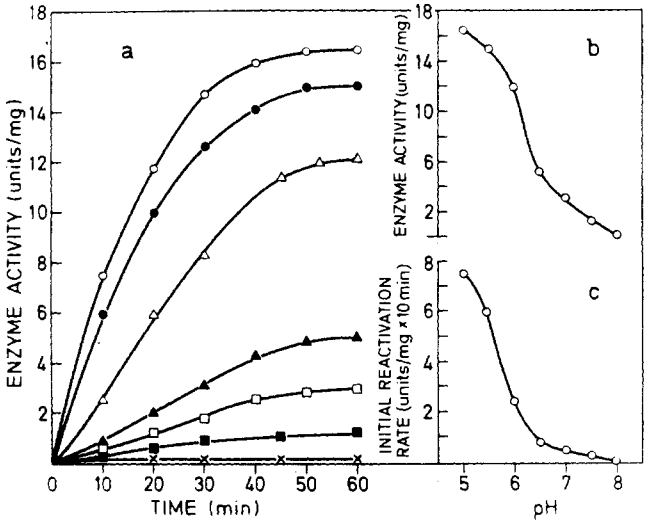

FIGURE 4: Effect of pH on reconstitution. (a) Kinetics of reconstitution at various pH: (○) pH 5; (●) pH 5.5; (△) pH 6; (▲) pH 6.5; (□) pH 7; (■) pH 7.5; (×) pH 8. By using Sephadex G-25 filtration, the buffer of the dissociated sample was changed to 5 mM potassium phosphate (pH 5.5). Aliquots of this sample were diluted with 50 mM potassium phosphate–2.5 mM ATP, having different pH values (final protein concentration 1 mg/ml). (b) Plots of maximal yield attained and (c) initial rate of reconstitution vs. pH.

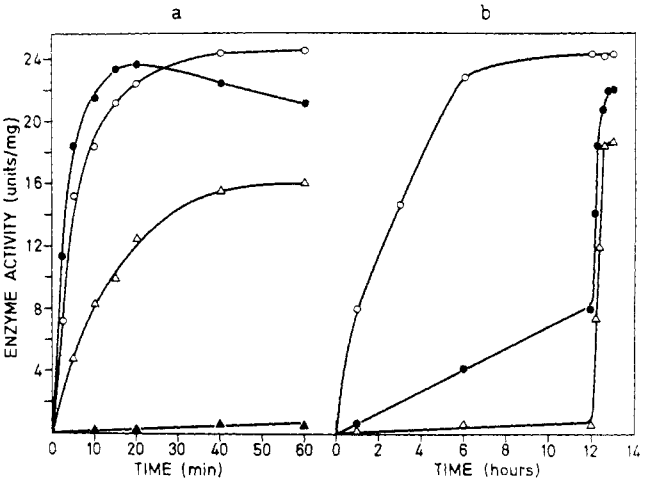

FIGURE 5: Effect of temperature on reconstitution. (a) Kinetics of reconstitution at various temperatures. Dissociated enzyme samples (1 mg/ml) were incubated in 50 mM Mes (pH 6.1) containing 2.5 mM ATP and 2.5 mM $MgCl_2$: (●) 37°C; (○) 25°C; (△) 20°C, and (▲) 14°C. (b) Kinetics of reconstitution measured at 4°C in the presence of: (○) methanol (20%); (●) glycerol (20%); (△) no addition. The samples were shifted to 25°C after 12 hr. All other conditions were identical with (a).

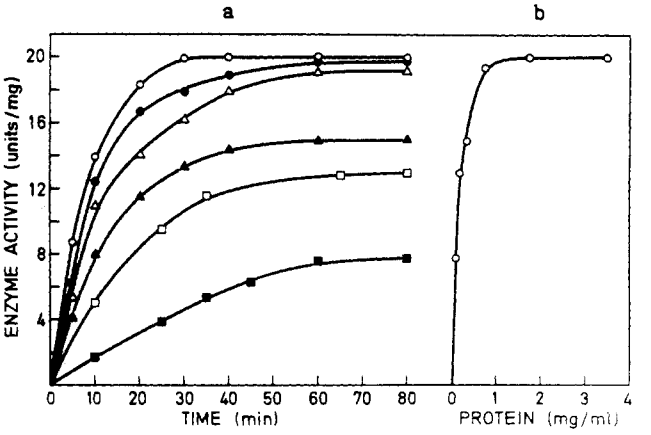

FIGURE 6: Effect of protein concentration on reconstitution. (a) Kinetics of reconstitution as a function of protein concentration: (○) 3.5 mg/ml; (●) 1.75 mg/ml; (△) 0.7 mg/ml; (▲) 0.35 mg/ml; (□) 0.18 mg/ml; (■) 0.07 mg/ml. (b) Plot of maximal activity yield against protein concentration. For experimental details see Materials and Methods.

enzyme activity at the characteristic of the higher temperature (Figure 5b). It was also shown (Figure 5b) that the enzyme is reconstituted in the presence of 20% methanol or 20% glycerol even at 4°C. Methanol was more effective than glycerol in overcoming cold lability.

Effect of Protein Concentration. Data from a typical experiment on reconstitution as a function of protein concentration, under otherwise optimal conditions, are shown in Figure 6. The reassociation appeared to be enhanced by increasing protein concentrations in the region from 70 μg to 1 mg/ml, at which concentration maximal reactivation was obtained (Figure 6b). The rate of increase of reconstitution with increasing protein concentration suggests that no nonspecific aggregation at fairly high concentration takes place. Since the half-time of the reaction is dependent on the initial protein concentration the kinetics of the reconstitution process is not first order. The kinetic data were not accurate enough to determine the reaction order with any certainty.

Effect of ATP and Mg^{2+} on the Reassociation Process. To determine whether ATP and Mg^{2+} affected the reassociation of the subunits the enzyme was dissociated and passed through a Sephadex G-25 column to remove Mg^{2+} and ATP. Incubation of the dissociated enzyme in the presence of 5 mM ATP and 5 mM $MgCl_2$ effected maximal reconstitution of activity (Figure 7). Preincubation with ATP alone gave no reassociation at all, whereas preincubation with $MgCl_2$ restored at best about 40% of enzyme activity compared to the sample containing both reagents. The ef-

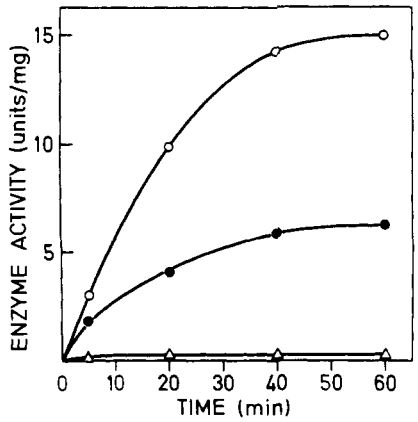

FIGURE 7: Effect of ATP and Mg^{2+} on reconstitution. The dissociated sample was desalted by passage through a Sephadex G-25 column equilibrated with 50 m*M* Mops (pH 7). Aliquots of the dissociated enzyme were subsequently incubated at a protein concentration of 0.8 mg/ml in the same buffer containing: (△) 5 m*M* ATP; (●) 5 m*M* $MgCl_2$; (○) 5 m*M* ATP; and 5 m*M* $MgCl_2$.

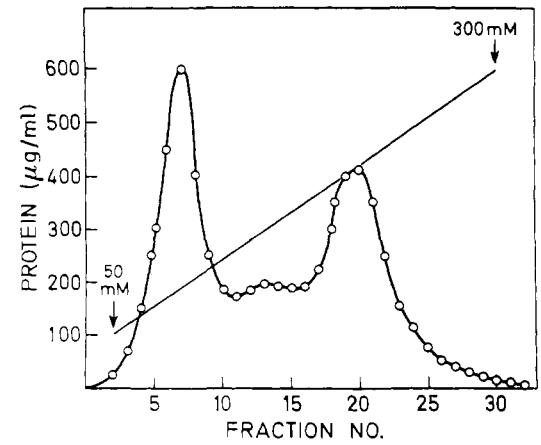

FIGURE 8: Separation of reconstitutively active subunits of ATPase on DEAE-cellulose by salt gradient elution (50–300 m*M* potassium phosphate). Details are described in Materials and Methods.

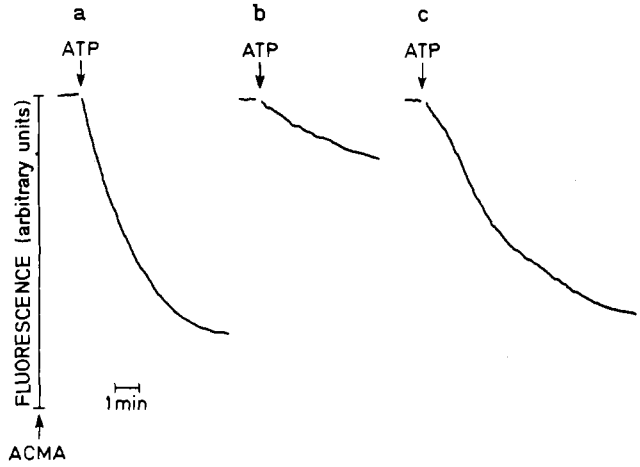

FIGURE 9: Quenching of ACMA fluorescence by ATPase-depleted vesicles (0.2 mg) after preincubation for 10 min with: (a) native ATPase (50 μg); (b) fractions I_A (30 μg) and II (30 μg); (c) fractions I_A (30 μg), I_B (10 μ), and II (30 μg). Fractions I_A, I_B, and II are ineffective when added singly (not shown here). Details of fluorescence quenching assay are described in Materials and Methods.

fect of Mg^{2+} is relatively small at acid pH but becomes more pronounced at alkaline pH.

Isolation and Partial Characterization of Subunits. Isolation. Since the two fragments formed in the dissociation procedure are different in charge, a separation by ion-exchange chromatography was attempted. The reconstitution experiments indicated that it is necessary to isolate the subunits at low temperature to prevent reassociation and at acid pH to prevent irreversible inactivation during the separation procedure.

Figure 8 shows a typical elution profile obtained by salt gradient elution of a dissociated sample on a DEAE-cellulose column. Generally two main protein peaks were observed. The first fragment (I_A) was eluted at an ionic strength of 60–90 m*M* potassium phosphate and the second one (II) at an ionic strength of 170–220 m*M* potassium phosphate. A minor fraction containing antigenic material (I_B) was eluted between these two major peaks.

From 11 mg of ATPase, 1.8 mg of fraction I_A, 0.4 mg of fraction I_B, and 1.7 mg of fraction II were recovered. Reconstitution of ATPase activity from isolated subunits was carried out by incubating equimolar amounts of the various fragments at room temperature. The specific activity obtained was normally 15–30 units/mg, which corresponds to about 80% of that of the original ATPase. Figure 9 demonstrates that the ability of the reassociated ATPase to restore ATP-driven quenching of ACMA fluorescence is only reconstituted after incubation of fractions I_A and II in the presence of fraction I_B, which contained the δ subunit.

Stability of Isolated Subunits. After addition of 20% glycerol, the concentrated subunit solutions were stable for several months when stored in liquid nitrogen. At 2°C about 50% of the potential activity was lost after 1 week. This loss of activity was mainly due to instability of subunit II, as indicated by comparison of activity restored after crosswise incubation of aged and freshly prepared subunit fractions. No systematic studies were done to examine the factors involved in stability of reconstitutively active subunits.

Molecular Weights. A preliminary molecular weight estimation of the ATPase and of its subunits was done by pore gradient acrylamide electrophoresis (Rodbard et al., 1971). Molecular weights were estimated by comparison with standard proteins and were found to be about 340000 for the ATPase, about 100000 for subunits I_A and I_B, and about 54000 for subunit II. These results are in good agreement with the values estimated from the polypeptide composition of the reconstitutively active subunits, except in the case of the I_B fraction. It is possible that the δ subunit is split off during electrophoresis and subsequently migrates out of the gel.

The value of 340000 estimated for the native ATPase is in good agreement with values found by gel filtration experiments (Davies andBagg, 1971; Giordano et al., 1975) but is somewhat higher than that of 296000 found by equilibrium centrifugation (Futai et al., 1974). It is possible that partial dissociation during the experimental conditions led to underestimation of the molecular weight by this technique.

Immunochemical Comparison of ATPase and Its Subunits. One of the most convenient tests for homogeneity of polypeptide preparations is the double diffusion Ouchterlony technique. A photograph of a typical assay is given in Figure 10. Anti-ATPase γ-globulin was applied to the center well and the various subunits and the native enzyme were arranged peripherally. Fragments I_A and I_B behave identically. Fractions I_A and II yielded single precipitin

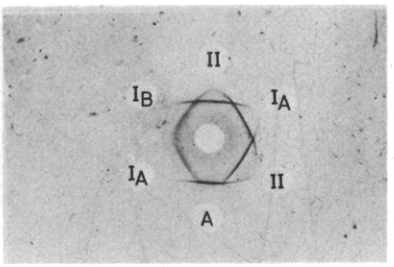

FIGURE 10: Ouchterlony double diffusion pattern of ATPase (A) and fragments I_A, I_B, and II against anti-ATPase γ-globulin (center well).

lines that were not homologous either in position or quality of the precipitates. The absence of any serological relationship demonstrates the differences in the primary structure of the subunits. The precipitin lines from both subunits fuse completely with the ATPase precipitin line, which on the other hand has more antigenic sites than each of the subunits as indicated by the spurs.

Arrangement of the Polypeptide Chains in the Reconstitutively Active Subunits. Each fraction was analyzed electrophoretically in the presence of detergent to determine its polypeptide composition. A summary of the results is given in Figure 11. Subunit I_A contains the α, γ, and ϵ peptide chains (Figure 11b). The γ-polypeptide chain was not stable under the separation conditions. It gave rise to a γ' chain with a molecular weight of 29000, which is 3000 lower than the original γ peptide. After aging subunit I_A for 1 week at 2°C only the γ' peptide chain was observed (Figure 11c). The minor fraction I_B contained the δ polypeptide in addition to the α, γ, and ϵ polypeptides found in I_A (Figure 11d). The γ chain is also unstable in this fraction as observed after aging. Finally, fraction II contains only the β polypeptide chain (Figure 11e).

Stoichiometry. The stoichiometry of the complex as determined by molecular weight estimation and by the arrangement of polypeptide chains in the reconstitutively active subunits was also verified by direct titration of one subunit with the other. The titration curve (Figure 12) indicates that maximal enzyme activity occurred at a protein ratio of about 1:0.5 of I_A to II. This corresponds to an equimolar mixture of the subunits.

Discussion

Reversible Dissociation. Cold lability is an attribute of many multimeric enzymes. Numerous examples were compiled by Jarabak et al. (1966), and Irias et al. (1969). Freezing in salt solution has been shown to cause dissociation or denaturation of a number of protein complexes (Chilson et al. (1965). Although the nature of this process is not clear, it was suggested that during the cooling period drastic changes of pH and solute composition take place in the remaining liquid phase leading to dissociation and inactivation. The increased salt concentration at the eutectic point may alter the p*K* of ionizable groups and modify the solvent structure which in turn affects protein–solvent interactions. Hydrophobic interactions are significantly weakened as the temperature is lowered and thus a high degree of hydrophobic bonding may be involved in stabilizing the native conformation of cold labile enzymes. Several other parameters might be affected including the activity coefficients of peptide bonds, specific ion binding effects, and disulfide bridging or cleavage.

In this communication we provide evidence that some of

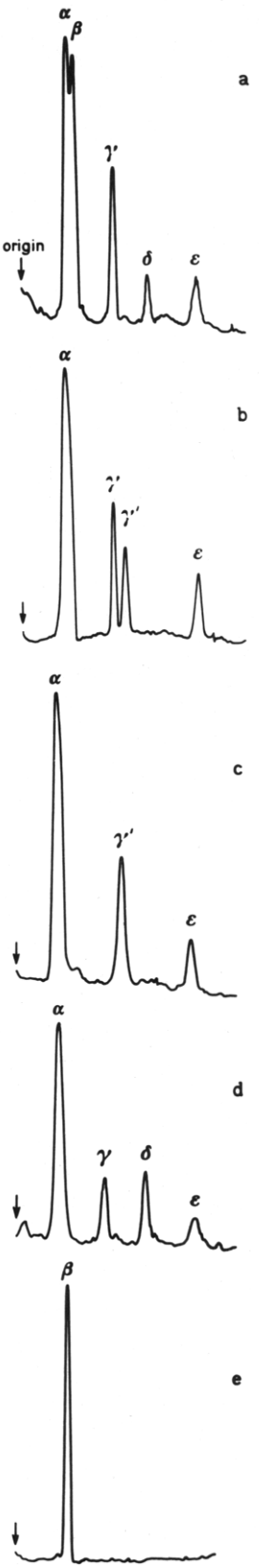

FIGURE 11: Densitometric traces after dodecyl sulfate gel electrophoresis of ATPase and its various subunits. Each sample was incubated for 1 min at 100°C in a medium containing 1% dodecyl sulfate and 1% 2-mercaptoethanol. Electrophoresis in polyacrylamide gel (7.5%), in the presence of 0.1% dodecyl sulfate, was carried out for about 6 hr at 6 mA/tube. (a) Native ATPase. Molecular weights of each polypeptide chain were estimated by comparison with standard proteins. The approximate molecular weights found were: α, 56000; β, 52000; γ, 32000; δ, 21000; ϵ, 11500. (b) Fraction I_A immediately after isolation. (c) Fraction I_B after aging for 1 week. (d) Fraction I_B. (e) Fraction II.

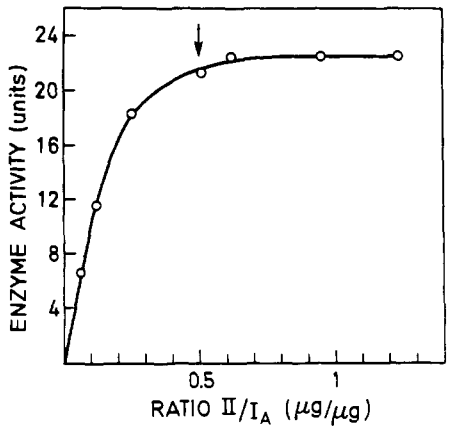

FIGURE 12: Titration curve of fragment I_A with fragment II for reconstitution of ATPase activity; 1 mg/ml of fragment I_A was incubated for 2 hr with increasing amounts of fragment II at 25°C in 50 m*M* Mes (pH 6.1) containing 2.5 m*M* ATP, 2.5 m*M* $MgCl_2$, and 10% glycerol. Aliquots of these mixtures were withdrawn and assayed for ATPase activity.

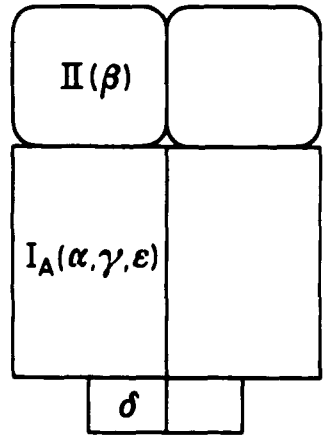

FIGURE 13: Model depicting the arrangement of the various subunits in the ATPase complex.

these factors are involved in the reversible dissociation of the *E. coli* ATPase. The observed temperature dependence and the protection by methanol and glycerol against inactivation point out the importance of hydrophobic bonding and protein–solvent interactions in maintaining the native structure of the enzyme. The sharp decrease in reconstitution above pH 6.5 strongly suggests that ionic interactions are also involved. The separated subunits are irreversibly denatured at alkaline pH and magnesium ions partially preclude this inactivation. From these observations it is probable that a single ionizable group (with a p*K* of about 6.5) in a positively charged form is required for the proper assembly of the subunits. Mg^{2+} ions may be acting as a Lewis acid to stabilize this group even under alkaline conditions. The reactivation is significantly enhanced by the Mg^{2+}–ATP complex which is the true substrate, whereas the uncomplexed ATP is ineffective in this respect. The requirement for Mg^{2+} to obtain optimal reassociation may in fact suggest that magnesium is required as a participant in the formation of the enzyme–substrate complex and also has a role as a structural component of the enzyme. The possibility that peptide bonds are affected by this treatment is demonstrated by the behavior of the γ polypeptide chain which breaks down into the smaller γ' peptide on aging of the separated subunits. This γ' peptide was never observed when the original ATPase was subjected to dodecyl sulfate electrophoresis.

Structural Arrangement of the Competent Subunits. The ATPase activity was reconstituted from the two major fractions, I_A and II which were eluted at different ionic strengths from a DEAE-cellulose column. Both of these fractions behave as homogeneous proteins under various electrophoretic conditions. The nonidentity of these subunits was demonstrated by immunological comparison. Subunit I_A is composed of the α, γ, and ϵ polypeptides and subunit II contains only the β peptide. The molecular weight of subunit I_A (100000) and of the corresponding polypeptides (α = 56000, γ = 32000, ϵ = 11500), and of subunit II (54000) and the β peptide (52000), were in good agreement. In addition to the two major fractions a third minor fraction (I_B) was obtained. This fraction contained the δ subunit in addition to the α, γ, and ϵ peptides from which I_A is assembled. The isolation of subunits I_A and II is also possible by electrophoretic separation on cellulose acetate blocks (G. Vogel, unpublished results). Excellent separation of subunits was obtained at pH 8.3 but the separation subunits were not reconstitutively active. Under these conditions the δ subunit was unexpectedly found to be associated with subunit II when subjected to dodecyl sulfate electrophoresis. This suggests that the δ subunit may be only loosely bound to the ATPase complex. This conclusion is supported by the fact that the δ subunit is lost during purification unless special precautions are observed. The reconstitution experiments support the previous conclusions of Smith and Sternweis (1975) that the δ subunit is directly involved in the attachment of the ATPase to the membrane.

The molar ratio of subunits I_A and II in the reconstituted complex was found to be 1:1 on the basis of titration experiments and by calculation from the molecular weight data, suggesting a stoichiometry of $(I_A)_2(II)_2$ for the basic ATPase complex. The five polypeptide chains observed in dodecyl sulfate electrophoresis would then be suggested to have the stoichiometry of $(\alpha,\gamma,\epsilon)_2(\beta)_2(\delta)_{1-2}$ in the native membrane-bound enzyme. This stoichiometry is not in agreement with the formula $\alpha_3\beta_3\gamma\delta\epsilon$ found by Bragg and Hou (1975).

An attractive speculative model for the structure of the ATPase would be a tetrametric arrangement of subunits I_A and II, as shown in Figure 13, linked to the membrane by the δ subunit. This model is different from previously proposed models, where a hexagonal planar arrangement for the α and β peptides has been suggested (Bragg and Hou, 1975; for review see, Senior, 1973). On the other hand, our data are consistent with the data presented for the cysteine content and the distribution of sulfhydryl groups and disulfide bonds in the subunits of the beef heart mitochondrial ATPase (Senior, 1975).

The method described in this communication for fragmentation and reconstruction of the *E. coli* ATPase may be applicable to ATPase complexes from other sources as well. The isolation of the reconstitutively active subunits allows closer investigation of the structure–function relationship of the ATPase subunits and of the mechanism of ATP synthesis.

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