PROTEOLYSIS OF THE DELTA SUBUNIT IS REQUIRED FOR RELEASE OF THE Ca^{2+}, Mg^{2+}-ACTIVATED ATPase FROM THE CELL MEMBRANE OF *ESCHERICHIA COLI*

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1. Introduction

The ATPase complex of the cell membrane of *Escherichia coli*, like the analogous enzymes of mitochondria, chloroplasts and chromatophores, is composed of two distinct portions [1]. The extrinsic portion can be readily detached from the membrane and can be purified as a Ca^{2+}- and Mg^{2+}-activated ATPase made up of 5 different types of subunits (α–ϵ) [2]. The other portion of the complex is an intrinsic membrane protein (F_o) which contains the pathway by which protons can be translocated across the membrane. The Ca^{2+}, Mg^{2+}-activated ATPase of *E. coli* interacts with F_o through the δ subunit [3,4].

The ATPase is generally released by washing the membranes with a buffer of low ionic strength containing EDTA [5]. The protease inhibitor *p*-aminobenzamidine was found [6] to prevent the release of ATPase by the washing procedure. This result suggested that proteolysis of a portion of the ATPase molecule might be required for release of the enzyme.

Here we show that release of the ATPase from the cell membrane of *E. coli* by the washing procedure is associated with the proteolytic cleavage of a portion of the δ subunit of the enzyme.

2. Methods

Unless indicated otherwise the preparation of the membranes and of the ATPase was conducted at 0–4°C.

2.1. *Preparation of ATPase in the presence of protease inhibitors*

E. coli K12 grown to the late exponential phase on a high peptone medium was obtained from Grain Processing Corp., Muscatine, Ohio. Frozen cell paste (20 g) was suspended in 50 ml 10 mM Tris–HCl buffer (pH 8) containing 10 mM $MgCl_2$, 6 mM *p*-aminobenzamidine hydrochloride and 40 mM ϵ-amino-*n*-caproic acid. The cell suspension was disrupted by passage twice through a French press at 8000 p.s.i. Unbroken cells were removed from the disrupted cell suspension by centrifugation at 10 000 × *g* for 10 min. Membranes were recovered from the supernatant by centrifugation at 177 000 × *g* for 2 h. The membranes were suspended in 50 ml 10 mM Tris–H_2SO_4 buffer (pH 7.5) containing 10 mM EDTA, 10% (v/v) glycerol, 6 mM *p*-aminobenzamidine hydrochloride and 40 mM ϵ-amino-*n*-caproic acid (buffer 1) and recentrifuged as above. The washed membranes suspended in 40 ml 1 mM Tris–HCl buffer (pH 7.5) containing 0.5 mM EDTA, 10% (v/v) glycerol, 6 mM *p*-aminobenzamidine hydrochloride and 40 mM ϵ-amino-*n*-caproic acid were stored at −70°C for 16 h. After thawing, the suspension was kept at 25°C for 1 h before being centrifuged at 177 000 × *g* for 2 h. The pellet (low ionic strength-washed membranes) was suspended to 50 ml in buffer 1. The suspension in 10 ml portions was mixed vigorously for 30 s with one-half its volume of chloroform using a vortex mixer, then centrifuged at 10 000 × *g* for 15 min to separate the two phases. The aqueous phase was recentrifuged at 250 000 × *g* for 2 h. The supernatant was further purified in one of two ways.

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(i) The supernatant (40 ml) was concentrated to 1.2 ml by ultrafiltration through an Amicon XM100A filter to give 'inhibitor-protected coupling factor'. Aliquots (0.4 ml) of the coupling factor were applied to a 15–25% (w/v) gradient of sucrose in 50 mM Tris–H_2SO_4 buffer (pH 7.8) containing 0.5 mM EDTA, 0.1 mM dithiothreitol, 6 mM *p*-aminobenzamidine hydrochloride and 40 mM ϵ-amino-*n*-caproic acid. The gradients were centrifuged for 15 h at 45 000 rev./min in a Beckman SW 50L rotor [5]. Fractions (30 drops) were collected by puncturing a hole in the bottom of the centrifuge tube and assayed for ATPase activity.

(ii) To the supernatant (39 ml) was added 9.75 ml methanol, 2.43 ml M $MgCl_2$ (47 mM final conc.) and 12.4 mg *p*-aminobenzamidine hydrochloride. The precipitate which formed was removed by centrifugation at 10 000 × *g* for 20 min. The supernatant was concentrated to 4.8 ml by ultrafiltration through an Amicon XM100A filter to give a partially purified preparation of the ATPase.

2.2. *Preparation of ATPase in the absence of protease inhibitors*

Washed membranes from 20 g cells were prepared as in section 2.1 except that *p*-aminobenzamidine and ϵ-amino-*n*-caproic acid were omitted from the buffers. The ATPase was released from the washed membranes by suspending them in 20 ml 1 mM Tris–HCl buffer (pH 7.5) containing 0.5 mM EDTA, 0.1 mM dithiothreitol and 10% (v/v) glycerol, and dialysed overnight against 1.5 l of the same buffer. The dialyzed suspension was diluted with an equal volume of the buffer and centrifuged at 250 000 × *g* for 2 h. The supernatant was recentrifuged under the same conditions and then concentrated to 2 ml of 'non-protected coupling factor' by ultrafiltration through an Amicon XM100A filter. The ATPase was further purified as in [2,5]. The pellet resulting from centrifugation of the dialyzed suspension was washed in the dialysis buffer and used as 'ATPase-stripped membranes'.

2.3. *Gel electrophoresis*

One-dimensional SDS–polyacrylamide gel electrophoresis was performed on 0.75 mm gel slabs as in [7]. Two-dimensional polyacrylamide gel electrophoresis followed the method [8] as adapted [9]. In the first dimension the 0.75 mm slab gel contained 1.6% and 0.4% (v/v) LKB Ampholines of pH 5–7 and pH 3.5–10, respectively. Electrophoresis was carried out for 18 h at 300 V followed by 1.5 h at 400 V. The polarity of system was the reverse of that used in [8]. Strips (4 mm wide) were cut from the first-dimension gel and mounted horizontally at the top of the 13% polyacrylamide second-dimension slab gel (1.5 mm thick) containing 0.1% (w/v) SDS. The second dimension gel was run at 40 mA for 2.75 h. Gels were stained with Coomassie blue [10] following removal of Ampholines by soaking the gel in several changes of 50% (v/v) ethanol containing 7% (v/v) acetic acid.

2.4. *Assays*

Energy-dependent quenching of the fluorescence of quinacrine with NADH and ATP as substrates [11] and ATPase activity measured in the presence of 2.5 mM $CaCl_2$ [2] were assayed as described.

3. Results

The result [6] showing that the ATPase was not released in the presence of the protease inhibitors, *p*-aminobenzamidine and ϵ-amino-*n*-caproic acid, by washing cell membranes with a low ionic strength buffer containing EDTA were verified (table 1). However, treatment of membranes with chloroform in the presence of the protease inhibitors liberated the enzyme.

The ATPase released by chloroform treatment was either used directly in the following experiments or was partially purified in the presence of the protease inhibitors. Purification to homogeneity was not attempted since the inhibitors would have been removed during ion-exchange chromatography [5]. For comparison, the ATPase was also prepared by washing membranes with low ionic strength buffer in the absence of protease inhibitors [5].

One-dimensional SDS–polyacrylamide gel electrophoresis showed a clear difference between the ATPases prepared in the absence and presence of the protease inhibitors (fig.1). The α, β, γ and ϵ subunits in the two preparations appeared to be identical. However, the δ subunit in the inhibitor-protected enzyme migrated more slowly than that of the ATPase

Table 1
Effect of protease inhibitors on the release of ATPase from *E. coli* membranes

Fraction	ATPase (total units[a])	
	–Inhibitors	+Inhibitors
Membranes[b]	173	223
Membrane pellet from treatment with low ionic strength buffer	43	215
Supernatant from treatment with low ionic stength buffer	156	0
Membrane pellet from $CHCl_3$ treatment	–	18
Supernatant from $CHCl_3$ treatment	–	68

[a] μmol.min^{-1}
[b] From 20 g cells

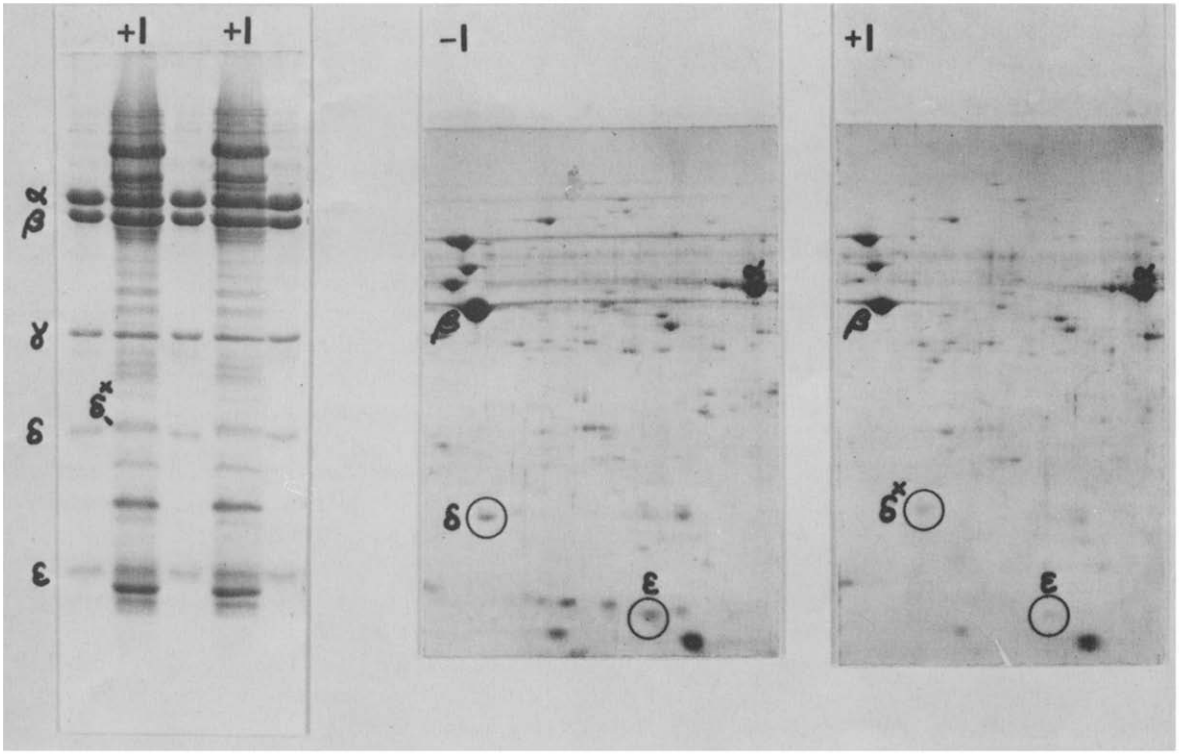

Fig.1. Polyacrylamide gel electrophoresis of ATPase prepared in the presence and absence of protease inhibitors. Left panel: One-dimensional SDS–polyacrylamide (13%) gel electrophoresis of ATPase purified in the absence of protease inhibitors and of ATPase partially purified by methanol–$MgCl_2$ treatment in the presence of protease inhibitors (+I). Centre and right panels: Two-dimensional isoelectric focussing (horizontal direction) – SDS (vertical direction) polyacrylamide gel electrophoresis of coupling factor (crude ATPase) prepared in the absence (–I) and presence (+I) of protease inhibitors. The subunits of the ATPase are indicated by α–ϵ. δ^X, δ subunit unmodified by proteolysis.

in which proteolysis could still occur. The apparent molecular weights of the δ subunits in the two cases were 21 400 and 20 700, respectively. Confirmation of the difference between the δ subunits of inhibitor-protected and non-protected ATPases was obtained using the two-dimensional gel system [8,9] (fig.1). Besides the difference in molecular weight, there was a small difference (0.1 pH unit) between the isoelectric points of the two forms of the δ subunit. The similarity of the molecular weights and isoelectric points of the α, β and ε subunits of the enzyme was confirmed by electrophoresis of mixed samples of the two ATPases (data not shown).

Removal of the ATPase from the cell membrane of *E. coli* by washing with low ionic strength buffer without protease inhibitors is reversible [5]. The enzyme is rebound if the ionic strength is increased. Removal of the ATPase results in loss of such energy-dependent functions as the NADH oxidation-dependent and ATP-dependent quenching of the fluorescence of quinacrine [1,11]. These functions are restored when the ATPase is rebound. We have examined the effectiveness of the inhibitor-protected ATPase in the restoration of fluorescence quenching in membranes from which the ATPase had been stripped by washing with low ionic strength buffer in the absence of the protease inhibitors. Membranes stripped by chloroform treatment could not be used in these experiments since this solvent caused extensive protein denaturation. As shown in fig.2, non-purified ATPase prepared in the presence of protease inhibitors (coupling factor, CF) or following purification on a sucrose gradient (ECF_1) restored respiration- and ATP-dependent fluorescence quenching to the extent of 73–85% and 45–65%, respectively, of the values obtained at a saturating level of coupling factor prepared in the absence of protease inhibitors. The difference is not due to the presence of protease inhibitors since these had no effect on the reconstitution of fluorescence quenching by inhibitor non-protected ATPase (data not shown). Although not shown in fig.2, at least 2–3-fold more inhibitor-protected than non-protected ATPase units have to be added to achieve maximal levels of reconstitution.

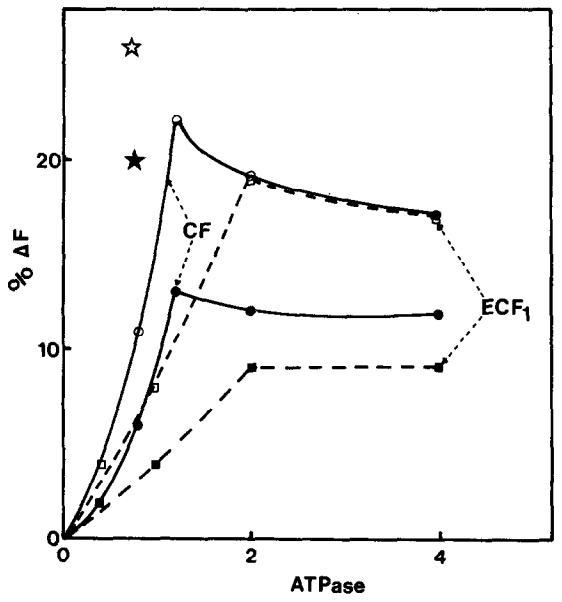

Fig.2. Reconstitution of the energy-dependent quenching of the fluorescence of quinacrine in ATPase-stripped membranes by protease inhibitor-protected coupling factor (crude ATPase) (CF) and ATPase partially purified by sucrose gradient centrifugation (ECF_1). The stars indicate the extent of fluorescence quenching given by a saturating level of coupling factor prepared in the absence of protease inhibitors. Reconstitution was carried out by incubating the indicated number of units of ATPase with 0.15 mg stripped membrane protein for 5 min at 22°C in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonate buffer (pH 7.5) containing 200 mM KCl and 4 mM $MgSO_4$. Fluorescence quenching (ΔF) is expressed as a percentage of the total fluorescence. Units of ATPase are expressed in μmol ATP hydrolysed.min^{-1}. Open points, fluorescence quenching dependent on the oxidation of NADH. Closed points, fluorescence quenching dependent on the hydrolysis of ATP.

4. Discussion

Release of the Ca^{2+}, Mg^{2+}-activated ATPase from the cell membrane of *E. coli* at low ionic strength is prevented when protease inhibitors are present. The release process appears to require proteolytic cleavage of the δ subunit of the ATPase with the removal of a portion (~700 mol. wt) of the polypeptide chain. The enzyme responsible for the cleavage is probably the protease which is associated with the outer membrane of *E. coli* and would be present in our membrane preparations [12]. It is not known if the small fragment removed from the δ subunit still remains attached to the membrane. Our reconstitution experiments imply that this is so, since reconstitution with

ATPase containing the non-cleaved δ subunit occurs less effectively than with that in which the δ subunit has been partially digested. The presence of the cleaved fragment in the stripped membranes used in these experiments could interfere with the proper interaction of the uncleaved δ subunit with the membrane.

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