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MEMBRANE ATPase OF *ESCHERICHIA COLI* K 12

SELECTIVE SOLUBILIZATION OF THE ENZYME AND ITS STIMULATION BY TRYPSIN IN THE SOLUBLE AND MEMBRANE-BOUND STATES*

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SUMMARY

ATPase (EC 3.6.1.3) of *Escherichia coli* has been solubilized from two morphologically distinct membranes (vesicles and "ghosts"). Maximum ATPase release is attained with 3 mM EDTA in NH_4HCO_3, pH 9.0, and depends on protein concentration. After solubilization, the total enzyme activity is increased by 300% with respect to the membrane-bound enzyme. The released soluble ATPase accounts for more than 90% of this activity. Its specific activity is at least 10 times higher than the original value. Membrane treatment with buffers of various ionic strengths without EDTA and detergents is less selective. The molecular sieving properties (gel electrophoresis and Sephadex G-200 filtration) confirm the soluble nature of the preparation. A molecular weight close to 300 000 has been estimated for it.

The membrane-bound ATPase is stimulated by trypsin by 70–100%. Most of the soluble ATPase maintains a trypsin activation of the same order. Exceptions are the preparations obtained at high protein dilution and extracted with sodium dodecyl sulphate and deoxycholate. The soluble ATPase is more labile than the membrane-bound enzyme. Its sensitivity to different temperatures depends upon protein concentration and pH during storage. Inactivation seems to result from dissociation and/or proteolysis.

We suggest an ATPase link to the membrane through ionic divalent cation bridges. We also suggest that the enzyme possesses self-regulatory properties which would account for trypsin stimulation.

INTRODUCTION

The ATPases (EC 3.6.1.3) associated with bacterial membranes appear to provide a good system for studying the functional–structural relationships in that class of membranes. One of their interesting properties is the ease with which they can be solubilized more or less selectively[1–5]. It is then possible to study their

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properties in two physically distinct states. Our studies on *Micrococcus lysodeikticus* membrane ATPase have demonstrated the existence of different properties for the soluble and the membrane-bound enzyme[3,6,7]. These differences ("allotropy") are expressed by the latency of the enzyme in some of its membrane-bound states and its trypsin stimulation as well as by its response to divalent cations as activators[3,6]. It has been shown recently that the latency of *M. lysodeikticus* membrane ATPase is not simply the result of its crypticity and/or inaccessibility to the substrate, but it appears to be a consequence of distinct states of the membrane-bound enzyme which depend upon divalent cations as regulatory effectors (refs 6, 7; and Lastras, M. and Muñoz, E., unpublished).

By its trypsin activation, the ATPase of this strict aerobe resembles those of mitochondria and chloroplasts[8,9]. The ATPase of the fermentative *Streptococcus faecalis* is the best-studied bacterial ATPase[1,10,11]. However, so far as we are aware, there is no report on the trypsin activation of that membrane ATPase in any of its states (for a review, see ref. 11). We thus thought it was of interest to study the behaviour in this respect of the membrane ATPase from *Escherichia coli*, a more versatile organism in its metabolic capabilities.

The membrane-bound ATPase of different strains of *E. coli* has been the subject of several recent studies[11–16]. Some of these studies were carried out on the membrane-bound enzyme[16]. In other work, either a partial solubilization of the ATPase[14,15] or its extraction with sodium dodecyl sulphate[12,13] has been described. A partially solubilized preparation may not reflect all of the actual properties of the enzyme, whereas the use of dodecyl sulphate, even with careful manipulation, may not obviate changes in the different levels of the protein structure. None of these studies reported on the trypsin activation of the soluble enzyme.

The present report describes the selective and complete solubilization of the ATPase from two different classes of membrane (spheroplast "ghosts" (ref. 17) and vesicles[12]) of a strain of *E. coli* K 12. Similarities and some differences are found during the release of *E. coli* ATPase from each type of membrane. The enzyme is consistently stimulated by trypsin in its membrane-bound state. Activation with trypsin is also found in most of the soluble preparations. The possible significance of these findings is discussed. A preliminary account of this work was presented at the XIIth Meeting of the Sociedad Española de Bioquímica (S.E.B.), Madrid, May 1972.

MATERIALS AND METHODS

Bacterial strain

The *E. coli* K 12 strain used in these studies was number 414 (Hfr, *thr*$^-$) of the collection of Dr J. Puig (Facultad de Ciencias, Universidad de los Andes, Mérida, Venezuela). It was grown and harvested as previously described[17].

Preparation of membranes

Membrane spheroplast "ghosts", *i.e.* a membrane preparation which is mostly composed of membranous structures the size of the original cells, were obtained as reported before[17]. The treatment used is a modification of the standard technique for isolating membrane vesicles from *E. coli* strains[12,18]. The modifica-

tion includes a sequential treatment of exponentially growing cells with EDTA and lysozyme instead of the simultaneous action of both agents on the bacteria. The membrane vesicles used in these studies were prepared according to Evans[12].

Chemicals

Adenosine 5'-triphosphate, Na_2ATP was obtained from PL Biochemicals. Tris(hydroxymethyl)aminomethane was from Merck. EDTA, sodium deoxycholate and sodium lauryl sulphate (sodium dodecyl sulphate) were bought from Fisher Scientific Co. Trypsin (EC 3.4.4.4) (20 000 units/g) was from Schuchardt. Bovine serum albumin and Triton X-100 (octyl phenoxypolyethoxyethanol) were purchased from Sigma Chem. Co. Lysozyme (EC 3.6.1.3) and deoxyribonuclease (EC 3.1.4.5) were obtained from Calbiochem. Nonidet P-42 (Shell TP7143) was from B.D.H. Chemicals. Coomasie brilliant blue was from Colab., Sephadex G-200 from Pharmacia. All other chemicals were of reagent grade. NH_4HCO_3 from different chemical sources (Fisher, Merck) gave identical results.

Solubilization procedure

To solubilize the ATPase, the membrane "ghost" and vesicle preparations with initial protein concentrations ranging from 0.4–1 mg/ml were first centrifuged at $27\,000 \times g$ for 20 min in a refrigerated Sorval RC2-B at 0–4 °C. The resulting pellets were resuspended in the same or greater volumes of the different extraction buffers as described in the text. The suspensions were then maintained at 37 °C for 30 min (see Results). After this time, the membranes were spun down for 20 min at $27\,000 \times g$ at room temperature in the Sorval centrifuge. The resulting supernatants were considered as the soluble material, whereas the residual pellets were suspended in the original volume. Aliquots of both fractions were taken off to determine ATPase and protein content. Results are corrected to refer all the enzyme preparations to the original volume of the suspensions.

Enzyme assay

ATPase activity was measured in a reaction mixture containing, in 400 μl: 4 μmoles ATP, 2.5 μmoles Mg^{2+} and 12 μmoles Tris, pH 7.5. To initiate the reaction, variable amounts (100–40 μg) of membrane protein in 100 μl of 20 mM Tris (pH 7.5)–1 mM Mg^{2+} or 3–18 μg of soluble proteins in the same volume of the appropriate extraction buffer were added. When the solubilization buffers contained EDTA, equimolar concentrations of Mg^{2+}, in addition to the 2.5 μmoles of the standard assay mixture, were added to neutralize EDTA. In the assays with trypsin (0.5 mg/ml enzyme assay), the protease was added prior to the addition of enzyme. Incubations were carried out at 37 °C for 30 min. The reaction was stopped by immersing the test tubes in an ice-cold water bath (0 °C)and by the immediate addition of the reagents for P_i determination. The amount of P_i determined was measured by the method of Vambutas and Racker[9] as reported previously[3]. One unit of enzyme activity is defined as that amount which liberates 1 μmole of P_i per 30 min at 37 °C. Specific activity is expressed in units per mg of protein. Protein content was measured by the method of Lowry *et al.*[19] with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Analytical electrophoresis of the soluble preparations was carried out on separating gels (4 cm × 0.6 cm) with 7% acrylamide and 0.183% *N,N'*-methylene-bisacrylamide. No stacking gels were used. The electrophoreses were run in Tris–glycine buffer, pH 8.5±0.2, at 2 mA per gel for 30 min and for another 30 min at 5–6 mA per column until the tracking dye (bromophenol blue) reached 3.5–3.7 cm. Proteins were stained with Coomasie blue according to Fairbanks *et al.*[20]. Enzymatic staining of the ATPase zone in the gels was performed with a modification of the technique of Weinbaum and Markman[21,2].

Gel filtration

Sephadex G-200 was equilibrated in 50 mM NH_4HCO_3–5 mM EDTA, pH 7.5, and packed on a column (2.5 cm × 92 cm) from Pharmacia Fine Chemicals. The column was eluted with the same buffer at room temperature. The void volume (V_0) was determined from the elution volume of dextran blue and the $V_0 + V_i$ from the elution volume of NaCl (detected with $AgNO_3$). The values were $V_0 = 135$ ml, $V_0 + V_i = 470$ ml. The flow rate was kept at 25 ml/h.

RESULTS

Table I shows the trypsin stimulation of the *E. coli* membrane ATPase. Small differences are found in the percentages of trypsin activation of the ATPase associated with each type of membrane. Note the similar specific activities of both types of membrane. The mechanism of ATPase activation by the protease is not known at the present time. We shall discuss this aspect of the study later on.

TABLE I

TRYPSIN STIMULATION OF THE MEMBRANE-BOUND ATPase FROM *E. COLI*

The membrane preparations have been obtained as indicated in the text. ATPase activity was assayed as specified under Materials and Methods either in presence or absence of trypsin. Definition of ATPase units and other experimental details are also given in that section. Units are referred to 1 ml of the membrane suspension. Protein shows the amount in the enzyme assay. Results are the means of at least duplicate experiments.

	Protein (*mg*)	*ATPase* (*units/ml*)	*Spec. act.* (*units/mg*)	% *increase*
Membrane "ghosts"	0.50	8.4	8.4	—
Membrane "ghosts" + trypsin	0.50	15.8	15.8	88
Membrane "ghosts"	0.25	11.01	22.02	—
Membrane "ghosts" + trypsin	0.25	19.42	38.84	76
Membrane vesicles	0.20	8.05	20.6	—
Membrane vesicles + trypsin	0.20	16.84	42.8	108

Effect of ionic perturbations on the solubilization of the E. coli membrane ATPase

Membrane washing with Tris buffers (pH 7.5) of different ionic strength (100, 30 or 3 mM) partially released the ATPase into solution. Fewer units (approx.

10% of the initial membrane-bound activity) were obtained in the supernatants after washing with 100 mM Tris. The specific activities of these supernatants were equal to or lower than those of the initial membrane suspension. Higher values were found in the supernatants of membrane washes with 30 or 3 mM Tris (the maximum values being 20 and 30% of the initial units, respectively). The specific activities of these fractions were 2–3 times greater than those of the original membranes. However, in all experiments the residual pellets contained at least 50% of the initial activity. The efficacy of these washes seemed thus to be limited. Since the most effective treatment for this partial release appeared to be the wash with hypotonic buffers, we then examined the pH and protein concentration effects in the 3 mM Tris extraction buffer. No marked effect of pH was found. However, an ATPase increase in the supernatants was found when the 3 mM extraction was carried out at a dilution of the suspension with 7 vol. of extraction fluid. These supernatants showed specific activities 3–4 times higher than those of the original membranes. Nevertheless, the residual pellets still contained appreciable amounts of bound ATPase (again 50% of the initial activity). These results confirm the partial solubilization of *E. coli* membrane ATPase previously reported[14,15], and suggest an increase in the total ATPase units. It seems therefore reasonable to conclude that the ATPase released by ionic perturbation of *E. coli* membranes corresponds to a portion of the total membrane-bound ATPase.

ATPase solubilization with EDTA

Hence, we attempted a more drastic removal of cations with EDTA and studied its subsequent effect on *E. coli* ATPase solubilization. Preliminary experiments with 5 mM EDTA showed an augmentation in the number of the ATPase units solubilized. We then decided to examine the conditions for maximum ATPase solubilization by this agent. The effect of pH in the ATPase release by 5 mM EDTA in 50 mM NH_4HCO_3 buffer is illustrated in Fig. 1. The protein concentrations during these extractions were kept the same as the initial values. The increase of

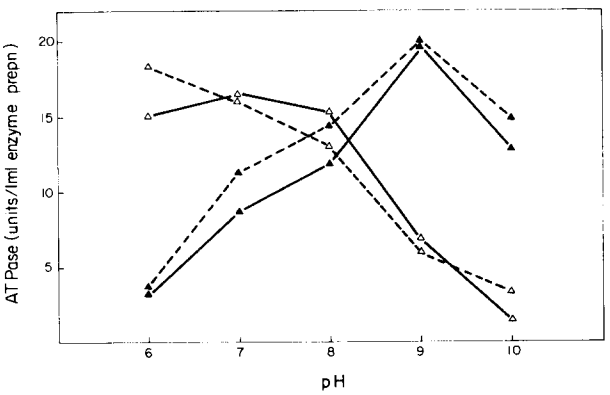

Fig. 1. Effect of pH in the ATPase solubilization by 5 mM EDTA–50 mM NH_4HCO_3. The two membrane preparations were extracted with 50 mM NH_4HCO_3 solutions of different pH values containing 5 mM EDTA. The pH values were adjusted after EDTA addition. ATPase was assayed in the presence of trypsin. Soluble ATPase: ▲—▲, from "ghosts"; ▲- - -▲, from vesicles. Residual membrane ATPase: △—△, ghosts; △- - -△, vesicles.

ATPase activity into soluble fractions is parallelled by a reduction of residual ATPase in the membrane pellets. The solubilization attains a maximum for alkaline pH values (9.0). The results are very similar for both types of membrane. The membrane (protein concentration 0.4–1 mg/ml) when washed with EDTA-free buffers, solubilized approx. 6 units/ml of enzyme preparation. The presence of EDTA increases the value up to 20 units/ml. Therefore, EDTA favours the ATPase release.

Activation by HCO_3^- of ATPases from various sources has been recently reported[22–24]. We have examined the effect of this anion in the *E. coli* ATPase assay and found a 20% increase in activity. To test its effect on the ATPase release, we replaced 50 mM Tris by NH_4HCO_3 in the assay for the EDTA effect. Table II

TABLE II

DIFFERENT EFFECTS OF TRIS AND NH_4HCO_3 ON THE SOLUBILIZATION OF *E. COLI* MEMBRANE-BOUND ATPase

Membranes (protein concentration as indicated in Table II) were extracted as described in Materials and Methods with either 50 mM Tris or 50 mM NH_4HCO_3, pH 9.0, containing 5 mM EDTA. The protein concentrations in these buffers were kept the same as the original ones.

Preparation	*ATPase (units/ml)*		*% increase*		*Spec. act. (units/mg)*		*% increase*	
	"Ghosts"	*Vesicles*	*"Ghosts"*	*Vesicles*	*"Ghosts"*	*Vesicles*	*"Ghosts"*	*Vesicl*
Supernatant Tris	19.14	10.43	—	—	134.15	158.8	—	—
Supernatant NH_4HCO_3	22.71	15.86	18.6	50.1	167.0	396.5	24.4	149.8

shows the comparative results, which show that the washing of membrane "ghosts" with 5 mM EDTA in NH_4HCO_3 liberates more ATPase units into solution. The specific activity is also greater than that for the assay including Tris. Since the increase is of the same order as the enzyme stimulation in the assay, we cannot conclude that HCO_3^- potentiates the EDTA effect. The results are nevertheless more clear-cut with the soluble ATPase from the vesicle preparation. The 50% increase in the total enzyme activity and the 150% augmentation of its specific activity would seem to point a stimulating effect of HCO_3^- on the ATPase release by EDTA. The other extractions were therefore carried out in NH_4HCO_3, unless otherwise stated.

We have previously indicated the effect of protein concentration in the *E. coli* ATPase release by 3 mM Tris. Therefore we thought it was of interest to study the same effect under the optimal conditions for ATPase release by EDTA. The results are illustrated in Table III. Several striking facts emerge from these results. First, it is noteworthy that 5 mM EDTA in NH_4HCO_3 at pH 9.0 solubilizes almost all ATPase units, as compared with the initial activity in the membranes. The yields of the soluble fractions range from 85–90% of initial activity at 1:1 (v/v) dilution (protein concn 0.4–1 mg protein/ml of extraction buffer) to 250% at 1:7 (v/v) dilution. Both membrane preparations behave in the same way.

TABLE III

EFFECT OF PROTEIN CONCENTRATION IN THE RELEASE OF *E. COLI* MEMBRANE ATPase BY EDTA–NH_4HCO_3

The pellets after centrifuging aliquots (1 ml) of membrane suspensions, were resuspended in different volumes, as indicated below, of 5 mM EDTA–50 mM NH_4HCO_3, pH 9.0. The resultant soluble fractions were assayed for ATPase activity in the presence of trypsin. The units of enzyme activity are referred to 1 ml of the initial volume of the suspensions (see the text).

Preparation	*Dilution by volume*	*ATPase (units/ml)*		*% enzyme activity*		*Spec. act. (units/mg)*	
		"Ghosts"	*Vesicles*	*"Ghosts"*	*Vesicles*	*"Ghosts"*	*Vesicles*
Initial Suspension	—	23.7	17.5	100	100	23.7	43.6
1st sol. fraction	1:1	21.1	14.5	89.0	83.0	163.6	431.0
1st sol. fraction	1:3	32.0	28.1	135.0	161.0	213.4	325.3
1st sol. fraction	1:7	51.7	44.8	218.1	256.0	282.4	319.5
2nd sol. fraction	1:1	6.0	3.5	25.3	19.8	61.2	130.5
2nd sol. fraction	1:3	9.1	3.4	38.4	19.6	70.5	95
2nd sol. fraction	1:7	12.8	5.9	54.0	34.0	80	106.3
Residual pellet from	1:1	6.0	1.3	25.3	7.3	8.5	7.4
Residual pellet from	1:3	5.2	1.0	21.9	5.6	8.1	6.0
Residual pellet from	1:7	3.6	0.7	15.2	4.1	6.3	5.8

The high specific activities of the EDTA-soluble fractions are also noteworthy. They are higher than those previously determined (see preceding results in this paper). This is an indication of the specific action of EDTA in releasing the ATPase from *E. coli* membranes. To explain the high yields of ATPase units it can be argued that this is the result of an enzyme activation by sample dilution. However, the residual pellets still showed high levels of ATPase (from 50–35% of the original activity in the membranes). The sum of the two fractions did therefore result in a net increase of enzyme units. Since the residual membranes are not diluted, this does not seem to be the sole explanation of the great yields of ATPase release. We would suggest that ATPase activity increases during its solubilization and/or dilution. The precise mechanism of this augmentation is not known. If the residual membranes were submitted to a second wash with the same EDTA-containing buffer, a new release of ATPase units into solution was observed. It must be noted that these second EDTA-soluble fractions possess a much lower specific activity, indicating a less selective protein solubilization. The residual pellets after the second extraction still gave appreciable ATPase activity. But in no case did this residual ATPase amount to more than 20% of the total units (actual figures vary from 2–20%), which gave in all cases more than 100% of the ATPase originally measured in the membranes.

We have thus demonstrated that EDTA promotes the selective and almost complete solubilization of *E. coli* membrane ATPase as well as an increase of its total activity. Since the 5 mM EDTA used in these studies was selected *a priori*, we considered it of interest to define the optimal EDTA concentration for *E. coli* membrane ATPase release. The results are shown in Fig. 2. As can be seen, a bell-shaped curve is obtained with a maximum at 3 mM EDTA. This maximum is more

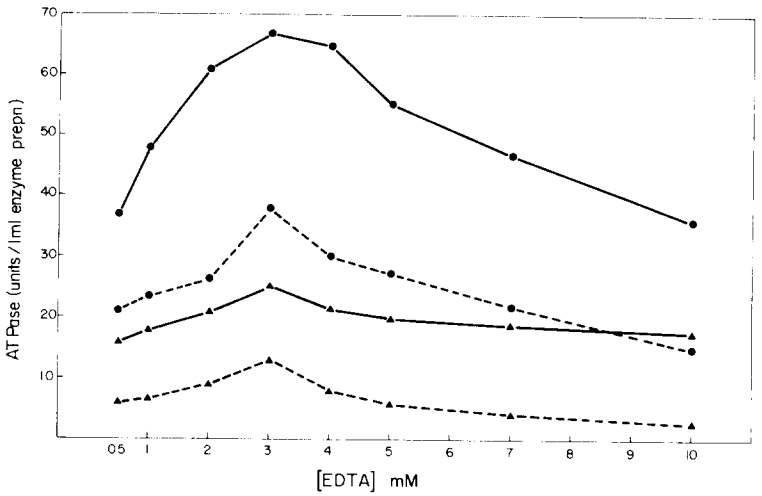

Fig. 2. Effect of EDTA concentration on the ATPase solubilization in 50 mM NH_4HCO_3, pH 9.0. The two membrane preparations were extracted at two dilutions, 1:1 and 1:7, by vol. The ATPase activity of the soluble fractions was assayed in the presence of trypsin. 1:1 dilution: ▲——▲, soluble fraction from "ghosts"; ▲- - -▲, soluble fraction from vesicles. 1:7 dilution: ●——●, soluble fraction from "ghosts"; ●- - -●, soluble fraction from vesicles.

apparent at the high protein dilution. In these conditions, 300% of the initially measured ATPase units in the two kinds of membrane are found in the first EDTA-soluble fraction. Less than 5% of the initial ATPase activity (*i.e.* less than 2% of the total, unmasked activity) remained in the residual pellets. We can therefore conclude that *E. coli* membrane ATPase is completely solubilized by washing the membranes (0.4–1 mg protein) with 7 ml of 3 mM EDTA–50 mM NH_4HCO_3, pH 9.0. Henceforth, we shall refer to this fraction as the soluble ATPase.

Characterization of the soluble ATPase by gel electrophoresis and gel filtration chromatography

To confirm the soluble nature and obtain some indications of the molecular characteristics of our EDTA-soluble ATPase, we examined its behaviour on molecular sieving.

The electrophoretic protein profile of the soluble ATPase and its ATPase zymogram are illustrated in Fig. 3. The protein staining shows (Gel a) one major protein component which corresponds to the band stained intensely for ATPase (Gel b). It must be noted that a second, faster, protein component, which also stains for ATPase but with less intensity, is identified in the gels. This resembles the result obtained with some *M. lysodeikticus* ATPase preparations[3]. Since the ATPase appears to be a major protein component of the electrophoretogram, we should like to point out the selectivity of the procedure for ATPase solubilization. It is worth noting, however, that some protein-staining material remains at the top of the gels. Moreover, if increasing amounts of protein (up to 200 μg) are applied to the gels, some other bands appear as constituents of the EDTA-soluble ATPase.

In any case, it is reasonable to conclude that the ATPase behaves as a soluble component in electrophoresis. It possesses mobility properties similar to those of *M. lysodeikticus* ATPase[2,3].

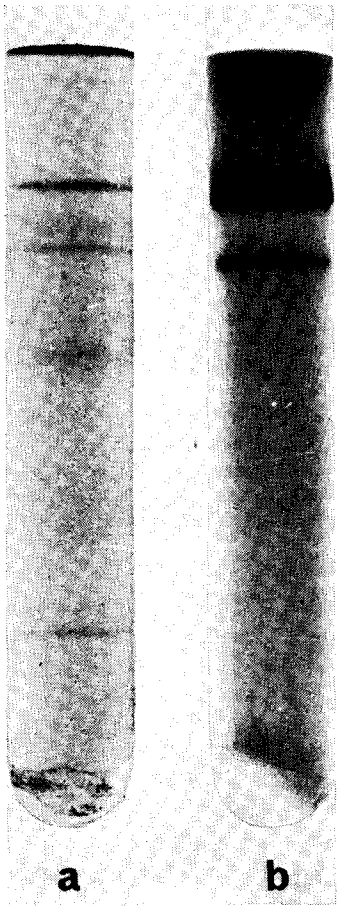

Fig. 3. Polyacrylamide electrophoresis of the soluble ATPase from *E. coli*. Aliquots (30–40 μg protein) of the soluble fraction after extracting the membranes with 3 mM EDTA–50 mM NH_4HCO_3, pH 9.0, were electrophoresed as indicated under Materials and Methods. Migration was towards the anode (bottom of the gels). Gel a shows protein staining, Gel b illustrates the ATPase stain.

Similar behaviour of the soluble ATPase has been observed by gel filtration on Sephadex G-200. The results are illustrated in Fig. 4. The ATPase elutes after the V_0 of the column. Its relative elution volume $V_e/V_0 = 1.27$ and its $K_d = 0.11$ are a confirmation of the soluble state of the ATPase and suggest a molecular weight close to 300 000, as calculated from the experimental relationships of Determann and Michel[25]. Molecular weights of $350\,000 \pm 30\,000$ (ref. 14) or higher than 400 000 (ref. 15) have been reported for other soluble ATPases from *E. coli*. A molecular weight of 100 000 has been described for the dodecyl sulphate-solubilized ATPase[12,13]. In no case was there a careful study of this parameter. The discrepancies

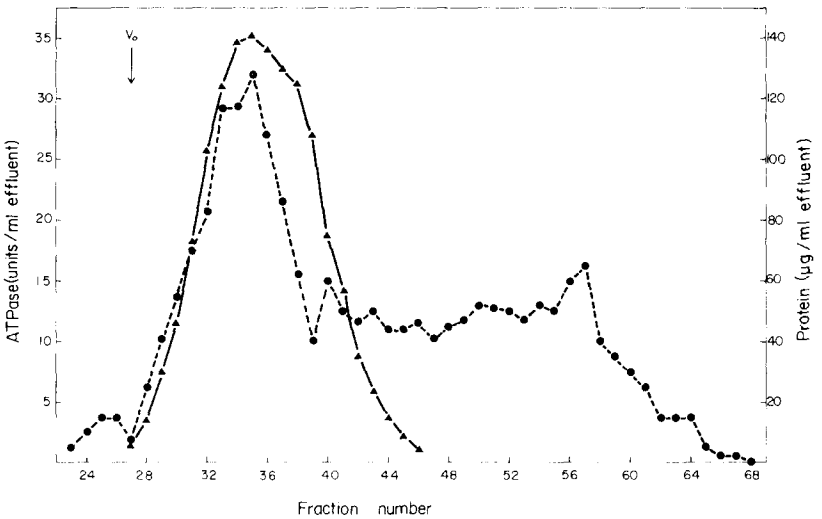

Fig. 4. Gel filtration of *E. coli* soluble ATPase. The soluble fraction (120 ml) obtained by extracting the membrane "ghosts" with 3 mM EDTA–NH_4HCO_3, pH 9.0 was concentrated to 5 ml by ultrafiltration on an XM 50 Diaflo ultrafilter. The 5 ml (16 mg protein) were charged onto a Sephadex G-200 column of the characteristics specified under Materials and Methods. Fractions of 5 ml were collected in a Packard Model 231 autofraction collector, and assayed for protein (●- - -●) and ATPase (▲——▲) in the presence of trypsin.

might reflect either the differences between the preparations or the lack of accuracy of the procedures employed. The last low value could be attributed to a partial dissociative action of the denaturant.

Trypsin stimulation of soluble ATPase preparations

We have previously described the trypsin activation by approx. 100% of *E. coli* membrane-bound ATPase. It was of interest to see whether or not this property was maintained through the solubilization of the enzyme. If the trypsin stimulation was due to a simple problem of crypticity, one would expect this stimulation to disappear in the soluble preparations. Table IV illustrates the results of trypsin stimulation of various soluble ATPase preparations. As can be seen, most of these preparations show trypsin stimulation. Low values of ATPase trypsin increase are found for the EDTA-extracted fractions at dilute concentration and the 100 mM Tris supernatant. Similar results are found for soluble preparations from the two types of membrane. When the dilute soluble fractions were concentrated, their trypsin stimulation of the ATPase reappeared, even after a partial purification, *e.g.* the eluate from Sephadex G-200. Of particular interest is the fact that the same trypsin activation is found for the soluble ATPase in Tris or HCO_3^-. This is a strong indication that HCO_3^- activation is not the result of the suppression of trypsin activation. It affects the ATPases, both trypsin-independent and trypsin-dependent, and therefore increases the total enzyme activity.

These results would suggest that trypsin activation is due to the protease destruction of some protein inhibitor and/or regulator which is intimately associated with the ATPase molecule and probably forms part of its quaternary structure. They also confirm that the crypticity hypothesis is very unlikely.

TABLE IV

TRYPSIN STIMULATION OF SOLUBLE PREPARATIONS OF THE MEMBRANE ATPase FROM *E. COLI*

The fractions were obtained as described in the text. They are identified with the buffers used for ATPase solubilization.

Fraction	*% increase enzyme activity*	
	"Ghosts"	*Vesicles*
100 mM Tris, pH 7.5, sol.	2.5	1.4
30 mM Tris, pH 7.5, sol.	31.0	5.4
3 mM Tris, pH 7.5, sol.	91.0	38.4
(1:1) 3–5 mM EDTA, 50 mM Tris, pH 9.0, sol.	55.0	69.0
(1:7) 3–5 mM EDTA, 50 mM Tris, pH 9.0, sol.	7.5	0.0
(1:1) 3–5 mM EDTA, 50 mM NH_4HCO_4, pH 9.0, sol.	59.0	69.0
(1:7) 3–5 mM EDTA, 50 mM NH_4HCO_3, pH 9.0, sol.	6.7	7.0
Sephadex G-200 eluate	70.0	—

ATPase solubilization with detergents

Detergents and surfactants have been in wide use to extract membrane-bound enzymes selectively (for a review see ref. 26). In order to compare our extraction procedure with the degree and characteristics of ATPase extraction by these agents, we treated the two types of membrane with several detergents. The results are illustrated in Table V. Low yields of soluble ATPase are obtained with this extraction as compared with the EDTA-alkaline solubilization. Although the specific activities are greater than those of the initial membrane suspensions, they are much lower than those of EDTA-soluble ATPase preparations. Interestingly enough, the residual pellets after dodecyl sulphate and deoxycholate extraction show very low contents of ATPase activity. The total ATPase is thus very low in these cases. On the other hand, Triton X-100 and Nonidet show an ATPase partition between the two fractions with regular recoveries of total enzyme activity (approx. 100% of the initial ATPase) and similar specific activities in both the soluble and the residual membrane fractions. It must be noted that these results are obtained with appropriate detergent concentrations. The 0.04% dodecyl sulphate is the same as that previously used before by Evans[13]. Deoxycholate and Nonidet were used at the usual concentrations[26], whereas the Triton X-100 concentrations were relatively reduced to obviate the difficulties created by this detergent in the P_i determination. It is also worth noting that the dodecyl sulphate and deoxycholate-soluble ATPases do not show the trypsin stimulation property while Nonidet and Triton-extracted enzymes, as well as their residual membranes, still give the 70–80% stimulation by the protease. All these results probably reflect the distinct denaturant and dissociative properties of these agents. It must be emphasized that the denaturant action of dodecyl sulphate and deoxycholate seems particularly evident for the *E. coli* vesicle preparation. In any case, we may reasonably conclude that the ATPase release by EDTA at alkaline pH is the most selective and complete procedure as far as optimum recovery of enzyme activity and specific enzyme activity are concerned.

TABLE V

THE DETERGENT SOLUBILIZATION OF THE MEMBRANE ATPase FROM *E. COLI*

Aliquots of membrane suspensions (protein concn as indicated in Table II) were extracted keeping the original protein concn with 30 mM Tris, pH 7.5, containing each one of the following detergents: sodium dodecyl sulphate (0.04% w/v), sodium deoxycholate (1% w/v), Nonidet (1% v/v) and Triton X-100 (0.2% v/v). ATPase was assayed either in the presence or absence of trypsin.

Preparation	*ATPase (units/ml)*		*Spec. act. (units/mg)*	
	"Ghosts"	*Vesicles*	*"Ghosts"*	*Vesicles*
Dodecyl sulphate, sol.	4.86	3.66	64.8	40.1
Dodecyl sulphate, sol. + trypsin	4.71	3.88	62.8	42.3
Dodecyl sulphate, pellet	3.29	0.57	6.1	3.1
Dodecyl sulphate, pellet + trypsin	3.57	0.43	6.7	2.3
Deoxycholate, sol.	6.00	2.57	55.0	16.2
Deoxycholate, sol. + trypsin	5.86	2.66	53.8	16.7
Deoxycholate pellet	2.57	0.14	5.0	1.1
Deoxycholate pellet + trypsin	2.43	0.57	4.7	4.2
Nonidet, sol.	8.71	2.71	22.3	13.6
Nonidet, sol. + trypsin	14.29	4.80	36.6	24.0
Nonidet, pellet	8.57	3.00	22.5	20.8
Nonidet, pellet + trypsin	12.86	4.57	34.9	31.7
Triton, sol.	10.43	4.71	28.6	27.0
Triton, sol. + trypsin	15.00	6.94	41.6	39.0
Triton, pellet	9.14	3.43	23.0	22.1
Triton, pellet + trypsin	14.29	5.43	36.0	34.9

Stability of the soluble ATPase preparations

It has been consistently reported that the ATPase from *E. coli* becomes unstable as it is detached from the membranes[13–15]. This lability is greater at low than at high temperatures. In this lability, the *E. coli* enzyme resembles the mitochondrial ATPase[8], but differs partially from that of *M. lysodeikticus*[3]. The mechanism of the inactivation of this bacterial enzyme is not known, as far as we are aware. To gain some information on this point and compare our preparation with those previously obtained, we have carefully examined the stability properties of the ATPase soluble in 3 mM EDTA–50 mM NH_4HCO_3, pH 9.0. Like the previous ones, this soluble ATPase is very labile. However, our preparation is far more stable than the dodecyl sulphate-soluble ATPase and perhaps than some other previous preparations. Moreover, the stability of our preparation varies with protein concentration, as regards its temperature dependence. At proteinc oncentrations ranging from 100–200 μg soluble protein/ml, the enzyme was more stable at low temperatures (0–4 °C) than at 37 °C. At low protein concentrations (20–30 μg/ml) the enzyme became more stable at high temperatures. The best stability was obtained at room temperature (20–24 °C). For example, more than 30% residual activities were found for soluble preparations after 144 h storage at 4 °C for 100–200 μg protein/ml and at 24 °C for 20–30 μg protein/ml. Conversely, the activity was completely destroyed after 144 h at 37 °C (100–200 μg/ml) and 4 °C (20–30 μg/ml). It must also be noted that the pH was very critical for the enzyme lability. At both

extremes of the 7.0–9.0 pH range the enzyme was rapidly destroyed, irrespective of the above-discussed conditions for storage.

It has been reported that glycerol overcomes the cold lability of the enzyme[14,15]. In our hands, the glycerol (10% w/v) did partially overcome the enzyme lability at 4 °C and pH 9.0. Moreover, if the enzyme was stored in EDTA–NH_4HCO_3 at a milder pH (7.5–8.0), the enzyme was equally stable in the absence or presence of glycerol. The glycerol effect might then be pH dependent, at least for our preparation.

These results indicate that the inactivation of the present ATPase preparation probably depends on a double mechanism. One process may be predominant at low temperatures and protein concentrations, and could be assigned to dissociation. The second one, however, may be preponderant at 37 °C and high protein concentrations, and should be tentatively ascribed to an enzymatic action (proteolysis). In any case, the ATPase should be moving towards lower molecular weight components. The analysis by gel electrophoresis of soluble preparations which have been stored at 24 °C for increasing times, revealed (see Fig. 5) the progressive

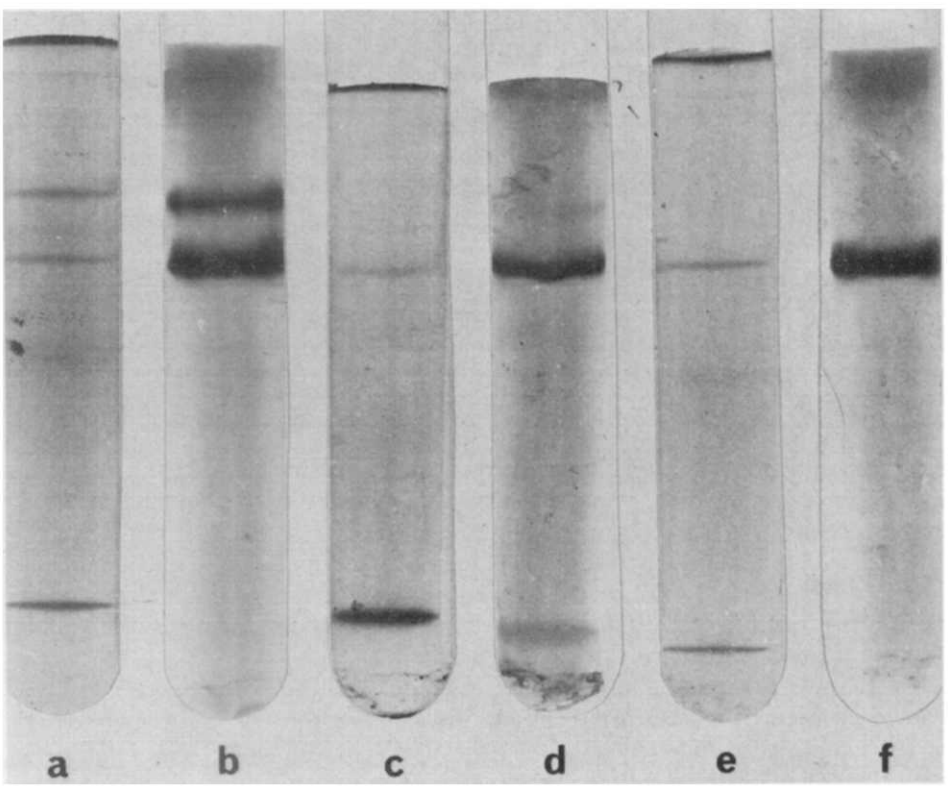

Fig. 5. Analysis by polyacrylamide electrophoresis of the inactivation of *E. coli* soluble ATPase. The soluble ATPase from EDTA extraction was stored at 24 °C. At different times of storage, aliquots containing 30–40 μg protein were electrophoresed as previously indicated. Gels a and b show the protein and ATPase stain of the enzyme after 44 h of storage; Gels c and d show the protein and ATPase profiles as 96 h and Gels e and f illustrate the protein and ATPase staining after 148 h.

disappearance of the slow-migrating ATPase and the concomitant appearance of a fast-moving component (approx. twice as rapid). This fast component showed a prominent staining for ATPase by the histochemical procedure, although it did not give any ATPase activity in the test tube. The reasons for this striking finding are not known, but work is in progress in this direction. The rapid inactivation of the dodecyl sulphate soluble ATPase may be explained in terms of an acceleration of the inactivation mechanism, such as dissociation and/or proteolysis, which both should be favoured by the detergent–protein interaction.

After the suggestion of an inactivation mechanism of the enzyme by proteolysis, we wondered what the nature of the ATPase solubilization process might be, since we carried out all the extractions at 37 °C (see Materials and Methods). This temperature was preselected on a theoretical basis, given the reported cold lability of the enzyme and the temperature dependence of the release of some protein components from other cell membranes, *e.g.* the "spectrin" from red cell "ghosts" (ref. 20). We then decided to examine the effect of time and temperature upon ATPase release from *E. coli* membranes. The velocity of ATPase release was not affected by incubating the membranes either at 24 or 37 °C. Moreover, this release was not dependent on time after 15 min incubation and up to 30 min at any of these temperatures. This indicates that the ATPase solubilization results from the transition of an interaction and not from the rupture of a covalent link. For experimental purposes the ATPase solubilization can then be carried out at any of these temperatures. Owing to the great stability of the enzyme at 24 °C, this temperature seems to be the most appropriate.

DISCUSSION

The systematic study carried out in the present work has led to the development of a procedure for the selective and complete release of the ATPase from *E. coli* membranes. The procedure is based upon the action of EDTA at alkaline pH values. After ATPase solubilization, we observed an increase in the total enzyme activity. The soluble ATPase contained more than 90% of the increased total units and a specific activity at least 10 times higher than that of the original membrane-bound enzyme. The other treatments did not equal the effectiveness of EDTA alkaline release by any of the above-mentioned criteria. This strongly suggests that the ATPase is held to the *E. coli* membrane complex by ionic interactions through divalent cation bridges. However, we cannot rule out the possibilty that the ATPase release is facilitated by the breaking down of some precise structural element of the cell envelope.

It is worth noting that the procedure for ATPase solubilization has been worked out with two types of membrane. As stated before by Roisin and Kepes[16], we suspected that membrane-associated enzymes could undergo changes depending upon the nature of the membrane preparation. This was our rationale in attempting the isolation of membrane sheets or "ghosts" from *E. coli* strains[17]. To our surprise, the ATPases from the vesicle and "ghost" preparations behave in a similar manner, in relation to their general properties as membrane-associated enzymes. The small differences found are not indicative of great changes that one would expect for morphologically different membrane preparations. From this similar behaviour

we should also like to point out that the vesicles and "ghosts" must have the same orientation. Comparative studies of this type should thus be of value in characterizing and correctly defining a membrane preparation.

We should also like to point out that both membrane preparations were prepared by EDTA *plus* lysozyme action. In spite of this early EDTA treatment, the ATPase remained membrane associated. Further EDTA action was needed to solubilize it selectively from the membranes. This indicates that the presence of EDTA during membrane isolation does not essentially modify the interactions within the membrane. Hence, we think that the previous statement by Davies and Bragg[14] in regard to EDTA use must be considered with caution. We have shown in this paper that the membrane-bound ATPase from *E. coli* is stimulated by trypsin. In this respect it resembles that of *M. lysodeikticus*. However, important differences are found between the two ATPases. These differences may reflect the physiological peculiarities of each microorganism. Among these differences we should like to point out the following: (i) the basal ATPase activity from *E. coli* membranes is higher than that of any kind of *M. lysodeikticus* membrane[3,6,7]; (ii) trypsin stimulation of this activity being 100% at the maximum; (iii) most of the *E. coli* soluble ATPase preparations conserve the trypsin stimulation in contrast with *M. lysodeikticus* soluble ATPases. Hence, we have tentatively suggested the presence of some protein component(s), which regulate(s), in a yet undefined way, the hydrolytic activity of the ATPase. This (these) hypothetical regulator(s) must be firmly associated with the intrinsic ATPase molecule; and by definition, would only be dissociated from the ATPase core by protein dilution and/or relatively high salt concentrations (see Results). On the other hand, we are suggesting that the regulation of *M. lysodeikticus* ATPase is much more dependent on its association with the membrane complex (Lastras, M. and Muñoz, E; Andreu, J. M., Tacoronte, E. and Muñoz, E., unpublished). However, a similar mechanism to that proposed by us for *E. coli* ATPase regulation has been recently proposed for *M. lysodeikticus* ATPase[27]. If this hypothesis is true, the differences in behaviour between the two enzymes could be explained in terms of distinct association affinities of the proposed regulator(s) for the ATPase core. We hope that future work will clarify these problems.

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