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Inhibition, by a Protease Inhibitor, of the Solubilization of the F_1-Portion of the Mg^{2+}-Stimulated Adenosine Triphosphatase of *Escherichia coli*

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The effects of two protease inhibitors on the solubilization of the membrane-bound Mg^{2+}-adenosine triphosphatase (Mg-ATPase) of *Escherichia coli* were investigated. *p*-Aminobenzamidine prevented the solubilization of the Mg-ATPase during treatment of membranes with low-ionic-strength buffers containing ethylenediaminetetraacetic acid. *p*-Aminobenzamidine did not prevent subsequent solubilization of the Mg-ATPase by treatment of the membranes with chloroform. This method of solubilization yielded a preparation of similar apparent molecular weight but with a 10-fold-increased specific activity as compared with the Mg-ATPase solubilized by washing with low-ionic-strength buffer. However, in contrast to the latter preparation, the chloroform-solubilized Mg-ATPase did not reconstitute ATP-dependent energization of stripped membranes, which were prepared by low-ionic-strength washing in the absence of *p*-aminobenzamidine. Another protease inhibitor, ε-amino-*n*-caproic acid, did not affect the solubilization of the Mg-ATPase, but did inhibit the loss of activity occurring during concentration, by ultrafiltration, of the Mg-ATPase solubilized by the low-ionic-strength treatment.

The solubilization of the membrane-bound Mg^{2+}-stimulated adenosine triphosphatase (Mg-ATPase) activity in bacterial cells by washing membranes with low-ionic-strength buffers was first reported by Abrams (1) working with the gram-positive organism *Streptococcus faecalis*. This method has since been used extensively with both gram-positive and gram-negative organisms (11) to solubilize the F_1 portion of the Mg-ATPase as a preliminary step in the purification of the enzyme for studies of its structure and function. The Mg-ATPase from *Escherichia coli* K-12, solubilized using low-ionic-strength buffer, appears to have a polypeptide composition of five dissimilar subunits (3). Such preparations are active in the reconstitution of energy-linked activities in washed membranes from normal or Mg-ATPase-deficient mutant strains (5).

MacGregor (15) has shown that solubilization of the nitrate reductase from *E. coli* membranes after heat treatment is dependent on a membrane-bound protease. This solubilization could be prevented by the addition of the protease inhibitor *p*-aminobenzamidine, whereas the solubilization of other membrane proteins at lower temperatures could be prevented by the addition of ε-amino-*n*-caproic acid.

This paper describes the effect of these protease inhibitors on the solubilization of the membrane-bound Mg-ATPase activity by washing with low-ionic-strength buffers containing ethylenediaminetetraacetic acid (EDTA). *p*-Aminobenzamidine was found to inhibit the solubilization. However, the Mg-ATPase activity could be solubilized from inhibitor-treated membranes by the chloroform treatment procedure described by Beechey et al. (2) for the solubilization of the Mg-ATPase of beef heart mitochondria.

MATERIALS AND METHODS

Materials. Acrylamide, *N,N'*-methylene-bisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Eastman Kodak Co., Rochester, N.Y. Sodium dodecyl sulfate (especially pure) was from British Drug Houses, Poole, England. Coomassie brilliant blue R250 was from Serva, Heidelberg, Germany. *p*-Aminobenzamidine hydrochloride was from Sigma Chemical Co., St. Louis, Mo., and ε-amino-*n*-caproic acid was from Fluka A.G., Buchs, Switzerland. Phosphorylase A and β-galactosidase were from Worthington Biochemicals Corp., Freehold, N.J. Bovine serum albumin, ovalbumin, catalase, rabbit muscle adenylate kinase, and ribonuclease A were from Sigma. Bovine liver glutamate dehydrogenase and rabbit muscle triosephosphate isomerase were from Boehringer, Mannheim, Germany. Horse liver alcohol de-

hydrogenase, and horse heart cytochrome *c* were from Calbiochem, La Jolla, Calif. All chemicals were of the highest purity available commercially and were not further purified.

Growth of cells. *E. coli* K-12 strain AN248 (*ilvC argH entA*) was grown in the glucose-mineral salts medium described previously (9) to which had been added 5% (vol/vol) Luria broth (14) and the required growth factors, thiamin hydrochloride (0.2 μM, final concentration), 2,3-dihydroxybenzoate (40 μM), L-arginine hydrochloride (0.8 mM), L-isoleucine (0.3 mM), and L-valine (0.3 mM). One-liter cultures were grown overnight and used to inoculate 10 liters of medium in 14-liter New Brunswick fermentors. The cells were grown at 37°C and harvested as described previously (7).

Preparation of cell membranes. Membranes were prepared as described previously (5). Briefly, washed cells were disintegrated by using a Sorvall Ribi cell fractionator, cell debris was removed by centrifugation, and the membranes were separated by ultracentrifugation. The membranes were then resuspended in a 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES) buffer system (pH 7.0) containing magnesium acetate, sucrose, and ethyleneglycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid. The protease inhibitors *p*-aminobenzamidine and ε-amino-*n*-caproic acid were added to buffers as indicated in the text at final concentrations of 6 and 40 mM, respectively, including the TES buffer system in which the cells were suspended before disintegration.

Solubilization of Mg-ATPase. The membrane suspensions (about 400 mg of protein in 8 ml) were diluted to 35 ml with 50 mM TES buffer (pH 7.0) containing 15% glycerol and centrifuged at 160,000 × *g* for 2 h. The resulting pellet was resuspended in 5 ml of the 50 mM TES buffer containing 15% glycerol to give the "membrane" preparation. The membrane preparation (8 ml) was then diluted to 70 ml with 5 mM TES buffer (pH 7.0) containing 15% glycerol, 0.5 mM dithiothreitol, and 0.5 mM EDTA and centrifuged at 160,000 × *g* for 2 h. The supernatant was retained, and the pellet was resuspended in the 5 mM TES buffer system. The centrifugation and resuspension of the pellet was repeated twice more, and the supernatant fractions from the last three centrifugations were pooled and concentrated, using an XM-50 Diaflo filter.

If the washing procedure was carried out in the presence of the protease inhibitors, then the membrane pellet from the last centrifugation was resuspended in the 5 mM TES buffer system to a protein concentration of about 10 mg/ml. Chloroform (0.5 volume) was added to the membrane suspension, and the mixture was shaken vigorously for 30 s. The emulsion was broken by centrifugation, and the aqueous layer was removed and centrifuged at 160,000 × *g* for 2 h. The supernatant was then concentrated to about 4 ml by filtration, using an XM-50 Diaflo filter.

Estimation of protein. Protein concentrations were determined by using the Folin phenol reagent (13), with bovine serum albumin (fraction V; Sigma Chemical Co.) as standard.

Assay of Mg-ATPase. The reaction mixture contained 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 9.0), 20 mM ATP, and 10 mM $MgCl_2$ in a final volume of 1 ml. The reaction was started by the addition of 5 to 20 μl of enzyme preparation. After incubation at 30°C in a water bath, 0.5-ml samples of the reaction mixture were taken at 0, 3, and 6 min and added to 9.5 ml of King's reagent (12). After 15 min, the absorbancy at 660 nm was measured.

Measurement of the ATP-dependent transhydrogenase activity. The reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) was assayed by coupling the reaction to the NADPH-dependent glutathione reductase and measuring the decrease in absorbance at 340 nm (8). Details of the technique used have been described previously (6).

Measurement of atebrin fluorescence. Atebrin fluorescence was measured at 30°C as described previously (10) by using an Aminco-Bowman fluorimeter with the excitation wavelength set at 450 nm and emission wavelength set at 510 nm.

Gel electrophoresis. Electrophoresis under either dissociating or non-dissociating conditions was performed in polyacrylamide-gradient slab gels. Gradients were poured using a standard two-chambered gradient mixer.

For non-dissociating gels the back chamber contained 2.4% (wt/vol) acrylamide, 0.14% *N,N'*-methylene-bisacrylamide, 0.38 M Tris-hydrochloride (pH 8.8), 2 mM $MgSO_4$, 0.03% TEMED, and 0.075% ammonium persulfate; the front chamber contained 8.5% (wt/vol) acrylamide, 0.49% *N,N'*-methylene-bisacrylamide, 0.38 M Tris-hydrochloride (pH 8.8), 2 mM $MgSO_4$, 0.03% TEMED, 0.04% ammonium persulfate, and 15% (vol/vol) glycerol. The gel was allowed to polymerize for at least 1 h before use. The electrode buffer used for both top and bottom chambers contained 5 mM Tris, 38.4 mM glycine, and 2 mM $MgSO_4$. Gels were run at 22 mA for 12 h. Mg-ATPase activity was detected as a white precipitate in the gel after incubation at room temperature in a buffer containing 35 mM Tris, 270 mM glycine, 14 mM $MgSO_4$, 0.2% $Pb(NO_3)_2$, and 8 mM ATP. The gels were photographed through a polarizing filter, using a diffuse light source polarized at 90° to the filter.

Electrophoresis in sodium dodecyl sulfate was performed essentially as described by O'Farrell (18), except that a gradient of 7.5 to 22.5% acrylamide was used: the 7.5% acrylamide mixture contained, in a volume of 11 ml, 3.5 mg of ammonium persulfate and 7 μl of TEMED; the 22.5% acrylamide mixture contained, in a volume of 11 ml, 1.4 g of glycerol, 1.5 mg of ammonium persulfate, and 3 μl of TEMED. Gels were run at 14 mA for 12 h without a dye marker. Proteins were fixed in the gels by incubation at 60°C for 30 min in 5% (wt/vol) trichloroacetic acid, 5% (wt/vol) sulfosalicylic acid, and 10% (vol/vol) methanol. Gels were then stained for 60 min at room temperature in 0.12% Coomassie brilliant blue R250 in ethanol-acetic acid-water (25:8:67, vol/vol/vol) and destained in the same ethanol-acetic acid-water solvent mixture.

Samples for sodium dodecyl sulfate electrophoresis (at a final concentration of about 2 mg of protein per ml) were solubilized at 100°C for 3 min in 40 mM Tris-hydrochloride buffer, pH 6.8, containing 4% (vol/vol) mercaptoethanol, 1.6% sodium dodecyl sulfate, and 10% glycerol.

RESULTS

The Mg-ATPase activity in membranes prepared from the normal strain AN248, in the absence of protease inhibitors, was solubilized by successive washings of the membranes with the 5 mM TES buffer system, giving about a 60% recovery of Mg-ATPase activity (Table 1). About 70% of the membrane protein was solubilized by this washing procedure (Table 1). However, if the protease inhibitors ϵ-amino-*n*-caproic acid and *p*-aminobenzamidine were included in the buffer systems used, no Mg-ATPase activity could be detected in the supernatant fractions, although about 55% of the membrane protein was solubilized. There was, however, an overall loss of about 25% of activity during the washing in the presence of the inhibitors.

The Mg-ATPase activity, present in the membrane fraction after washing of the membranes in the presence of ϵ-amino-*n*-caproic acid and *p*-aminobenzamidine, could be solubilized by the chloroform extraction method of Beechey et al. (2). A suspension of the washed membranes was shaken with chloroform, and, after separation of the two phases, the aqueous phase was centrifuged at 160,000 × *g* for 2 h. The supernatant fraction was concentrated by filtration and found to contain about 50% of the Mg-ATPase activity present in the membranes before chloroform treatment (Tables 1 and 2). No activity was detected in the pellet.

The chloroform method gave a final yield of about 35% of Mg-ATPase activity compared with about 20% using the low-ionic-strength washing procedure. In the latter method about 40% of the activity was lost during the concentration by filtration of the pooled washes (Table 2). The specific activity of 15 of the chloroform-solubilized Mg-ATPase was considerably higher than the specific activity of 0.5 of the Mg-ATPase in the concentrated low-ionic-strength washes obtained in the absence of protease inhibitors (Table 2).

Inhibition of the solubilization of the Mg-ATPase activity also occurred when *p*-aminobenzamidine alone was added to the buffers (Table 1). Thus, both the loss of protein from the membranes and the retention of the Mg-ATPase activity on the membranes were similar regardless of whether ϵ-amino-*n*-caproic acid was present. However, if only ϵ-amino-*n*-caproic acid was added, the Mg-ATPase activity was solubilized as described for the procedure in which no protease inhibitors were added, but the loss of activity during the concentration of the low-ionic-strength washes did not occur (Table 2).

Mg-ATPase solubilized by either low-ionic-strength wash or chloroform treatment have

TABLE 2. *Specific activity and recovery of solubilized Mg-ATPase preparations*

Fraction	Mg-ATPase		Recovery of Mg-ATPase activity (%)
	Total units (μmol/min)	Sp act (μmol/min per mg of protein)	
Pooled low-ionic-strength washes (no protease inhibitor)	304	1.0	60
Concentrated low-ionic-strength washes (no protease inhibitor)	87	0.5	18
Pooled low-ionic-strength washes (plus ϵ-amino-*n*-caproic acid)	351	0.8	58
Concentrated low-ionic-strength washes (plus ϵ-amino-*n*-caproic acid)	346	1.1	57
Concentrated aqueous extract after chloroform treatment	147	15	33

TABLE 1. *Effect of the addition of the protease inhibitors ϵ-amino-n-caproic acid (EACA) and p-aminobenzamidine (PAB) on the solubilization of the membrane-bound Mg-ATPase*

Fraction	Mg-ATPase (total units, μmol of P_i released/min)			Protein (total mg)		
	No protease inhibitors	Plus EACA + PAB	Plus PAB	No protease inhibitors	Plus EACA + PAB	Plus PAB
Membranes	495	436	553	473	434	620
Membranes after first wash	163	367	383	292	319	467
Membranes after second wash	38	316	338	167	243	362
Membranes after third wash	17	316	330	133	196	252
First wash	202	0	0	179	147	204
Second wash	108	0	0	135	78	130
Third wash	2	0	0	10	47	91

similar apparent molecular weights, as indicated by electrophoresis under non-dissociating conditions (Fig. 1a and a'). The difference in specific activities of the Mg-ATPase solubilized by the two procedures (see Table 2) is reflected in the protein profiles of the Mg-ATPase preparations subjected to electrophoresis under non-dissociating (Fig. 1b and b') or dissociating (Fig. 1c and c') conditions.

As reported previously (17), removal of the Mg-ATPase from membranes by low-ionic-strength washes results in a loss of both ATP-dependent and NADH-dependent membrane energization, as judged by the loss of quenching of acridine dye fluorescence. The Mg-ATPase from the low-ionic-strength wash readily reconstituted both the NADH- and ATP-induced acridine fluorescence quenching (Fig. 2a). However, no reconstitution of either activity occurred when Mg-ATPase prepared by the chloroform treatment method was used (Fig. 2b). About 50% more Mg-ATPase activity was added in the latter experiment compared with the former.

DISCUSSION

It is clear from the results described above that the known protease inhibitor *p*-aminobenzamidine (16) inhibits the solubilization of the Mg-ATPase by the low-ionic-strength EDTA washing procedure. It is concluded, therefore, that the Mg-ATPase is not a so-called "extrinsic" membrane protein (20) but that the washing

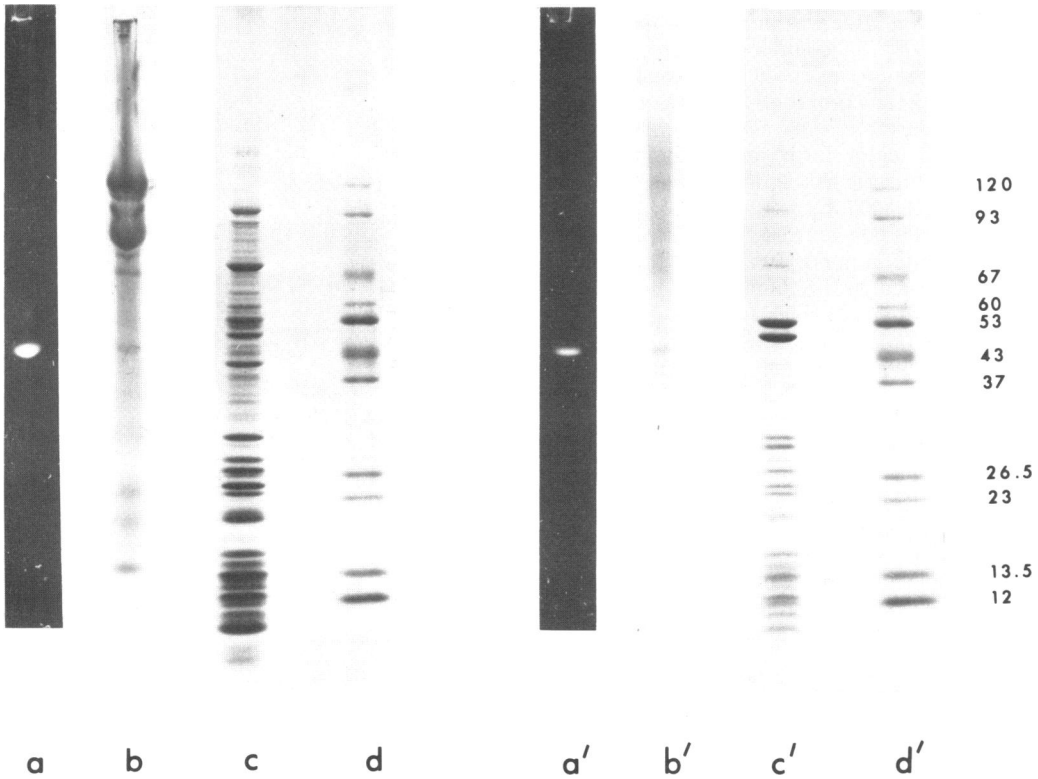

FIG. 1. *Electrophoresis of Mg-ATPase solubilized by low-ionic-strength washing (left, a–d) or by chloroform treatment (right, a'–d'). Low-ionic-strength wash (530 μg of protein) was subjected to electrophoresis under non-dissociating conditions and stained for Mg-ATPase activity (a) and then protein (b). (c) Low-ionic-strength wash (22 μg of protein) was subjected to electrophoresis under dissociating (sodium dodecyl sulfate [SDS]) conditions and stained for protein. Protein (40 μg) was solubilized by chloroform treatment, subjected to electrophoresis under non-dissociating conditions, and stained for Mg-ATPase activity (a') and then protein (b'). (c') Protein (9 μg) was solubilized by chloroform treatment, subjected to electrophoresis under dissociating (SDS) conditions, and stained for protein. Molecular weight standards (d and d') were subjected to electrophoresis under dissociating (SDS) conditions and stained for proteins: β-galactosidase (120,000), phosphorylase A (93,000), bovine serum albumin (67,000), catalase (60,000), glutamate dehydrogenase (53,000), ovalbumin (43,000), alcohol dehydrogenase (37,000), triosephosphate isomerase (26,500), adenylate kinase (23,000), ribonuclease A (13,500), cytochrome c (12,000). Molecular weights ($\times 10^{-3}$) of the protein standards are indicated.*

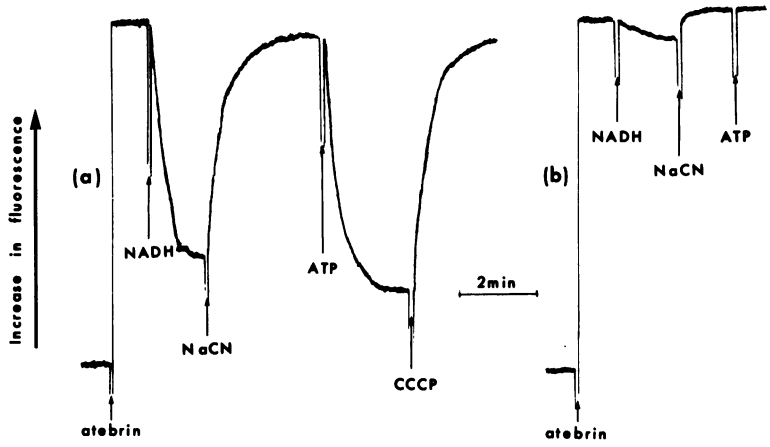

FIG. 2. *Reconstitution of electron transport- and ATP-induced atebrin fluorescence quenching by solubilized Mg-ATPase preparations. Stripped membranes (0.9 mg of protein), prepared by low-ionic-strength washing in the presence of ε-amino-n-caproic acid, were preincubated with 20 mM $MgCl_2$ for 10 min at 30°C with either (a) Mg-ATPase (5 mg of protein containing 5.5 U of Mg-ATPase activity) solubilized by low-ionic-strength washes in the presence of ε-amino-n-caproic acid or (b) Mg-ATPase (0.6 mg of protein containing 9 U of Mg-ATPase activity) solubilized by chloroform treatment of membranes in the presence of p-aminobenzamidine and ε-amino-n-caproic acid. After preincubation for 10 min at 30°C, the volume was made up to 2.5 ml by the addition of 2.2 ml of 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.5) containing 300 mM KCl and 20 mM $MgCl_2$. Atebrin was added to give a final concentration of 4 μM, NADH to 1 mM, NaCN to 2.5 mM, ATP to 0.8 mM, and carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) to 4 μM. In a control experiment containing stripped membranes only, a trace similar to (b) was obtained.*

procedure exposes the Mg-ATPase to a protease, or activates a protease, allowing the Mg-ATPase to be solubilized. Regnier and Thang (19) have reported that *E. coli* contains membrane-bound proteases that are activated by sonic treatment.

p-Aminobenzamidine has little effect on the total amount of protein solubilized from the membrane; thus, about 55% of the membrane protein is solubilized in the presence of *p*-aminobenzamidine compared with approximately 70% in its absence. Despite the loss of protein during washing with low-ionic-strength buffer in the presence of *p*-amino-benzamidine, the membranes can still be readily energized by the addition of NADH or ATP (J. A. Downie, unpublished data). This indicates that the washed membranes are not "leaky" to protons and that the Mg-ATPase is still functional.

Whether or not the protease responsible for the solubilization of the Mg-ATPase is the same as that reported by MacGregor (15) for the solubilization of nitrate reductase from *E. coli* membranes is not known. Although both proteases are inhibited by *p*-aminobenzamidine, solubilization of the nitrate reductase occurs at 60°C and at alkaline pH, somewhat different conditions than those required for the solubilization of the Mg-ATPase.

The chloroform extraction method of Beechey et al. (2) applied to membranes washed in the presence of the protease inhibitors gives a purer preparation of solubilized Mg-ATPase than does the low-ionic-strength EDTA washing procedure in the absence of protease inhibitors. The lack of reconstitution of ATP-dependent atebrin fluorescence quenching by the addition of chloroform-solubilized Mg-ATPase to stripped membrane particles would suggest that the structure of this Mg-ATPase may be different from that obtained by the low-ionic-strength EDTA washing procedure. Such structure-function relationships have not yet been made. Furthermore, the chloroform-solubilized Mg-ATPase preparation does not reconstitute the NADH-induced fluorescence quenching. This indicates that such Mg-ATPase preparations do not bind normally to stripped membranes, which consequently remain permeable to protons. Preliminary observations (D. R. H. Fayle, unpublished data) indicate that in the chloroform-solubilized Mg-ATPase preparations the δ-subunit is absent.

The marked loss of activity occurring during concentration of the Mg-ATPase solubilized by the low-ionic-strength washing of the *E. coli* membranes is similar to that reported previously (4) for the Mg-ATPase of *Micrococcus lysodeikticus*. The prevention of this loss of activity by ε-amino-*n*-caproic acid alone would suggest that a protease, other than the enzyme required for

the solubilization of the Mg-ATPase, is responsible for the loss of Mg-ATPase activity during concentration. The actual changes occurring in the structure of the Mg-ATPase leading to the loss of activity remain to be determined.

It is clear from the results of MacGregor (15) and from those described above that the presence of protease activity in membranes can significantly affect the solubilization of membrane-bound enzymes and presumably alter their subunit structure. Caution should therefore be exercised in interpreting the results of studies on structure-function relationships, especially when protease inhibitors are not used.

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