The topology of the proton translocating F_0 component of the ATP synthase from *E. coli* K12: studies with proteases

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The accessibility of the three F_0 subunits *a*, *b* and *c* from the *Escherichia coli* K12 ATP synthase to various proteases was studied in F_1-depleted inverted membrane vesicles. Subunit *b* was very sensitive to all applied proteases. Chymotrypsin produced a defined fragment of mol. wt. 15 000 which remained tightly bound to the membrane. The cleavage site was located at the C-terminal region of subunit *b*. Larger amounts of proteases were necessary to attack subunit *a* (mol. wt. 30 000). There was no detectable cleavage of subunit *c*. It is suggested that the major hydrophilic part of subunit *b* extends from the membrane into the cytoplasm and is in contact with the F_1 sector. The F_1 sector was found to afford some protection against proteolysis of the *b* subunit *in vitro* and *in vivo*. Protease digestion had no influence on the electro-impelled H^+ conduction *via* F_0 but ATP-dependent H^+ translocation could not be reconstituted upon binding of F_1. A possible role for subunit *b* as a linker between catalytic events on the F_1 component and the proton pathway across the membrane is discussed.
Key words: protein pathway/F_1 ATPase binding/F_0 subunits *a*, *b* and *c*/ATPase mutants

Introduction

ATP synthases are composed of a hydrophilic membrane-associated component F_1 and a membrane-integrated moiety F_0. The ATP sites are located on F_1 whereas F_0 catalyzes the proton conduction across the membrane. In all analyzed organisms, F_1 is composed of the five subunits α, β, γ, δ and ϵ (Abrams and Smith, 1974; Penefsky, 1974; Sebald, 1977). However, the subunit composition of F_0 has been unequivocally determined only in the case of *E. coli*. Preparations of F_0 generally contain three different subunits (Negrin *et al.*, 1980; Friedl and Schairer, 1981), and three genes coding for these subunits have been identified in the *E. coli atp* operon (Downie *et al.*, 1981; Hansen *et al.*, 1981). The sequence of these genes has been determined (Gay and Walker, 1981; Kanazawa *et al.*, 1981; Nielsen *et al.*, 1981) and with the help of protein sequence data the complete amino acid sequences of the three gene products were established (Nielsen *et al.* 1981). F_0 consists of subunit *a* (mol. wt. 30 000) which is a very hydrophobic protein, subunit *b* (mol. wt. 17 200) which is an amphophilic protein and subunit *c* (mol. wt. 8300), again a very hydrophobic protein. Subunit *c* is also referred to as the proteolipid subunit, because of its solubility in organic solvents, or as the dicyclohexylcarbodiimide

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(DCCD)-binding protein (Cattel *et al.*, 1971; Sebald *et al.*, 1980). The following stoichiometries for the F_0 subunits have been suggested $a:b:c = 1:2:10$ (Foster and Fillingame, 1982) and $1:2:12-15$ (von Meyenburg *et al.*, 1982). So far, there is no unequivocal information about the orientation and the arrangement in the membrane of the polypeptide chains. We have used protease digestion to probe some topological features of the F_0 sector subunits. Large parts of subunit *b* could be digested without inhibition of electro-impelled proton conduction across the membrane while ATP-driven H^+ translocation was completely abolished.

Results

Protease sensitivity of the F_0 sector subunits a, b and c

F_1-depleted membranes were chosen rather than the isolated ATP synthase complex to avoid possible artefacts during reconstitution of the complex. In wild-type membranes the ATP synthase constituted $\sim 10\%$ of the protein mass. Thus, it was not possible to identify unequivocally the individual F_0 subunits and their possible cleavage products in conventional SDS-gel electrophoresis systems. Two systems were developed to monitor the F_0 subunits. (i) After SDS-gel electrophoresis the separated proteins were transferred onto nitrocellulose sheets and the F_0 subunits visualized by immunofluorescence (cf., Figure 3). This enabled us to study the extent of degradation and to identify cleavage products. As no antibodies directed against subunit *a* were available a second method was employed for detection of this subunit. (ii) The strain CM 2786-I harboured the cloned *atp* operon on a plasmid and the membrane consequently contained 4- to 5-fold more ATP synthase than the wild-type. This strain was thus ideal for a study of the effects of proteases because the individual F_0 subunits could be easily recognized after SDS-polyacrylamide electrophoresis of F_1-depleted membranes (Figure 1). A further improvement came from the use of very thin gels (0.3 mm) in combination with the silver strain method. The hydrophobic proteins *a* and *c* are more intensely stained compared with the Coomassie blue stain. Secondly, since only minute amounts were needed (2 μg of membrane protein), no distortion of bands in the low mol. wt. region due to the high content of phospholipids was observed.

Trypsin, chymotrypsin and V8 were chosen because they cleave at a limited number of relatively specific sites, while subtilisin was used because of its unspecific cleavage. Different ratios of protease protein to membrane protein were applied to study the specific sensitivity of each F_0 subunit.

Subunit b. This subunit was the most sensitive towards proteases. Except with chymotrypsin, degradation was already observed at a ratio of protease to membrane protein of 1/1000 (w/v) (Figure 1). After incubation with proteases at a ratio of 1/100 for 1 h at 37°C, no intact subunit *b* was detectable. Trypsin, subtilisin and protease V8 gave rise to small cleavage products only (Figure 1), indicating that large portions of the protein were accessible to the proteases. With chymotrypsin, however, a defined cleavage product of mol. wt. 15 000 was generated (Figure 1), as demonstrated by immuno-stain techniques (Figure 3C). The 15-kd fragment
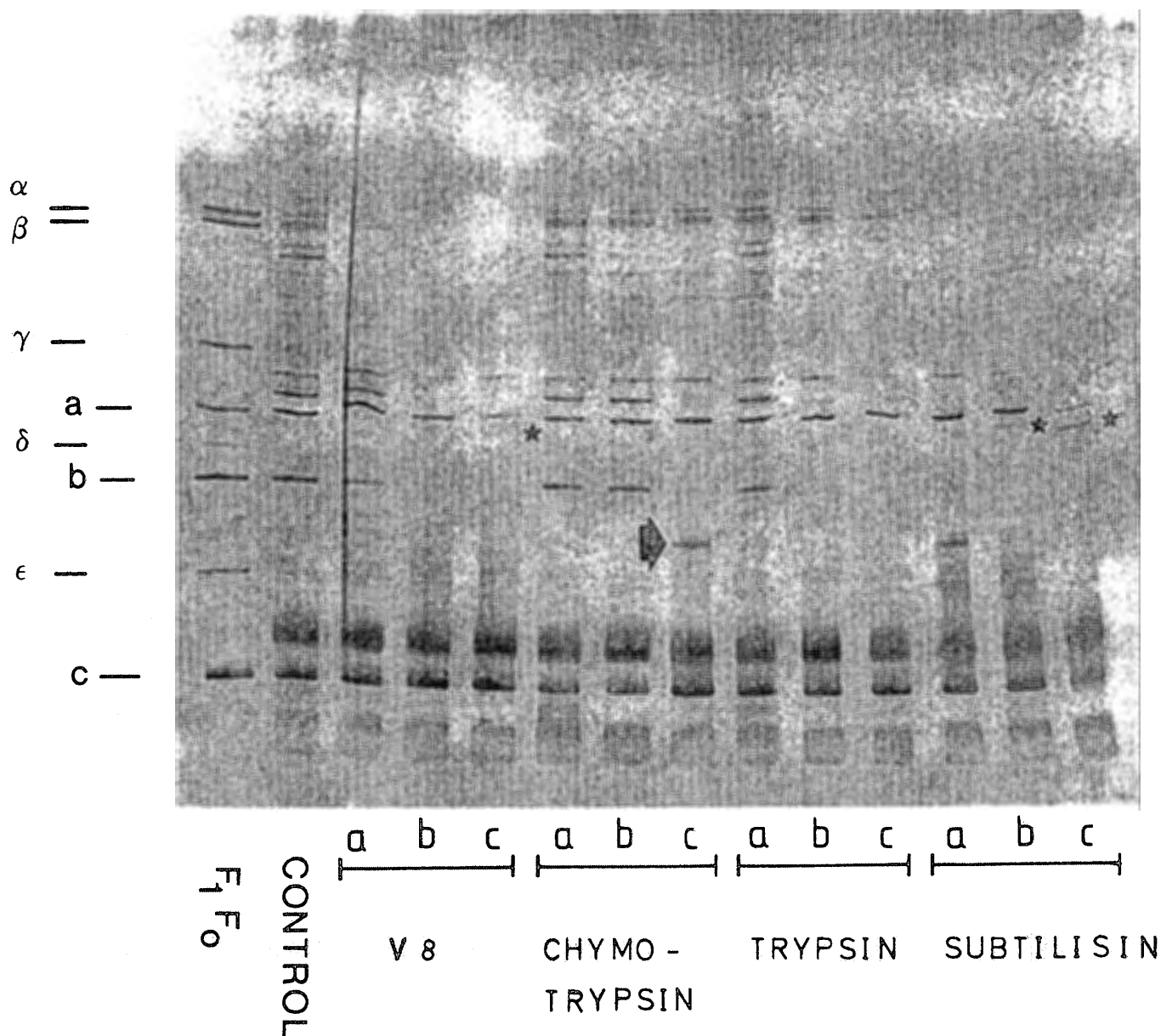
Fig. 1. Protease digest of F_1-depleted membranes. Protease digestion of F_1-depleted membranes from strain CM2786 was performed as described in Materials and methods. Incubation time was 1 h at 37°C at a ratio of protease to membrane protein of (a) 1/1000, (b) 1/100, (c) 1/20. For the membranes, 2 μg of protein were applied per lane, for F_1F_0 0.3 μg. Proteins were stained by the silver stain method. Arrows indicate chymotrypsin fragment of subunit *b*. (*) indicate possible cleavage products of subunit *a*.

appeared to be firmly bound to the membrane since treatment with urea (8 M), guanidine hydrochloride (6 M), KCl (4 M) or KSCN (2 M) failed to remove it from the membrane. Sequence analysis of the chymotryptic 15-kd fragment revealed an intact N terminus: fMet-Asn-Leu-Asn-Thr- (Nielsen *et al.*, 1981). Thus, the cleavage must have occurred ~20–25 residues away from the C terminus. Since the primary chymotrypsin cleavage sites such as Phe, Tyr, Trp or Met are all located within the 26 amino acid residues of the N terminus the cleavage by chymotrypsin must have occurred at an atypical secondary site.

These results suggested that the N terminus of the *b* subunit was not accessible to proteases it probably being embedded in the lipid bilayer of the cytoplasmic membrane, as also suggested by results obtained with hydrophobic photolabels

(Hoppe *et al.*, 1982).

Subunit a. Chymotrypsin and trypsin appeared not to cleave the *a* subunit while V8 and subtilisin at high concentrations resulted in an ~50–75% reduction in the amount of subunit *a* (Figure 1) with the concomitant appearance of a fragment with an apparent mol. wt. of 22 kd which is marked by an asterisk in Figure 1. Subtilisin at 1/100 for 1 h led to an almost complete disappearance of all other membrane proteins with mol. wts. >15 kd except the *a* subunit (Figure 1). Only at the higher concentration of subtilisin was *a* subunit reduced and a considerable amount of a '22' kd band appeared, which is thus likely to be a fragment of subunit *a*. These results show that the trypsin and chymotrypsin cleavage sites in the *a*-polypeptide are inaccessible; only a minor portion of the molecule (~15–20%) is accessible to the non-
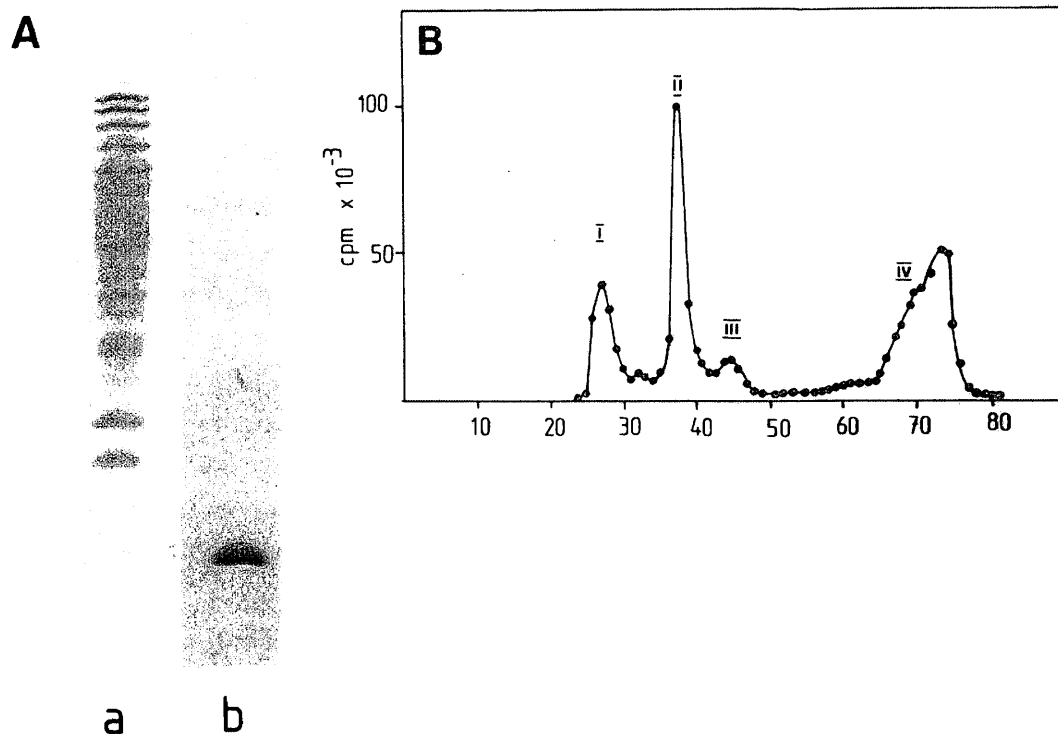
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Fig. 2. P30 chromatography of [¹⁴C]DCCD-labelled F₁-depleted membranes after digestion with subtilisin. F₁-depleted membranes from strain A 1 were labelled with [¹⁴C]DCCD as described in Materials and methods. (A) Polyacrylamide gel electrophoresis (Hoppe and Sebald, 1980) of 30 μg labelled membranes. (a) Coomassie stain, (b) fluorography (Bonner and Laskey, 1974). (B) Labelled membranes were incubated with subtilisin at a ratio of 1/10 for 5 days at 30°C. The sample was lyophilized, dissolved in 0.8 ml 80% formic acid and separated on a P30 column (0.8 x 150 cm) in 80% formic acid. Fractions of 0.5 ml were collected and analyzed for ¹⁴C-radioactivity.

specific subtilisin. Which end of the molecule is attacked has not been determined. On the basis of the amino acid sequence, however, one could argue that it was the least hydrophobic end, i.e., the N terminus (Nielsen *et al.*, 1981). It should in this context be noted that the *a* subunit was reported to correspond to the coding sequence *a'* by Nielsen *et al.* (1981). This conclusion is based on recent determination of the N-terminal amino acid sequence of the *a* subunit (J. Hoppe, unpublished results) and isolation and characterization of mutations in the beginning of the *a'*-coding sequence (J. Nielsen and K. von Meyenburg, unpublished results).

Subunit c. This subunit was not, or only slightly affected by any of the proteases (Figure 1). The resistance of subunit *c* to the action of proteases was studied in more detail taking advantage of its specific labelling by [¹⁴C]DCCD when F₁-depleted membranes were used (Figure 2A). During P30 chromatography of untreated membranes only 25% of the protein-bound radioactivity appeared in the void volume; ~75% was associated with a peak corresponding to the mol. wt. of subunit *c* (8300). This method thus gave quantitative data on the extent of degradation and should furthermore reveal low mol. wt. fragments which contain the [¹⁴C]DCCD. Figure 2B shows the P30 chromatogram of membranes treated with subtilisin (a non-specific endoprotease) at a ratio of 1/10 for 5 days at 30°C. In comparison with the control experiment ~65% of the radioactivity in peak II was recovered. Only minor amounts of radioactivity were noticed in the lower mol. wt. range of 8000 – 1000 (peak III) where cleavage products were expected. Instead a peak (IV) emerged very close to the position of free [¹⁴C]DCCD. This position corresponds to one or two amino acids (mol. wt. 400). Ap-

parently, no defined fragments were generated and the appearance of peak IV was due to a complete digestion of the protein, which might be the result of a general disruption of a small fraction of the membrane. Similar results were obtained using various other proteases up to a ratio of 1/5 (w/w) (trypsin, chymotrypsin, proteinase K) at various incubation times (16 h to 6 days). These results again demonstrate the resistance of protein *c* to proteolytic attack.

Protection of F₀ subunits by F₁ and F₁ proteolysis

The rate of chymotryptic degradation was reduced to some extent when F₁ was rebound to the F₀ sector prior to the addition of the protease, indicating a protective effect of the F₁ sector (Figure 3).

The protection by complete F₁ or its subunits also occurs *in vivo*, as shown by the analysis of mutant strains with an incomplete F₁ on the membrane (Figure 4). In strain NR70 the content of the F₁ subunit α was greatly reduced, while the amount of β was ~50% of that in wild-type membranes. Membranes from strain AS12/25 contained only very small amounts of subunit β. No subunit α was detectable. This strain is a heat-induced isolate of strain AS12 which contains a Mu phage integrated in the *atp*A gene coding for subunit α. Subunits γ, δ, ε are missing on the membranes from both strains, while subunit *c* is present in normal amounts (data not shown). Both strains contain a functional F₀ (Rosen, 1973; Schairer *et al.*, 1976).

Two observations suggest a protective effect of F₁, or its subunits, on the F₀ subunit *b*. First, under comparable growth conditions, the amount of subunit *b* on the membrane decreased with a decrease of the concentrations of subunits α and β. Second, the protective effect was also evident from a
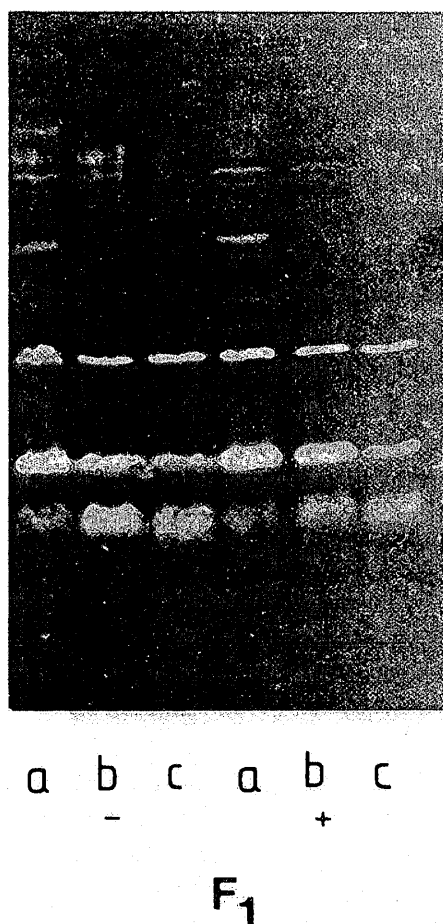
a b c a b c

－ ＋

F_1

Fig. 3. F_1 protects the subunit *b* against chymotryptic digestion. F_1-depleted membranes from strain A 1 were digested with chymotrypsin as described in Materials and methods. Membranes were incubated for 1 h at 37°C at a ratio of protease to membrane protein of (b) 1/100, (c) 1/10, (a) control without protease. F_1 was added to the membranes prior to the digestion at 10 U/mg membrane protein in a small volume. Non-bound F_1 and membranes were separated by centrifugation. 20 μg of membrane protein were subjected to gel electrophoresis (0.7 cm thick, 13.5% acrylamide). Proteins were transferred onto nitrocellulose. Subunit *b* and its cleavage products were visualized with *b*-specific antibodies subsequented with FITC-conjugated goat anti-rabbit antibodies (see Materials and methods).

comparison of the amount of subunit *b* in membranes from strains harvested at log phase with that from strains in the stationary phase.

The effect of endogenous proteolysis is expected to be more pronounced in resting cells (stationary phase). The results clearly showed that subunit *b* was not degraded by endogeneous proteases in wild-type cells. In contrast, the content of subunit *b* is reduced by ~50% in aged cells of the mutant strains NR 70, and AS12/25 compared with the amount in logarithmically growing cells.

Differential effect of proteolysis of subunit b on function

Untreated wild-type membranes exhibited the following enzymic activities: 100−150 U_{f_1}/mg for the electro-impelled H^+ conduction, and ~70 U_{f_1}/mg for the ATP driven H^+ conduction. These values agree with previously published data (Friedl *et al.*, 1980). Since protease treatment released roughly one third of membrane-bound protein, the results were therefore expressed on the basis of volume activities. Conditions were chosen where subunit *b* is 50−80% degraded without affecting subunit *a*. The protease treatment had
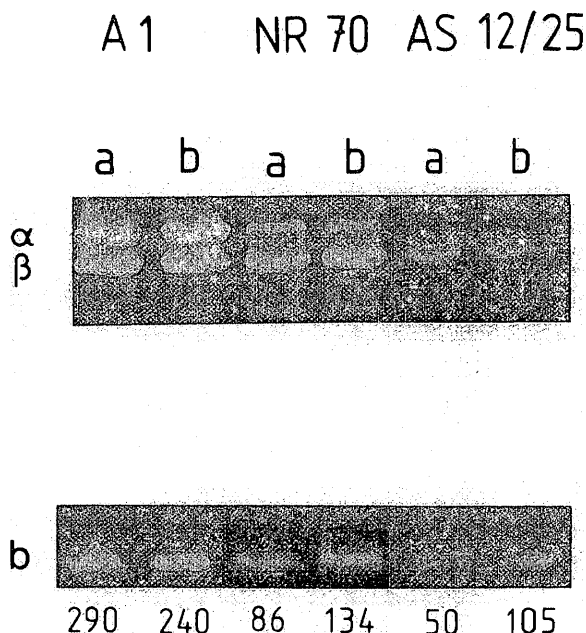
A 1　　NR 70　AS 12/25

a　　b　　a　　b　　a　　b

α
β

b

290　240　86　134　50　105

Fig. 4. Protection of subunit *b* by F_1 subunits *in vivo*. Cells from strains A 1, NR 70 and AS 12/25 were harvested at stationary phase (a) and mid-log phase (b). Membranes were prepared by disrupting the cells in the presence of *p*-aminobenzamidine, to recover maximum amounts of F_1 on the membranes. 20 μg of membrane protein were applied per lane. Subunits were visualized by immunostaining (c f., Figure 3). Negatives were scanned by a Shimadzu thin layer scanner. The values indicate peak areas (arbitrary units). Equal amounts of subunit *c* were found in all strains tested.
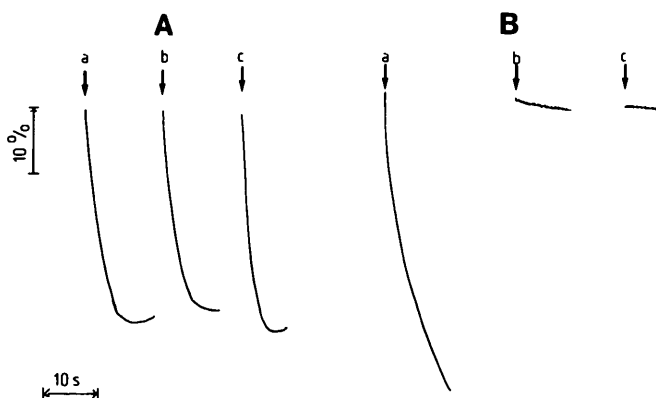
A　　　　　　　　**B**

a　　b　　c　　　　a　　b　　c

10 s

Fig. 5. Electro-impelled H^+ conduction and ATP-driven H^+ translocation in protease-treated membrane vesicles. Strain A 1 was used. About 40 μg of protein were used for both measurements. (a) Control, (b) F_1-depleted membranes treated with subtilisin (1/100) for 1 h at 37°C, (c) F_1-depleted membranes treated with chymotrypsin (1/100) for 1 h at 37°C.

(A) Electro-impelled H^+ conduction in membrane vesicles. The reaction was started by the addition of 2 μM valinomcyin. (B) F_1 was added to the membranes at 10 U/mg membrane protein in a small volume and incubated for 15 min at room temperature. The reaction was started by the addition of 2 mM ATP. The arrows indicate the addition of valinomcyin or ATP, respectively.

no effect on the electro-impelled H^+ conductance. This is shown in Figure 5A for subtilisin and chymotrypsin treated membranes (identical results for other proteases are not shown). In all cases, the electro-impelled H^+ conductance was completely inhibited by DCCD.

The F_1-binding activity of protease-treated membranes was largely retained, as more than 0.6 U/mg could be rebound

(untreated membranes rebound 1.0 U/mg). However, the ATPase activity of the rebound F_1 was insensitive to DCCD. Under conditions which resulted in a 75% inhibition of the ATPase activity in untreated membranes (40 nmol DCCD/mg protein, 0°C, 16 h), there was <5% inhibition of the ATPase activity in protease-treated membranes.

The ATP-dependent H^+ conduction was not found to be reconstituted correspondingly, although F_1 rebound to the protease-treated membranes in an amount equivalent to that which bound to untreated membranes.

Discussion

There are marked differences in the sensitivity of the individual F_0 subunits towards proteases. Subunit *b* is highly sensitive to all proteases tested, whereas subunit *c* is almost completely resistant. Subunit *a* is moderately susceptible to protease V8 and subtilisin.

Since subunit *c* appears not to be accessible to proteases we conclude that it is embedded in the membrane. Its general physical characteristics, such as its solubility in organic solvents and its low content of polar amino acids are in accordance with this assumption. It should, however, be noted that in the middle of its amino acid sequence there is a hydrophilic segment, which due to its high polarity is hardly in contact with the lipid bilayer (Sebald and Hoppe, 1981). This segment, which includes a proline residue, most probably forms a β-turn. Such conformations are often resistant to proteolytic cleavage. Indeed, subtilisin does not digest this hydrophilic segment of subunit *c* purified by chloroform/methanol extraction (J. Hoppe, unpublished data). The resistance of subunit *c* to proteases, therefore, does not necessarily mean that the subunit is completely embedded in the lipid bilayer.

The amino acid sequence of subunit *a* is typical for an integral membrane protein. There are several long stretches of hydrophobic amino acids and short segments containing hydrophilic amino acid residues. A long stretch of hydrophilic residues is found only at the N terminus from residue 1 to 35 in the coding sequence *a'* (Nielsen *et al.*, 1981). The polypeptide chain probably traverses the membrane several times, with the major protein mass being in the lipid bilayer. The relatively high resistance of subunit *a* to proteases also suggests that it is embedded in the membrane.

There is a hydrophobic segment of 32 amino acid residues at the N terminus of subunit *b*. The rest of the molecule is extremely hydrophilic (Nielsen *et al.*, 1981). This suggests that subunit *b* is bound to the membrane *via* its hydrophobic N terminus. Chymotrypsin did not attack any of the potential cleavage sites in the N-terminal segment but cleaved off a small fragment from the C-terminal end. We conclude that the N terminus is embedded in the membrane. On the other hand, the high susceptibility of subunit *b* to other proteases indicated that the major hydrophilic part of the protein in F_1-depleted membranes extends into the water phase.

The stoichiometry of the subunits of the F_0 sector was found to be *a:b:c* = 1:2:10 (Foster and Fillingame, 1982) to 1:2:12 − 15 (von Meyenburg *et al.*, 1982). Thus the *b* subunits may be arranged in one of the following three possible ways: (a) the hydrophilic ends of the two molecules may both extend into the cytoplasm or (b) they may extend into the periplasm or (c) the subunits may have opposite orientations. The protease digestion experiments showed that subunit *b* was efficiently attacked by all proteases used suggesting that

the hydrophilic domains of both molecule are orientated towards the cytoplasm. The hydrophilic domains, representing a protein mass of ~30 000, are most likley in contact with F_1 subunits since (a) absence or reduced levels of F_1 subunits *in vivo* resulted in loss of *b*-subunit and (b) addition of F_1 to F_1-depleted membranes resulted in a modest degree of protection from proteolysis *in vitro* (Figure 3).

The digestion of subunit *b* had no effect on the electro-impelled H^+ conduction. Similar results have been reported by Sone *et al.* (1978) for an F_0 preparation from the thermophilic bacterium PS-3. In their experiments they could digest a protein with a mol. wt. of 13 000 without affecting the electro-impelled H^+ conduction. This protein is probably homologous to subunit *b* in *E. coli*. In the case of the F_0 from the thermophilic bacterium PS-3, F_1 binding was lost after protease treatment. This result is in contrast to our data which showed that the F_1 binding capacity was largely retained after protease digestion. The ATPase activity of the rebound F_1, however, was no longer sensitive to DCCD. These results parallel the finding that ATP-dependent protein translocation could not be reconstituted after binding of F_1 to protease-treated membranes. Thus, ATPase activity was apparently uncoupled from energy transduction. These results differ from the observations of Pedersen *et al.* (1981), who found that after tryptic treatment of F_1-depleted membranes ATP-dependent H^+ translocation could not be reconstituted but the ATPase activity of rebound F_1 was still sensitive to DCCD. They postulated the existence of 'interphase' components which were necessary for the coupling of catalytic events on F_1 to energy transduction. Our experimental results indicate that subunit *b* is such an 'interphase' component.

Materials and methods

Strains of E. coli

A 1 (K12 $Y_{mel}(\lambda)$, F^- *lacl*, *fadR*, *but12*, *rha* NR 70 is a gift from Dr. B. Rosen (1973). AS 12/25 (*ilv*, *met*) was derived from strain AS 12 (*atpA623* :: Mu) (Friedl *et al.*, 1981, 1982) by curing of Mu phage (Schairer *et al.*, 1976).

Genotype of strain CM2786 (CM1470(pBJC706)): CM1470 *E. coli* K12 F^+ *asnB32 thi-1 relA1 spoT1 atp-706* (del *atpIBEFHA*) (Hansen *et al.*, 1981). PBJC706 *atp(BEFHAGDC)$^+$* on a dimer of pBR322. Expression of the ATP synthase genes on the plasmid is mainly due to transcription from a promoter in pBR322, resulting in ATP synthase levels 4- to 5-fold higher than in a wild-type strain (von Meyenburg *et al.*, 1982 and unpublished results).

Chemicals

Trypsin (3-fold crystallized) and subtilisin (bnb novo) were from Serva (Heidelberg), chymotrypsin A_4 and proteinase K from Boehringer (Mannheim) and *Staphylococcus aureus* protease V8 from Miles. Nitrocellulose sheet bond were from Schleicher & Schüll; fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was from Paesel (Mannheim).

Growth and media

Cells were grown on Vogel-Bonner minimal medium as described (Vogel and Bonner, 1956). Carbon source for strain A 1 was 1% succinate, or 1% glucose, for all other strains 1% glucose. The concentration of isoleucine, valine and methionine, was 0.1 mg/ml for strain AS 12/25; ampicillin was added to 0.1 mg/ml for strain CM 2786.

Preparation of membranes

The preparation of membranes (Friedl *et al.*, 1979), of F_1-depleted membranes (Friedl *et al.*, 1980) or F_1 ATPase (Vogel and Steinhart, 1976) and of antibodies against individual subunits (Friedl *et al.*, 1981) were as previously described. In some preparations of F_1-depleted membranes residual ATPase activity was removed by inclusion of 1 M KSCN in the final washing buffer. KSCN was then removed by two washes with low ionic strength buffer.

Assays

Assays of ATPase activity, ATP-driven proton translocation and electro-impelled proton conduction were performed as described previously (Friedl *et al.*, 1979, 1980).

Protease digestion

This was carried out at 37°C at various ratios of membrane to protease and various incubation times in 100 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.8 at 5 mg membrane protein per ml. The reaction was stopped by a 15 min incubation with 1 mM phenylmethyl sulphonylfluoride (PMSF) at room temperature and immediate centrifugation at 300 000 g for 45 min. Membranes were washed twice with 1 mM Tris-HCl, 0.5 mM EDTA, 10% glycerol to remove residual protease and PMSF.

F_1 binding

F_1 (40 U/ml) was added to the membranes (2.5 mg protein/ml) in 50 mM N-morpholino propane sulfonic acid, 175 mM KCl, 5 mM $MgCl_2$, 0.2 mM dithioerythritol, 0.2 mM EGTA, pH 7.0 at a ratio of 10 units per mg membrane protein. Membranes were washed once with the above buffer containing 5 mM p-aminobenzamidine and were taken up in the same buffer.

Polyacrylamide gel electrophoresis

A modified procedure of Laemmli (1970) was used. The separation gel contained 15% glycerol. The ratio of acrylamide to N',N'-methylenebisacrylamide was 30/0.8 (w/w). Thin gels (160 x 140 x 0.3 mm) were prepared and electrophoresis was performed at 800 V for 1 h with cooling of one plate by circulating water. Protein bands were then visualized by a silver stain (Ansorge, 1982). The detection limit of this method is ~1 ng per protein band.

Immunostaining

Protein blotting was made onto nitrocellulose (Towbin *et al.*, 1979) using gels of 0.7 mm thickness. The nitrocellulose was saturated with 10% horse serum, 2.5% bovine serum albumin (BSA, Sigma, fraction V) in phosphate-buffered saline (PBS) for 2 h. The nitrocellulose sheet was incubated for at least 12 h with antibodies directed against the individual subunit (1:200 dilution in PBS/horse serum/BSA). After briefly washing with PBS, the bound rabbit antibodies were visualized under u.v. light after reaction with FITC-conjugated goat anti-rabbit antibodies (1/100 in 10% horse serum 2.5% BSA in PBS for at least 5 h).

Isolation of peptides and sequencing

The chymotryptic fragment of *b* was isolated by preparative gel electrophoresis in the above system using 13.5% acrylamide gel. 10 mg of chymotrypsin-treated membranes from stain CM 2786 were separated on a gel 0.3 cm x 12 cm, 20 cm long. Protein bands were visualized with the Coomassie stain; the fragment of subunit *b* was eluted from the proper gel slice by a 16 h incubation with 10 ml 50 mM sodium phosphate buffer, pH 7.5 containing 0.2% SDS. The pH was readjusted with 1 M NaOH when necessary. The eluate was lyophilized, resolved in a small volume and dialysed against water. The fragment was coupled to porous glass beads as described (Nielsen *et al.*, 1981). Sequencing and identification of the phenylthiohydantoin amino acids was performed as described (Hoppe and Sebald, 1980; Hoppe *et al.*, 1980; Lottspeich, 1980).

[^{14}C]DCCD labelling

F_1-depleted membranes were incubated with 1 µg/ml [^{14}C]DCCD (55 mCi/mM) (Hoppe and Sebald, 1980) at 0°C for 16 h. Unbound [^{14}C]DCCD was removed by washing the membranes with 10% BSA in 50 mM Tris-HCl, pH 7.5. After six washes the specific radioactivity remained constant.

Chromatography

Chromatography on P30 was performed as described (Hoppe and Sebald, 1980; Hoppe *et al.*, 1980).

Acknolwedgements

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