

Energy-Transducing Adenosine Triphosphatase from *Escherichia coli*: Purification, Properties, and Inhibition by Antibody

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The membrane adenosine triphosphatase (E.C. 3.6.1.3) from *Escherichia coli* has been solubilized with Triton X-100 and purified to near homogeneity. The purified enzyme has a sedimentation coefficient of 12.9S in a sucrose gradient, corresponding to a molecular weight of about 360,000. On electrophoresis in gels containing sodium dodecyl sulfate, it dissociates into subunits with apparent molecular weights of 60,000, 56,000, 35,000, and 13,000. The purified enzyme loses activity and breaks down into subunits when stored in the cold. Guanosine 5'-triphosphate and inosine 5'-triphosphate are alternative substrates. Ca²⁺ and, to a small extent, Co²⁺ or Ni²⁺ will substitute for Mg²⁺ in the reaction. The K_m for Mg-adenosine triphosphate of the membrane-bound enzyme is 0.23 mM, and for the pure enzyme it is 0.29 mM. Azide is a noncompetitive inhibitor of both the membrane-bound enzyme and the pure enzyme. P_i is a noncompetitive inhibitor of the solubilized enzyme. An antibody to the purified enzyme was obtained from rabbits. The antibody inhibits the solubilized enzyme and virtually all of the adenosine triphosphate hydrolysis by membranes from cells grown aerobically or anaerobically. The antibody also inhibits the adenosine triphosphate-stimulated pyridine nucleotide transhydrogenase (E.C. 1.6.1.1) of the *E. coli* membrane.

Adenosine triphosphatase (ATPase) activity is associated with a variety of membranes which carry out oxidative phosphorylation or active transport. The ATPase from beef heart mitochondria which has been purified and extensively characterized by Racker has been found to be a coupling factor at all three sites of oxidative phosphorylation (16). It is also involved in the utilization of adenosine triphosphate (ATP) for the ATP-driven transhydrogenase reaction and for ATP-driven reversed electron transfer (17). Similar enzymes have also been purified from rat liver mitochondria (9, 32) and from yeast mitochondria (46). Although these enzymes catalyze the hydrolysis of ATP in vitro, their role in vivo is presumably that of energy transduction.

Several ATPases have also been described in bacterial membranes (20, 23, 30, 36, 38). The best characterized of these is from *Streptococcus faecalis* (48). Indirect evidence points to a role for this enzyme in K⁺ and amino acid transport (3, 22).

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Recently Butlin, Cox, and Gibson isolated mutants of *Escherichia coli* with greatly reduced levels of membrane ATPase (7). These mutants, although able to oxidize lactate, cannot couple such oxidation to phosphorylation of adenosine diphosphate (ADP). In addition, the transhydrogenase of these mutants is not stimulated by ATP (13) as it is in the parent strain. Kanner and Gutnick obtained similar results with a mutant isolated in their laboratory (26, 27).

Experiments of Pavaslova and Harold (42) indicated that the *E. coli* ATPase might function in active transport of galactosides. They reported that in cells grown anaerobically, thiomethylgalactoside accumulation was blocked by uncouplers of oxidative phosphorylation, although ATP was present at normal levels and could be used for other reactions. Thus, it appeared possible that in anaerobic cells, ATP from glycolysis was utilized by the membrane ATPase to provide energy for the active transport of galactosides.

Schairer and Haddock have presented genetic evidence for the involvement of this enzyme in

galactoside accumulation (45). Thiomethylgalactoside accumulation in an ATPase-less mutant was completely abolished by cyanide, whereas accumulation in the parent strain was only partially affected by cyanide, indicating that both respiratory enzymes and ATPase can be linked to active transport.

Because of the obvious advantages of working with *E. coli* in performing genetic studies aimed at a resolution of the energy transduction system, the properties of the ATPase complex from this particular organism are of great interest.

This paper reports on the solubilization, purification, and characterization of this ATPase. Immunological evidence is presented that this enzyme is responsible for all the ATP hydrolysis by membranes of aerobically and anaerobically grown cells and that the enzyme is involved in the utilization of ATP for the ATP-driven transhydrogenase reaction.

MATERIALS AND METHODS

ATPase assay. Method A: The assay system contained 40 mM tris(hydroxymethyl)aminomethane (Tris) sulfate (pH 7.8), 10 mM MgSO₄, and 0.4 mM ATP (containing about 10⁶ counts/min γ ³²P) in 1 ml at 30 C. The reaction was stopped by the addition of 0.5 ml of 5% perchloric acid. Norit A was added (final concentration 50 mg per ml), and the suspension was shaken at 4 C for 10 min. The addition of charcoal was repeated, and the suspension was shaken for another 10 min. The charcoal was removed by centrifugation, and 1 ml of the supernatant extract was added to Patterson-Greene solution (41) for scintillation counting. A blank was prepared by adding perchloric acid before the enzyme, and a standard, to which no charcoal was added, was counted.

Method B: Instead of adding charcoal to adsorb unreacted ATP, the ³²P_i released was extracted into isobutanol-benzene as described by Penniall (44). One milliliter of the upper layer was counted in Patterson-Greene solution.

Method C: The assay system contained 40 mM Tris sulfate (pH 7.8), 10 mM MgSO₄, ATP, 2 mM phosphoenolpyruvate, 0.1 M KCl, and 20 μ g of pyruvate kinase in 1 ml at 30 C. The reaction was stopped by the addition of 5% perchloric acid (0.5 ml). The P_i released was extracted into isobutanol-benzene and determined colorimetrically as described by Penniall (44).

Method D: The assay system contained 40 mM Tris sulfate (pH 7.8), 10 mM MgSO₄, ATP, 2 mM phosphoenolpyruvate, 0.1 M KCl, 30 μ g each of pyruvate kinase and lactate dehydrogenase, and 0.3 mM nicotinamide adenine dinucleotide, reduced form (NADH) in 3 ml at 30 C. The reaction was monitored by the decrease in absorbance at 340 nm using cuvettes of 1 cm path length and a Gilford 2000 spectrophotometer. Auxiliary enzymes were dialyzed into 50 mM Tris sulfate (pH 7.8) before use to prevent precipitation of magnesium ammonium phosphate.

Methods A and B gave identical amounts of P_i release. The reaction was not linear with enzyme or time because of inhibition by ADP, but the product of enzyme multiplied by time was constant. With Methods C and D, P_i and ADP release were linear with enzyme and time. Methods A and B were useful for checking effects of metal ions, alternative substrates, and inhibitors without the complications of coupling enzymes.

Transhydrogenase assay. The assay system contained 40 mM Tris sulfate (pH 7.8), 10 mM MgSO₄, 2 mM ATP, 0.1 mM nicotinamide adenine dinucleotide (NAD), 0.5 mM triphosphopyridine nucleotide, oxidized form, 10 mM dithiothreitol, 10 mM NaCN, 87 mM ethanol, and 0.3 mg yeast alcohol dehydrogenase in 1 ml. The reaction was initiated by the addition of alcohol dehydrogenase and, after the initial reduction of NAD, the steady state increase in absorbance at 340 nm was followed in cells of 1 cm path length with a Gilford 2000 spectrophotometer. The membrane suspension to be assayed was added just before the alcohol dehydrogenase, but the rest of the assay mixture was first incubated for about 10 min to allow the absorbance from the NAD-cyanide complex (28) to stabilize.

NADH oxidation. The assay system contained 40 mM Tris sulfate (pH 7.8), 10 mM MgSO₄, and 0.3 mM NADH in a volume of 3 ml. The decrease in absorbance at 340 nm was followed in cells of 1 cm path length.

Determination of sedimentation coefficient. Samples of 0.2 ml containing alcohol dehydrogenase (0.1 mg), pyruvate kinase (0.04 mg), phosphofructokinase (0.04 mg), and pure ATPase (0.02 mg) in 0.1 M phosphate buffer of pH 8 containing 2 mM dithiothreitol were layered over each of 2 sucrose gradients (10 to 25%) in this buffer and centrifuged for 10 h at 20 C and 40,000 rpm with the International SB-283 rotor. Fractions of 5 drops each were collected from the bottom of each tube and assayed for enzymic activity. Method A was used for ATPase. Alcohol dehydrogenase, pyruvate kinase, and phosphofructokinase were assayed by following absorbance changes at 340 nm. Pyruvate kinase was coupled to lactate dehydrogenase. Phosphofructokinase was coupled to lactate dehydrogenase through pyruvate kinase.

Protein determination. Protein was determined by the Lowry procedure (34) using 10% trichloroacetic acid to precipitate the samples, when necessary, and using bovine serum albumin as a standard. Alternatively, protein was determined by absorbance at 280 nm.

Gel electrophoresis. Standard gels at pH 7.4 contained acrylamide (7.5 or 4.7% wt/vol), bisacrylamide (1.7% of acrylamide concentration), imidazole (1.14% wt/vol), HCl (0.0453 N), *N,N,N',N'*-tetramethylethylenediamine (0.112% vol/vol), and ammonium persulfate (0.02% wt/vol) which was added last to initiate polymerization. The cathode buffer contained imidazole (0.0197% wt/vol) plus *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (0.530% wt/vol), and the anode buffer contained imidazole (0.136% wt/vol) adjusted to pH 7 with HCl.

Sodium dodecyl sulfate gels contained acrylamide 10% wt/vol), bisacrylamide (5% of acrylamide concentration), Tris (4.54% wt/vol), HCl (0.0300 N), *N,N,N',N'*-tetramethylethylenediamine (0.029% wt/vol), sodium dodecyl sulfate (0.1% wt/vol), and ammonium persulfate (0.07% wt/vol) which was added last. The buffer used for both reservoirs contained Tris (0.6% wt/vol), glycine (2.8% wt/vol), and 0.1% sodium dodecyl sulfate.

Protein samples were dialyzed against a 1:2 dilution of the cathode buffer used for the standard gels plus 20% glycerol. For the sodium dodecyl sulfate gels, mercaptoethanol (2 mM) and sodium dodecyl sulfate (1%) were also added, and the samples were heated at 60 C for 30 min. Gels were of 2 ml volume contained in tubes of 0.5 cm diameter. Samples were layered over the gels and run at constant voltage starting at 3 mA per gel (standard) or 6 mA per gel (sodium dodecyl sulfate). Gels were stained for protein with Coomassie blue and for ATPase activity by the method of Abrams and Baron (2). Gels were scanned with a Phorvot densitometer.

Bacterial strains and growth conditions. *E. coli* A-3245 is a K-12 strain. It was grown on the mineral medium of Cohen and Rickenberg (11), with thiamine (2 mg per liter) and a carbon source. Cells were grown aerobically on 1% glycerol at 37 C. They were grown anaerobically at 37 C on 2% glycerol, 0.6% fumarate, and 0.1% Casamino Acids or on 1% glucose plus 0.1% Casamino Acids in an evacuated filtration flask filled nearly to the top. These conditions were effectively anaerobic, as shown by the fact that the cells did not grow in the absence of fumarate, the required electron acceptor (29).

Preparation of antibody. Pure ATPase in 0.9% NaCl, 20 mM NaP_i (pH 7), was mixed with an equal volume of Freund complete adjuvant. Seventy micrograms of protein in 0.5 ml was injected into each of 2 male 5- to 6-pound (2 to 3 kg) rabbits. Six weeks after the first injection, an additional 70 μg of ATPase in 0.2 ml of 0.9% NaCl, 20 mM NaP_i (pH 7), was injected into the hind foot pads of each rabbit. Seven days after the second injection, about 60 ml blood was collected from each rabbit from an ear artery or by cardiac puncture. The bleeding was repeated 4 days later. The blood was allowed to clot, and the clot was removed by centrifugation. Serum from both rabbits gave a positive ring test (8). Gamma globulin was separated from this serum, and from control serum from rabbits that did not receive ATPase, by using diethylaminoethyl (DEAE)-cellulose by the method of Stanworth (51).

Separation of inner and outer membranes. A discontinuous sucrose gradient of the type designed by Schnaitman (47) was used. Cells were washed with and suspended in 50 mM Tris sulfate (pH 7.8), 1 mM ethylenediaminetetraacetic acid (EDTA). They were passed once through a French pressure cell and centrifuged for 10 min at 5,000 × *g* to remove unbroken cells. One milliliter was layered over the gradient. Phosphatidylserine decarboxylase, determined by release of ¹⁴CO₂ from radioactive phosphatidylserine, was used as a marker for the inner membrane (25).

Materials. Materials were obtained from the following sources: [³²P]-ATP, New England Nuclear Corp.; pyruvate kinase and alcohol dehydrogenase, Boehringer; DEAE-cellulose, Whatman; DEAE-Sephadex A-25, Pharmacia Fine Chemicals, Inc.; Triton X-100 (*t*-octylphenoxy polyethoxy ethanol), Rohm and Haas; ultrafiltration membranes, Amicon Corp.; and frozen *E. coli* B grown on a glycerol-salts medium to late log phase, Grain Processing Company, Muscatine, Iowa. Phosphofructokinase was a gift from H. A. Lardy.

RESULTS

Purification of ATPase. Frozen cells of *E. coli* B (40 g) were thawed and suspended in a Waring blender in 80 ml of a solution containing 50 mM Tris sulfate (pH 7.8), 10 mM MgSO₄, and a few mg of deoxyribonuclease (Table 1). The suspension was divided into three portions, immersed in ice water, and the cells were disrupted with a MSE sonicator by using the 0.9 cm probe and four 30-s pulses at an amplitude setting of 7 to 8 μ, interrupted by 30-s cooling periods. The broken cell suspension was centrifuged for 30 min at 100,000 × *g*. The supernatant extract was discarded, and the pellet was suspended at room temperature in 80 ml of 50 mM Tris sulfate (pH 7.8) containing 10% Triton X-100 by using a Waring blender at low speed. All subsequent steps were performed at room temperature. After standing overnight, the membrane suspension was centrifuged at 100,000 × *g* for 30 min.

The Triton extract was applied to a column (8 × 2.5 cm) containing DEAE-cellulose (DE-52; 40 ml in 50 mM Tris sulfate, pH 7.8). The column was washed with 120 ml of this buffer to remove the detergent. The enzyme was eluted at 225 ml per h with 160 ml of 50 mM Tris

TABLE 1. Purification of the membrane-bound ATPase

Fraction	Activity ^a (μmol/ min)	Protein (mg)	Specific activity (μmoles/ min/mg)	Recovery
1. Membranes ^b	244	2,340	0.104	
2. Extract ^b	179	1,160	0.154	73%
3. DE 52	61	256	0.238	30%
4. A 25	29.4	20.2	1.45	14%
5. Sucrose gradient	8.45	1.69	5.00	4.2%

^a Assayed by method C.

^b These fractions were diluted for assay so that the concentration of Triton X-100 was 0.2%. This concentration of detergent reduced the absorbance of phosphate standards to 82% of the value without detergent, and the activity was corrected to account for this.

sulfate (pH 7.8) containing 0.25 M KCl. The enzyme began to emerge in the first yellow fraction. All subsequent fractions were saved and concentrated to a volume of 25 ml with an ultrafiltration cell using a 76 mm XM-50 membrane under 2.5 atmospheres of N_2 .

Saturated ammonium sulfate (25 ml adjusted to pH 7 with concentrated ammonium hydroxide) was added with stirring. The suspension was allowed to stand on ice for 30 min, and then was centrifuged for 15 min at $10,000 \times g$. (The suspension of the enzyme in ammonium sulfate can be stored at 4 C without loss of activity.) The pellet was dissolved in 25 ml of 50 mM imidazole chloride (pH 7) and dialyzed for 3 h against 4 liters of this buffer. The turbid dialyzed enzyme solution was applied to a column (16 \times 2.5 cm) containing DEAE-Sephadex A-25 (80 ml in 50 mM imidazole chloride, pH 7). The column was washed with 160 ml of a solution containing 50 mM imidazole chloride (pH 7), 2 mM ATP, 2 mM EDTA, and 0.15 M KCl. The enzyme was eluted by increasing the KCl concentration in this solution to 0.2 M. The flow rate of this column was 270 ml per h.

Two linear sucrose density gradients, each containing 10 to 25% sucrose (wt/vol) in 11.6 ml of buffer (50 mM Tris sulfate at pH 7.8) were prepared in 12 ml polyallomer tubes. A pool (40 ml) of the most active fractions from the DEAE-Sephadex column was concentrated by ultrafiltration through an XM-50 membrane, centrifuged to remove insoluble material, and 0.4 ml was layered over each of the two density gradients. The gradients were centrifuged for 14 h at 40,000 rpm with an IEC SB-283 rotor. The tubes were punctured at the bottom and fractions of 6 drops each were collected. The three or four fractions containing most of the activity were saved. If disk gels showed any minor contaminating bands at this stage, they could be removed by applying fraction 5 of Table 1 directly to a 4 ml DEAE-Sephadex column packed in 50 mM imidazole chloride (pH 7), and eluting with this buffer containing 2 mM ATP, 2 mM EDTA, and 0.2 M KCl.

The overall purification (Table 1) is about 48-fold when the pure enzyme is compared with the membranes suspended in Triton X-100. However, the specific activity of the membranes in buffer is about three times as great as that of the membranes suspended in detergent. The concentration of Triton X-100 in the assay of fractions 1 and 2 is 0.2%. If a membrane suspension not exposed to the detergent is assayed in the presence of 0.2% Triton, the

specific activity is also decreased by about 70%. It is not known whether Triton affects the assay or whether the decreased activity results from solubilization of the enzyme.

The purified ATPase migrated as a single band in gel electrophoresis (Fig. 1) at two different acrylamide concentrations and also ran as a single band in a pH 9 system (not shown). Using an ATPase activity stain on parallel gels, the positions of the bands were the same as those shown in gels 1 and 2 of Fig. 1.

Subunit structure and molecular weight. Gels containing sodium dodecyl sulfate (Fig. 1) revealed the presence of four sizes of subunits. Proteins running near the dye marker are not well-resolved; however, when the gel concentration was chosen so that the smallest subunit migrated about half as far as the bromphenol blue, no additional subunits could be detected (not shown in Fig. 1). In addition to these four major bands, the gels had numerous striations near the top which may be various aggregates of the subunits.

The molecular weights of the 4 subunits were estimated by electrophoresis of samples containing both ATPase and marker proteins and plotting $\log R_f$ versus molecular weight (Reference 39; Fig. 2). The hemoglobin chains obscured the position of the smallest subunit of the ATPase. The molecular weight of this subunit was obtained from gels containing ATPase alone, by using the three larger subunits as standards. The molecular weights of the four subunits were estimated to be 60,000, 56,000, 35,000, and 13,000. Similar values were obtained by plotting \log molecular weight ver-

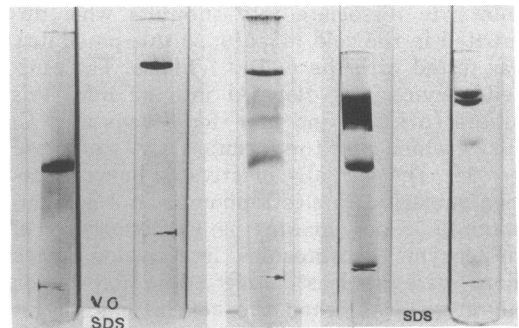


FIG. 1. Gel electrophoresis of purified ATPase. Gels are, from left to right: (i) 4.7% acrylamide, 12 μ g of protein; (ii) 7.5% acrylamide, 19 μ g of protein; (iii) 7.5% acrylamide, 34 μ g of cold-inactivated enzyme; (iv) 47 μ g of protein; (v) 25 μ g of protein. Gels i to iii are standard gels, and gels iv to v contain sodium dodecyl sulfate. The position of the bromphenol blue is marked with India ink.

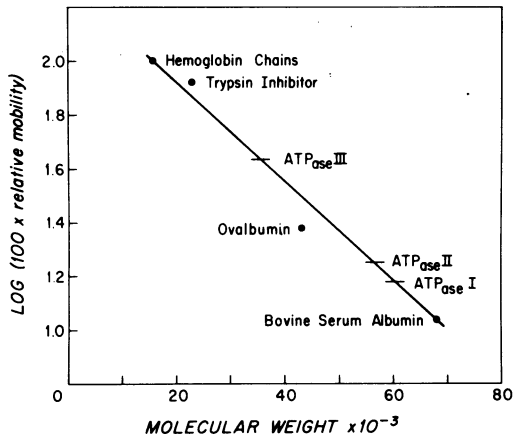


FIG. 2. Calibration curve for estimation of molecular weights of ATPase subunits in gels containing sodium dodecyl sulfate.

sus migration distance as originally described by Shapiro et al. (50).

The sedimentation coefficient of the pure ATPase was estimated to be 12.6S by the method of Martin and Ames (35). This is the mean of values of 11.4S, 13.4S, and 13.1S based, respectively, on sedimentation relative to yeast alcohol dehydrogenase, pyruvate kinase, and phosphofructokinase. By using the empirical relationship formulated by Paetkau and Lardy (molecular weight = $5.4 \times 10^3 [S]^{1.66}$; reference 40), an approximate molecular weight for the ATPase of 361,000 was calculated.

Cold lability. As seems to be typical for ATPases from other sources, the *E. coli* enzyme is cold-labile after it has been released from the membrane. The mitochondrial enzyme is known to dissociate into subunits when incubated in the cold (43, 19), so this possibility was tested with the *E. coli* ATPase. The purified enzyme was dialyzed into 50 mM Tris sulfate (pH 7.8) and kept for 6 days at 4 C, during which time the specific activity declined by 80%. The partially inactivated enzyme was then subjected to electrophoresis in a gel containing 7.5% acrylamide (Fig. 1). Dissociation of the enzyme accompanies inactivation, since there were at least three additional bands migrating faster than the holoenzyme, as revealed by a densitometer scan.

Substrate and metal specificity. With ATP as substrate, Mg^{2+} and Ca^{2+} were found to be the divalent cations producing the greatest activity of the purified ATPase (Table 2). Co^{2+} and Ni^{2+} functioned in the reaction to a small extent. Purine nucleoside triphosphates were more effective substrates than the pyrimidine

analogues. Pyrophosphate and ADP were not hydrolyzed. With the membrane-bound enzyme, using assay A, β , γ -methylene ATP (2 mM) and deoxyATP (2 mM) inhibited 30 and 40%, respectively; oligomycin (1 μ g per ml) and *N*-ethylmaleimide (2 mM) were not inhibitory.

Kinetic properties of the ATPase. Lineweaver-Burk plots for the purified ATPase and for the membrane bound form of the enzyme (Fig. 3) were linear. The linearity of the double reciprocal plot for membrane ATPase activity

TABLE 2. Specificity for metals and nucleotides

Metal	Nucleotide ^a	Rate with MgATP ^b (%)
Mg^{2+}	ATP	100
Mg^{2+}	GTP	61
Mg^{2+}	ITP	33
Mg^{2+}	UTP	11
Mg^{2+}	CTP	3
Ca^{2+}	ATP	108
Co^{2+}	ATP	19
Ni^{2+}	ATP	14
Mn^{2+}	ATP	5
None	ATP	0

^a ATP, adenosine triphosphate; GTP, guanosine 5'-triphosphate; ITP, inosine 5'-triphosphate; UTP, uridine 5'-triphosphate; CTP, cytidine 5'-triphosphate.

^b Method B was used except that phosphoenolpyruvate, KCl, and pyruvate kinase were omitted from the assay. For the experiment where nucleotides (2 mM) were varied, pure ATPase (7 μ g) was used, and the incubation time was 40 min. For the experiment where metals (10 mM, added as chlorides) were varied, pure ATPase (11 μ g) was used, and the incubation time was 20 min.

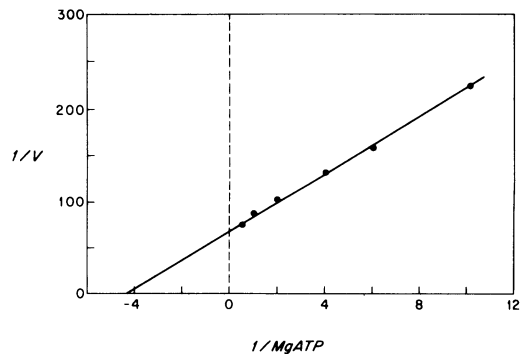


FIG. 3. Lineweaver-Burk plot for membrane ATPase. Method C was used with 54 μ g of membrane protein and variable ATP. $MgSO_4$ concentration was 10 mM plus the concentration of ATP. Values of *v* are expressed in μ mol of P_i per min, and MgATP is given in mM.

suggests a single enzyme, although several ATPases (6) have been reported. The Michaelis constants, 0.23 mM for the particulate enzyme and 0.29 mM for the pure enzyme, were not significantly different. The turnover number for the pure ATPase was 1,870 per min per 360,000 daltons of enzyme.

Phosphate (Fig. 4) was a noncompetitive inhibitor of the solubilized enzyme. The nonlinear Dixon plot suggests that the mechanism is random, since product inhibition is always linear for a nonrandom mechanism (10).

Azide (Fig. 5) was a noncompetitive inhibitor of both the membrane-bound and pure ATPase. With azide, inhibition was also nonlinear. Inhibition by azide is not sigmoidal as would be expected for an allosteric inhibitor, but, since it is a linear molecule (12), it does not resemble any part of ATP, and binding at the active site is unexpected. Cyanide does not inhibit the

enzyme as might be expected if the ATPase contained a transition metal.

Inhibition of the ATPase with an antibody.

An antibody was prepared for use as a specific inhibitor of the ATPase by injecting the pure enzyme into rabbits. The pure enzyme and the Triton extract of the membranes produced single precipitin lines at identical positions on the double-diffusion plates. No lines were observed between the wells containing control γ -globulin and those containing enzyme.

The antibody to the purified ATPase inhibited both the soluble and membrane-bound forms of the enzyme. With sufficiently high concentrations of antibody, it was possible to inhibit completely the ATPase activity of the membranes (Fig. 6). This indicates that the purified enzyme, although obtained in rather low yield, accounts for essentially all of the ATP hydrolysis by membrane preparations.

The mitochondrial ATPase has been shown to be coupled to the energy-linked transhydrogenase of that organelle (16). Experiments with inhibitors and the results with the ATPase mutants (13, 26) suggested that the *E. coli* ATPase performs a similar role. The antibody provided a means to test this hypothesis, and the results are shown in Fig. 6. The stimulation by ATP of the transhydrogenase is abolished parallel to ATPase activity as antibody concentration is increased. γ -Globulin from control rabbits had only a small inhibitory effect on ATPase and transhydrogenase.

The ATPase and transhydrogenase reactions are coupled, but two lines of evidence indicate that they are catalyzed by different enzymes. Pure ATPase was assayed for transhydrogenase, and this activity was not detectable. Membranes from cells grown anaerobically on glucose plus Casamino Acids have as great a specific activity of ATPase as those from aerobic cells. However, we observed, in agreement with Bragg et al. (5), that the ATP-stimulated transhydrogenase is present at only a small fraction of the activity found in cells grown aerobically without Casamino Acids, indicating that it is repressed independently of the ATPase.

The membrane preparation used for these experiments contains a NADH dehydrogenase activity which is completely inhibited by 10 mM NaCN. This activity is not inhibited by the antibody to the ATPase (data not shown) which indicates that the inhibition of the transhydrogenase has some specificity.

The antibody to the ATPase purified from *E. coli* B grown anaerobically on glycerol is also an inhibitor of the ATPase activity of membranes

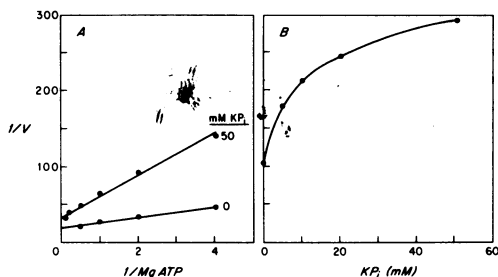


FIG. 4. Inhibition of solubilized ATPase by P_i . Assay D was used with 112 μ g of protein (purified through the DEAE-cellulose step). Values of v are expressed in micromoles P_i per min assuming a millimolar extinction coefficient of 6.22 for NADH at 340 nm. (A) MgATP is expressed in mM. KP_i concentration was 50 mM (top) and 0 (bottom). (B) MgATP concentration was 1 mM.

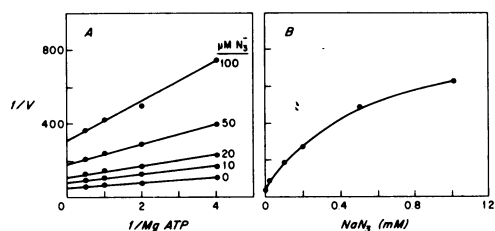


FIG. 5. Inhibition of ATPase by azide. (A) Method C was used with 3.6 μ g of pure ATPase. Values of v are given in micromoles of P_i per min, and MgATP concentration is expressed in mM. NaN_3 concentrations were, from top to bottom: (A) 100 μ M, 50 μ M, 20 μ M, 10 μ M, and 0. (B) Method D was used with 112 μ g of protein (purified through the DEAE cellulose step). Values of v are expressed in micromoles of P_i per min, assuming a millimolar extinction coefficient of 6.22 NADH at 340 nm. MgATP concentration was 1 mM.

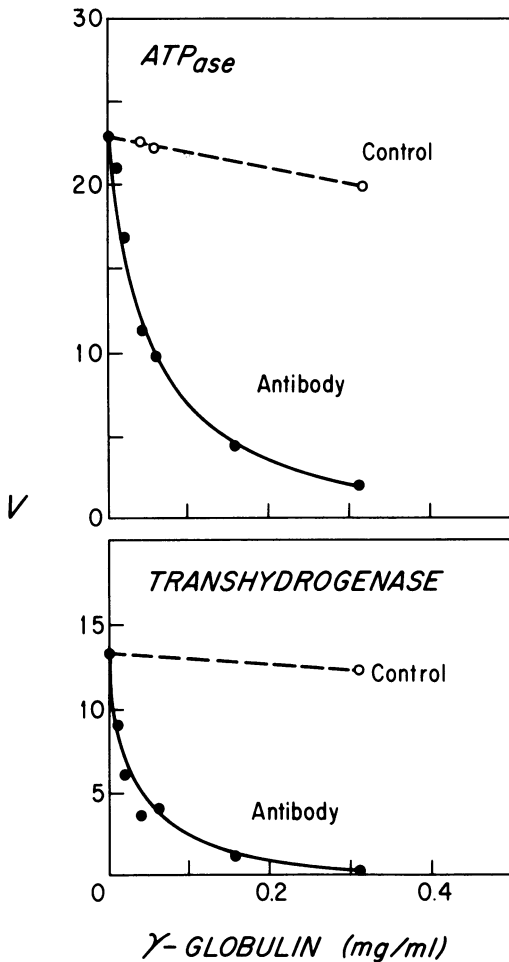


FIG. 6. Inhibition of aerobic ATPase and transhydrogenase by antibody. Method B (5 min incubation time) was used to assay ATPase; 144 μ g of membrane protein was used. V is nmol of P_i per min for ATPase and nmol of triphosphopyridine nucleotide (reduced form) per min for transhydrogenase. Values shown for transhydrogenase assays are corrected for the slight activity seen in the absence of added ATP.

from a K-12 strain grown anaerobically on glycerol, fumarate, and Casamino Acids (data not shown). The membrane ATPase of cells grown anaerobically on glucose plus Casamino Acids is also completely inhibited by the antibody. It is possible that the aerobic and anaerobic enzymes have only certain subunits in common. However, the ATPase from *E. coli* B grown aerobically on glycerol and the enzyme from the K-12 strain (A 324-5) grown aerobically on glycerol or anaerobically on glucose plus Casamino Acids were of similar size as judged by chromatography on gellarose 4%. All three activities, when extracted from the mem-

branes with 50 mM Tris sulfate (pH 7.8) at room temperature in the absence of Triton X-100, were eluted at about 1.5 void volumes. In addition, the enzyme from anaerobic cells when compared with the enzyme from aerobic cells was inhibited to a similar extent by phosphate, ADP, and azide.

Subcellular distribution of the ATPase. The distribution of azide-inhibitable ATPase activity in a discontinuous sucrose gradient was examined. This type of gradient was devised by Schnaitman (47) to separate inner and outer membranes of *E. coli*. In our experiments the entire broken cell suspension was layered on the gradient rather than just a resuspended membrane fraction as described by Schnaitman. The bulk of the ATPase activity was found in the inner membrane fraction along with phosphatidylserine decarboxylase, a marker enzyme (4, 54) for this fraction. There was also a peak of ATPase activity in the upper sucrose layer which must contain the soluble proteins. The peaks of ATPase activity in this gradient were tested with the antibody to the purified ATPase, and all of them were strongly inhibited, indicating that they were different states of the same enzyme.

It was noted that the distribution of ATPase in such sucrose gradients was markedly affected by the method used to break the cells and by the composition of the buffer in which the cells were suspended. Sonication resulted in a large peak of activity in the outer membrane fraction, possibly due to hybridization (52) of inner and outer membrane proteins. When the cells were broken with a French press, inclusion of 10 mM $MgSO_4$ in the buffer resulted in a large diminution of the size of the ATPase peak in the upper layer. However, the separation of inner and outer membranes depends on the use of a medium of low ionic strength containing EDTA (47). We conclude from studies carried out under the mildest conditions permitting separation of inner and outer membranes that the ATPase is primarily associated with the cytoplasmic membrane fraction. Miura and Mizushima reached a similar conclusion (37) based, however, on a less direct and convenient procedure.

DISCUSSION

The observation that the antibody to the purified ATPase inhibits all of the ATPase activity of the membrane is evidence that a single enzyme is responsible for this activity. This conclusion is further supported by the observation that mutation at a single site also abolishes nearly all hydrolysis of ATP by the membrane

fraction (7, 27). However, the enzyme can apparently be solubilized in several different forms (6).

Partial purification of *E. coli* ATPase has been reported in which the enzyme was solubilized with sodium dodecyl sulfate (15) or with dilute Tris buffer (14, 31). The use of Triton X-100 to solubilize the enzyme as reported here has the advantages that the extraction is a one step procedure, is nearly complete, and the activity is relatively stable in Triton. Extraction of other membrane proteins is more extensive than with Tris, but most of this protein is removed when the detergent is removed in the next step.

The ATPase extracted with sodium dodecyl sulfate was reported to have a molecular weight of about 100,000 (15), whereas molecular weights of 365,000 to 390,000 (14) and 400,000 to 600,000 (31) were reported for the enzyme extracted with Tris. The lower of the two values for the Tris-extracted enzyme is probably more accurate since it was obtained using a gel in which the ATPase is well included, whereas the higher value was estimated with Sephadex G-200 in which the enzyme elutes close to the void volume. The value of 365,000 to 390,000 for the Tris-extracted enzyme is in good agreement with the value estimated here for the Triton-extracted enzyme by sedimentation in a sucrose gradient. In light of the dissociation of the ATPase into subunits in sodium dodecyl sulfate reported here, it is possible that the enzyme extracted with sodium dodecyl sulfate represents only a part of the enzyme extracted with Triton or Tris. The enzyme extracted with sodium dodecyl sulfate or Tris, like the Triton-extracted enzyme, exhibited cold lability (14, 15, 31) and a preference for Ca^{2+} or Mg^{2+} (15, 31) as well as for purine nucleotides (14, 31).

The solubilization of the ATPase with Triton and the purification procedure appear not to have produced any gross alterations in the ATPase. The kinetic properties of the membrane-bound and solubilized forms of the enzyme are similar with respect to the K_m for ATP and inhibition by azide and P_i . Hammes and Hilborn (21) in a comparative study of the mitochondrial ATPase in the solubilized and membrane-bound forms also found only small differences in the K_m for ATP, although some other differences were noted. Further evidence that the structure of the enzyme has not been greatly changed by the purification procedure is the observation that the antibody to the pure ATPase inhibits the membrane-bound activity.

Disk gel electrophoresis of the cold-inac-

tivated ATPase showed that loss of activity is accompanied by dissociation of the enzyme to forms of lower molecular weight. It is not known whether the dissociation is the cause of the decreased activity or is a secondary consequence. It was noted, however, that when loss of activity was partial, dissociation was also partial. Similar observations have been made with mitochondrial ATPases (19, 32, 43).

In addition to the cold lability, other similarities of the *E. coli* ATPase to the mitochondrial enzyme are striking. The mitochondrial enzyme was originally thought to have a molecular weight of 284,000. However, this value has recently been revised to 360,000 (33). The larger subunits of the *E. coli* ATPase have their counterparts in the mitochondrial ATPase (9, 32, 49). There is some disagreement as to whether the mitochondrial enzyme has an additional small pair of subunits. One small subunit with a molecular weight of about 13,000 was found in the *E. coli* enzyme. Like the *E. coli* enzyme, the enzyme from mitochondria is inhibited by azide, and the inhibition is nonlinear (53). Kagawa and Racker have provided evidence that the stalked spheres lining the mitochondrial membrane are identical with the ATPase (24). Such particles have also been reported to be present on *E. coli* membranes (1).

These physical similarities would suggest that the *E. coli* enzyme would perform roles in energy metabolism similar to those of the extensively studied mitochondrial enzyme. The results with mutants lacking ATPase indicated that the *E. coli* enzyme is involved in the coupling of phosphorylation to oxidation (7) and in the utilization of ATP for the transhydrogenase reaction (13, 26). The experiments with the ATPase antibody also indicate that the ATPase is linked to the transhydrogenase. In this regard, it is interesting that Fisher et al. (18) have reported that mitochondrial energy transfer factors may be used to stimulate the *E. coli* transhydrogenase.

Respiratory enzymes are generally lower or absent in cells grown anaerobically (55), and transhydrogenase activity is depressed by growth on Casamino Acids (5), yet the specific activity of ATPase in anaerobic cells grown on glucose plus Casamino Acids is not depressed. This would suggest an additional role for this enzyme, although the possibility has not been excluded that anaerobic cells have phosphorylation by membrane electron transfer reactions not linked to O_2 (29). The experiments of Schairer and Haddock (45) suggest that this enzyme is involved in anaerobic transport.

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