

# STRUCTURE AND GENETICS OF THE H<sup>+</sup>-CONDUCTING F<sub>0</sub> PORTION OF THE ATP SYNTHASE

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## INTRODUCTION

The ATP synthase occurs in remarkably conserved form in procaryotic and eucaryotic cells. Thus, our present knowledge of ATP synthase is derived from studies of the enzyme from different organisms, each offering specific experimental possibilities. In recent times, research on the H<sup>+</sup>-conducting F<sub>0</sub> part of the ATP synthase has been greatly stimulated by two developments in the *Escherichia coli* system. Firstly, the purification and reconstitution of the whole ATP synthase as well as the proton conductor F<sub>0</sub> from *E. coli* have been achieved. These functionally active preparations are well defined in terms of subunit composition, similar to the thermophilic enzyme from PS-3 studied by Kagawa's group.<sup>1,2</sup> Secondly, the genetics and the molecular cloning of the genes of all the F<sub>0</sub> subunits from *E. coli* yielded information on the function of subunit polypeptides and essential amino acid residues. Furthermore, the amino acid sequence of hydrophobic F<sub>0</sub> subunits, which are difficult to analyze by protein-chemical techniques, could be derived from the nucleotide sequence of the genes.

These achievements, which shall be briefly summarized in the next part of this communication, provide the framework to study specific aspects of the structure and function of the F<sub>0</sub> subunits.

## SUBUNITS, GENES, AND FUNCTIONAL PROPERTIES OF ISOLATED F<sub>0</sub>

Due to the work of Foster and Fillingame and Friedl *et al.*,<sup>3,4</sup> a highly purified preparation of *E. coli* ATP synthase can now be prepared in large quantities. These preparations are at least 90% pure and show, after electrophoretic separation on dodecylsulfate gels (FIGURE 1), the eight polypeptide bands of F<sub>1</sub>-ATPase ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ ) and the proton-conducting F<sub>0</sub> ( $a$ ,  $b$ , and  $c$ ). The eight polypeptides are coded for by the eight genes that form the *atp* operon. The *atp* operon has been well defined genetically, especially by Downie, *et al.*,<sup>5</sup> and its nucleotide sequence has been determined. Thus, the complex subunit composition of isolated F<sub>1</sub>F<sub>0</sub> is proven.

The isolated ATP synthase exhibits an ATPase activity of about 20 units per mg protein, corresponding to turnover numbers of about 150/second. The ATPase activity is inhibited to 90% by dicyclohexylcarbodiimide (DCCD) under conditions where this hydrophobic carbodiimide binds specifically to the proteolipid subunit (subunit  $c$ ) of F<sub>0</sub>. After reconstitution with artificial phospholipid vesicles, ATP-dependent H<sup>+</sup> translocation as well as ATP-inorganic phosphate (P<sub>i</sub>) exchange can be measured.

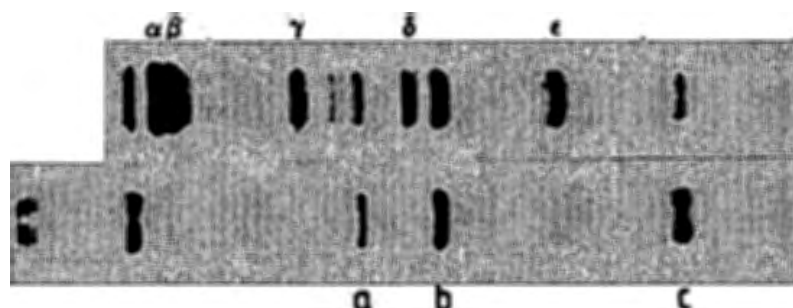
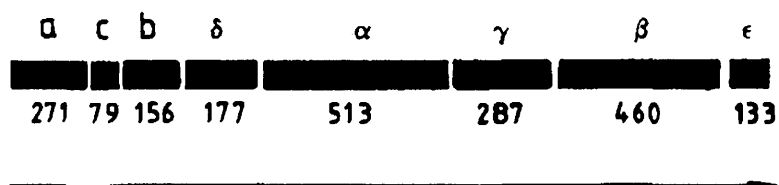
SubunitsGenes ( atp operon )

FIGURE 1. Subunits and genes of the ATP synthase from *E. coli*. The isolation of ATP synthase ( $F_1F_0$ ) and proton-conducting  $F_0$  is described by Friedl *et al.* and Friedl and Schairer.<sup>4,7</sup> The subunits were electrophoretically separated on 13.5% acrylamide gels and stained with Coomassie Blue. The order of the genes in the *atp* operon and the correlation of genes with ATP synthase subunits were established genetically<sup>5,45,46</sup> and by nucleotide-sequence analysis.<sup>11-13</sup> The numbers below the genes indicate the number of amino acid residues in the subunit polypeptides.

From whole isolated ATP synthase, the  $F_0$  part has been isolated by different procedures.<sup>8-8</sup> After reinsertion into artificial membranes,  $H^+$  conductance can be measured in response to a valinomycin-induced potassium diffusion potential (TABLE 1). At about 100 mV and a pH of 7.5, turnover rates of about 1/second are observed.<sup>7</sup> The  $H^+$  conductance is inhibited strongly by DCCD and by rebound  $F_1$ . Reconstituted  $F_0$  rebinds  $F_1$ -ATPase, and thereby ATP-dependent  $H^+$  translocation is restored. Thus, both  $H^+$  conductance and functional rebinding of  $F_1$  can be used to analyze  $F_0$ .

THE NUCLEOTIDE SEQUENCE OF THE *atp* OPERON

Initially, the *atp* operon comprising the genes for all ATP synthase subunits was cloned during studies on the origin of replication, *ori C*, of the *E. coli*

chromosome by Miki *et al.* and by von Meyenburg's group.<sup>9,10</sup> *Ori C* is located in the vicinity of the *atp* operon.

The nucleotide sequence of the genes of all three  $F_0$  subunits of *E. coli* was determined independently by three groups in 1981.<sup>11-13</sup>

In cooperation with von Meyenburg's group, J. Hoppe and P. Friedl isolated the  $F_0$  subunits and established the N-terminal amino acid sequences in order to prove the origins of the individual subunit genes.<sup>13</sup>

In eucaryotic cells, some of the  $F_0$  subunits are encoded on organellar DNA. The chloroplast DNA contains genes for  $F_0$  subunits *a* and *c*.<sup>14,15</sup> The mitochondrial DNA of yeast codes for subunits *a* (subunit 6) and *c* (subunit 9). They were sequenced by Macino and Tzagoloff in 1979 and 1980.<sup>16,17</sup> Mammalian<sup>18</sup> and fungal mitochondrial DNAs probably encode only subunit *a* (subunit 6). In passing it may be mentioned that in the case of procaryotic as well as organellar-

TABLE 1  
FUNCTIONAL PROPERTIES OF  $F_0$  ISOLATED FROM *Escherichia coli*

| Reconstituted Protein*    | H <sup>+</sup> Conduction†<br>(influx) |   | ATP-Dependent H <sup>+</sup> Translocation‡ |
|---------------------------|--|---|---|
|                           | pH Electrode<br>(nmol/minute per mg)   | Fluorescence Test<br>(U <sub>f</sub> /mg) |   |
| $F_0$                     | 870                                    | 2565                                      | 1   |
| $F_0$ plus DCCD           | 2                                      | 24  | —   |
| $F_0$ plus $F_1$          | 41                                     | 151                                       | 1910  |
| $F_1F_0$                  | 5                                      | 16  | 2330  |
| $F_1$ -depleted membranes | 44                                     | 65  | —   |
| Whole membranes           | —                                      | —   | 130   |

\* $F_0$ ,<sup>7</sup>  $F_1$ ,<sup>49</sup>  $F_1F_0$ ,<sup>4</sup> and  $F_1$ -depleted membranes<sup>48</sup> were prepared as previously described.

† $F_0$  and phospholipids were reconstituted to proteoliposomes by the dialysis method.<sup>50</sup> The vesicles were loaded with  $K_2SO_4$ . Addition of valinomycin catalyzed an electrogenic  $K^+$  efflux, which caused an equivalent  $H^+$  influx assayed as quenching of acridine dye fluorescence or by a pH electrode.

‡The fluorescence test of ATP-dependent  $H^+$  translocation was performed as previously described.<sup>50</sup>

coded  $F_0$  subunits, the primary translation products are not processed. In contrast, the nuclear-coded subunit *c* (proteolipid) from *Neurospora crassa*, which is imported into mitochondria, is initially synthesized with a 66-residue presequence of exceedingly polar and basic character. This has been recently demonstrated by molecular cloning and sequencing of the mRNA.<sup>19</sup> The organization of the genes and the biosynthesis of the  $F_0$  subunits raise several intriguing questions, e.g., how the different molar ratios of the subunits are produced from the most likely polycistronic mRNA in *E. coli*, and how in eucaryotic cells the expression of organelle genes, which are present in hundreds of copies, is coordinated with the expression of single nuclear genes. These problems will not be further discussed here. Rather, the established primary structures shall be evaluated in the following for the discussion of structural and functional questions.

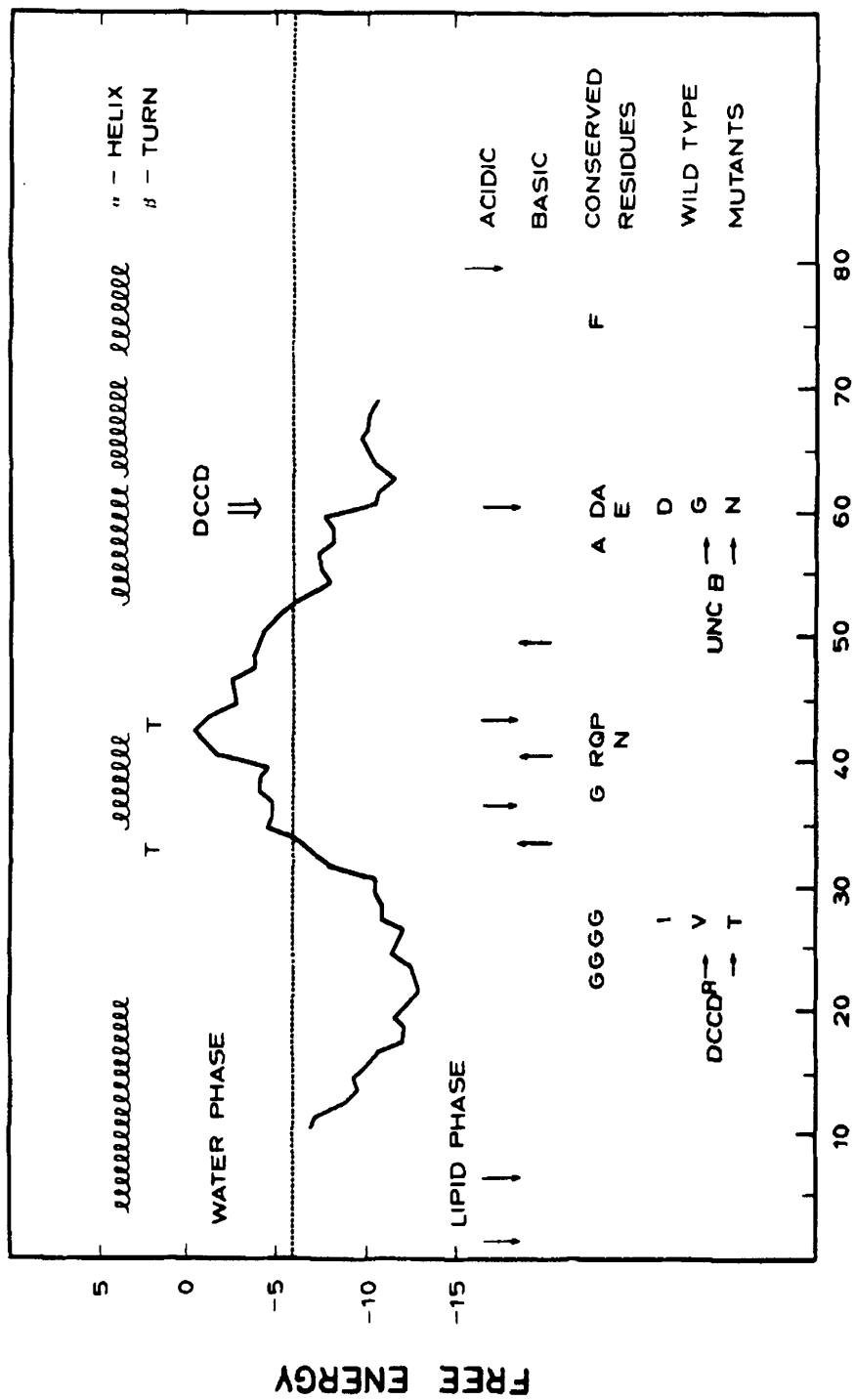
## SUBUNIT c

The amino acid sequence of subunit c (proteolipid, DCCD-binding protein, subunit 9) has been determined for nine different organisms.<sup>20</sup> The proteolipids from bacteria, mitochondria, and chloroplasts exhibit homologies similar to those observed with rRNA, cytochrome c, and ferredoxin from the corresponding organisms. The homologies occur over most of the 76 to 81 residues of the polypeptide chain. All proteolipids can be aligned without postulating any insertions or deletions.

FIGURE 2 summarizes our present knowledge of the conserved and typical properties of subunit c as well as data obtained by chemical and genetic modification of this subunit. The computer outprint in the upper part shows for the *E. coli* subunit c predictions for secondary structures and membrane-permeating segments.<sup>21-24</sup> For two segments of about 25 residues, a high tendency for helix formation in the lipid bilayer is predicted by the parameters given by von Heijne.<sup>25</sup> Different prediction methods for secondary structures yield a  $\beta$ -turn in the central polar loop of the proteolipid. These calculations are in accordance with a "hairpin"-like structure. Preliminary chemical modification data suggest that the C-terminal segment is pointing to the periplasmic side of the membrane.<sup>26</sup> A glycine-rich segment as well as a proline is strictly conserved in the proteolipid from all organisms. Invariant also are three polar positions, one positive (arg), one negative (glu or asp), and one neutral (asn or gln). The invariant acidic residue is selectively modified by the inhibitor DCCD.<sup>27,28</sup> Furthermore, two mutations in *E. coli* leading to a nonfunctional proton channel exhibit a substitution of this acidic residue by a glycine<sup>29</sup> or an asparagine.<sup>30</sup> This indicates that this acidic residue of the proteolipid is essential for a functional  $F_0$ . DCCD-resistant mutants of *E. coli* originate from substitutions of isoleucine 28 by either a threonine or valine.<sup>28</sup> In a "hairpin"-like conformation, this isoleucine is located in the vicinity of the carbodiimide-reactive aspartyl side chain. Oligomycin-resistant mutants from yeast and *Neurospora crassa* are affected at several amino acid positions in the C-terminal lipophilic segment in the vicinity of the DCCD-binding residue.<sup>20</sup> This suggests that at least part of this segment is oriented to the surface of  $F_0$  and is in contact with the core of the lipid bilayer. Preliminary data with membrane-permeating photoreactive labels (see below) suggest that several residues of this segment are accessible from the lipid phase (J. Hoppe, unpublished results).

## SUBUNIT a (SUBUNIT 6)

Subunit a, the largest  $F_0$  subunit, like the proteolipid is very hydrophobic. The sequence of the 271 amino acids of the *E. coli* subunit shows seven lipophilic sequences of about 20 to 25 residues (FIGURE 3), which possibly permeate the membrane. Homology between the *E. coli* subunit<sup>13</sup> and those from mitochondria of mammals<sup>18</sup> and of yeast<sup>17</sup> is restricted to a short, 40-residue sequence near the C-terminus. The rest of the polypeptide chain is completely unrelated. The conserved segment contains 4 invariant polar residues. An oligomycin-resistant mutant from yeast exhibits an amino acid substitution in this segment.<sup>17</sup> These data suggest that at least this part of subunit a plays an important role. It is unclear whether the structure of the rest of subunit a is of secondary importance, or whether the bacterial  $F_0$  and the mitochondrial  $F_0$  are constructed differently. The protein-chemical analysis of subunit a is difficult. Therefore, up to now the orientation of this polypeptide in the membrane is unknown, and the predictions for membrane-permeating segments are not yet confirmed experimentally.



### SEQUENCE POSITION

FIGURE 2. Prediction of secondary structures and membrane-permeating segments in  $F_0$  subunit c (proteolipid) of *E. coli*. Alpha-helical segments (coiled lines) and  $\beta$ -turns (T) were consistently calculated applying four different prediction methods.<sup>21-24</sup> The free-energy gains during a transition from a random coil in water to an  $\alpha$ -helix in the membranes were calculated for all amino acid sequence positions using the parameters given by von Heijne.<sup>25</sup> The location of the acidic (↓) and basic (↑) residues is indicated by arrows. Residues conserved in the amino acid sequences of the proteolipid from different organisms<sup>26</sup>—including bacterial, mitochondrial, and chloroplast membranes—are indicated in the amino acid one-letter code. Amino acid exchanges in the proteolipid from *DCCD*-resistant mutants<sup>28</sup> (*DCCD*<sup>R</sup>) and *unc B*-type mutants<sup>29,30</sup> (*unc B*) are shown in the lower part of the figure. Aspartyl residue 59 is covalently modified by the inhibitor *DCCD*.<sup>27,28</sup>



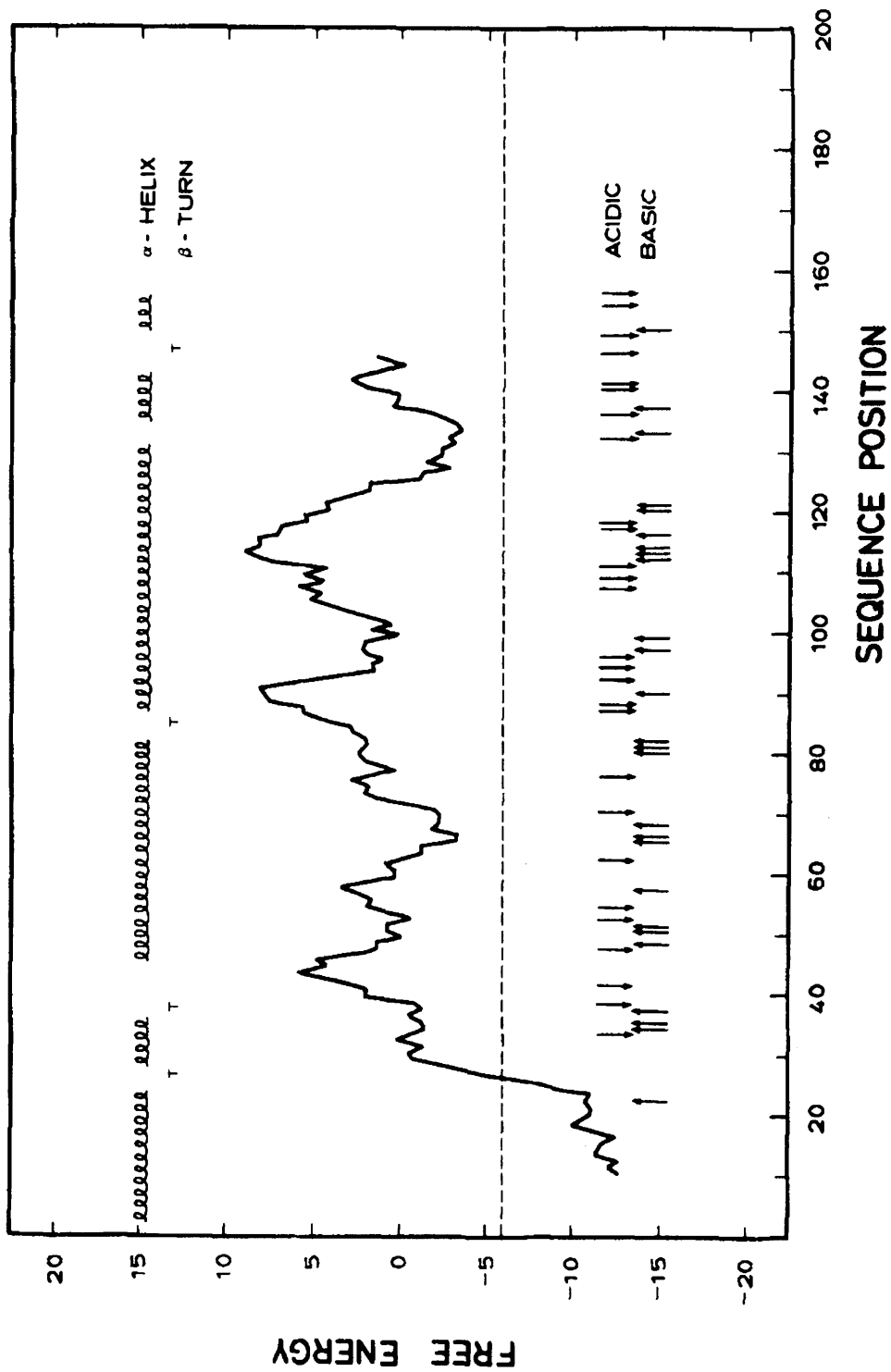


FIGURE 4. Prediction of membrane-permeating segment and secondary structures in the  $F_0$  subunit b from *E. coli*. Alpha-helical segments (coiled lines) and  $\beta$ -turns (T) were consistently predicted by four different prediction methods.<sup>21-24</sup> The free-energy gains for a transition from a random coil in water to an  $\alpha$ -helix in the membrane were calculated for all amino acid sequence positions using the parameters given by von Heijne.<sup>25</sup> The location of acidic ( $\downarrow$ ) and basic ( $\uparrow$ ) residues is indicated by arrows.

SUBUNIT *b*

*E. coli* subunit *b*, consisting of 156 amino acids, is for the most part hydrophilic (FIGURE 4). Only at the N-terminus does there occur a lipophilic segment of 22 residues. This "amphipathic" structure is reminiscent of cytochrome *b<sub>6</sub>*, for example, where a large polar functional domain is anchored in the membrane by a short lipophilic segment. In chloroplast  $F_0$ , a subunit with a molecular weight comparable to the *E. coli* subunit *b* exists.<sup>14</sup> A homologous subunit in the mitochondrial  $F_0$  has not yet been identified: oligomycin-sensitivity-conferring protein (OSCP), factor B, and  $F_6$  are possible candidates.<sup>31-33</sup>

## MEMBRANE-PERMEATING, PHOTOREACTIVE LABELS

The membrane-spanning segments of the  $F_0$  subunits, which are predicted from the amino acid sequences, have to be proven experimentally. J. Hoppe has used lipophilic, membrane-permeating, photoreactive labels to modify specifically amino acid residues located in a hydrophobic environment. Up to now, definite results at the level of individual modified residues have been obtained for the *b* subunit only.

The labels used were iodonaphthylazide (Gitler's reagent)<sup>34</sup> and trifluoromethyl-iodophenyl-diazirine (Brunner's reagent).<sup>35</sup> These reagents were reacted with isolated *E. coli* ATP synthase solubilized with aminoxide detergent. In parallel, membranes were used from an *E. coli* strain that overproduces the ATP synthase five- to sixfold due to the presence of the whole *atp* operon on a multicopy plasmid. The strain was obtained from K. von Meyenburg.

Illumination of the ATP synthase in the presence of iodonaphthylazide leads to the labeling of mainly the subunit *b* (FIGURE 5, lanes A and B). When the enzyme is dissociated with dodecylsulfate and then reacted with the photoactivated label, all subunits become labeled, but  $F_0$  subunits *a* and *c* are only marginally attacked. Amino acid sequence analysis revealed that in subunit *b*, a cysteinyl residue at position 21 was affected almost exclusively (data not shown). Apparently, the reactive nitrene is not aggressive enough to attack to a significant extent other lipophilic residues occurring in the membrane.

Illumination in the presence of the diazirine leads to the labeling of all three  $F_0$  subunits (FIGURE 5, lanes C and D), both in isolated ATP synthase and in the  $F_1$ -depleted membrane from the overproducing strain. Protein-chemical analysis of subunit *b* established that only the lipophilic N-terminal segment had been attacked. Amino acid sequence analysis of subunit *b* revealed a labeling of individual residues as shown in FIGURE 6. Cysteine-21 still had been modified predominantly, but in addition, aliphatic side chains had been attacked.

Labeled subunit *b* isolated from  $F_1$ -depleted membranes exhibited a quantitatively similar distribution of modified residues (data not shown). The observed labeling pattern is explained if the lipophilic segment of subunit *b* forms in the membrane an  $\alpha$ -helical structure that is accessible from two opposite sides.

## PROTEASE TREATMENT

In order to study the orientation of the  $F_0$  subunits as well as the accessibility from the water phase,  $F_1$ -depleted membranes (inside out) from the overproducing strain were treated with various proteases, such as trypsin, chymotrypsin, subtilisin, and staphylococcal V8 protease. The proteins were then separated by



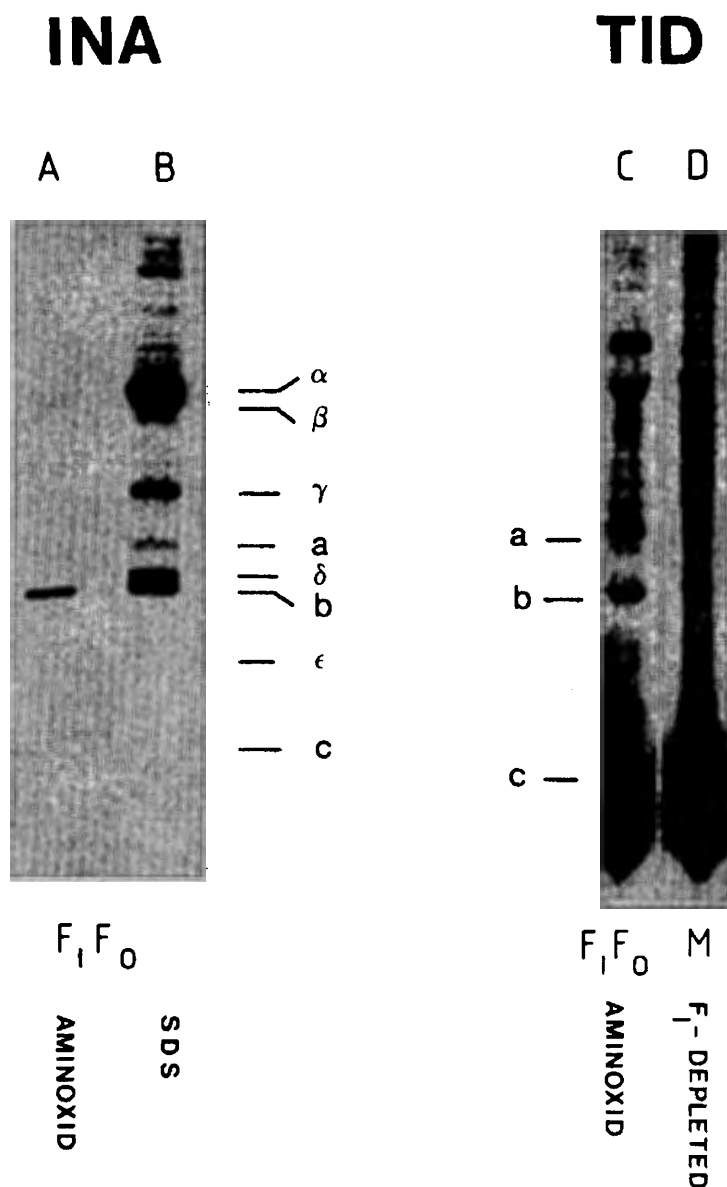


FIGURE 5. Labeling of isolated ATP synthase and  $F_1$ -depleted membranes with membrane-permeating photoreactive probes. (A) One milligram of isolated  $F_1F_0$  dissolved in 0.2 ml 25 mM Aminoxide WS 35 [50 mM tris-HCl, pH 7.5, 100 mM KCl, 2 mM  $MgCl_2$ , 0.2 mM dithiothreitol, 0.2 mM ethylene glycol-bis(2-aminoethyl ether)- $N,N'$ -tetraacetic acid (EGTA), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 20% methanol] was illuminated (340–380 nm, 250 watt xenon lamp) at room temperature for five minutes with 40  $\mu$ Ci of  $^{125}I$ -iodonaphthylazide<sup>34</sup> (INA, 5 Ci/mmol). (B) Isolated  $F_1F_0$  was reacted under similar conditions as in (A) with INA, but the Aminoxide was omitted and subunits had been dissociated in the presence of 2% dodecylsulfate. (C) One milligram of isolated  $F_1F_0$  dissolved in 0.2 ml Aminoxide buffer (see A) was reacted with 100  $\mu$ Ci of  $^{125}I$ -trifluoromethyl-iodophenyl-diazirine<sup>35</sup> (TID, 10 Ci/mmol). (D)  $F_1$ -depleted membranes (1 mg) suspended in 0.2 ml 1 mM tris-HCl, pH 7.8, 0.5 mM EDTA, 10% glycerol, were reacted with  $^{125}I$ -trifluoromethyl-iodophenyl-diazirine as the isolated  $F_1F_0$ . All proteins were submitted to dodecylsulfate gel electrophoresis. Gels were stained with Coomassie Blue, and radioactive proteins were visualized by autoradiography. In the  $F_1$ -depleted membranes, labeled subunit c is obscured by the large amount of labeled membrane lipids.

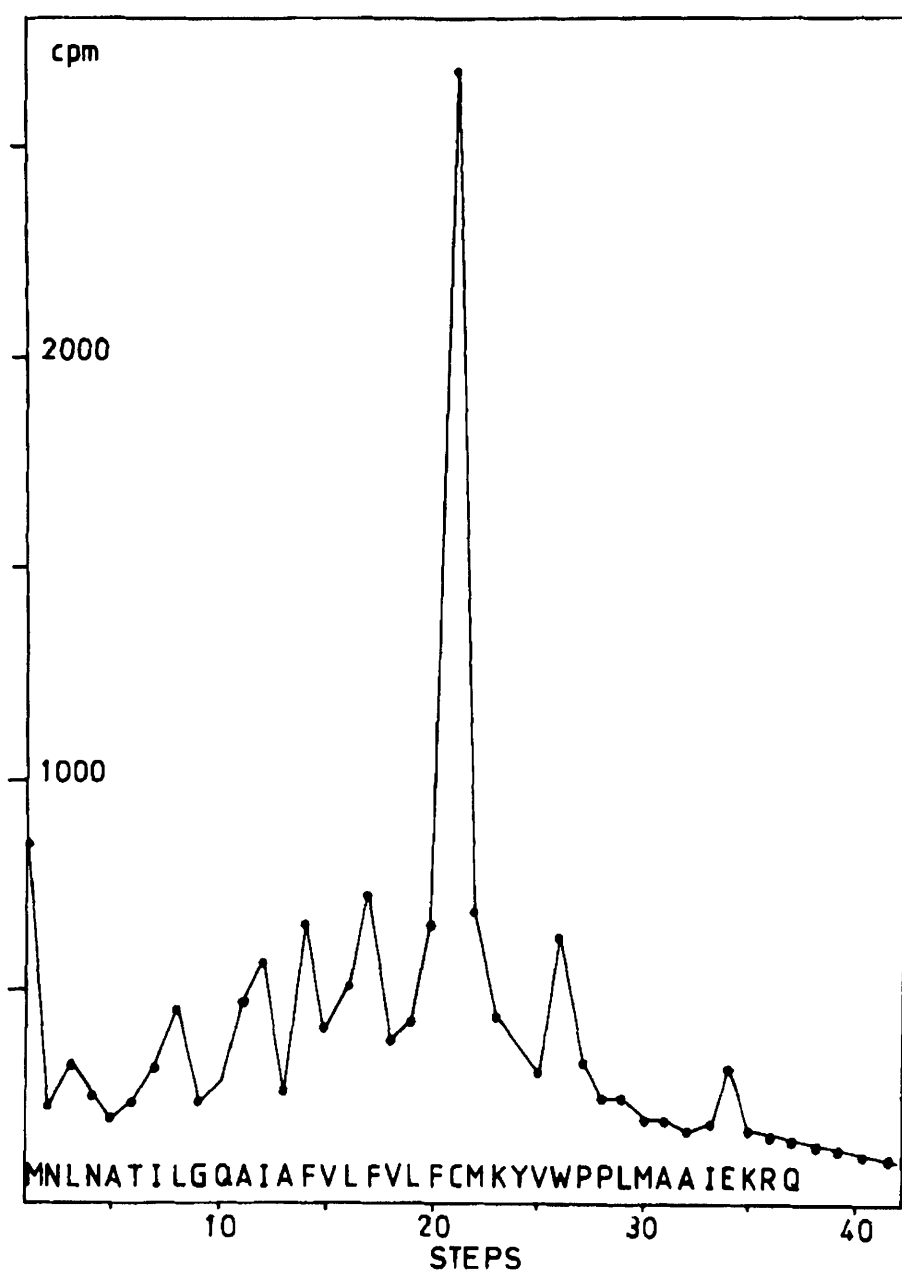


FIGURE 6. Labeling of individual amino acid residues in subunit *b* by membrane-permeating photoreactive  $^{125}\text{I}$ -trifluoromethyl-iodophenyl-diazirine. Subunit *b* of *E. coli* ATP synthase, labeled with TID as described in FIGURE 5, lane C, was isolated by high-performance liquid chromatography on a G 3000 SW column ( $0.8 \times 60$  cm) in 200 mM sodium phosphate, pH 7.0, 0.1% dodecylsulfate. The protein was coupled to thioisocyanato glass,<sup>13</sup> deacylated,<sup>28</sup> and submitted to 41 cycles of automated Edman degradation.<sup>28</sup> The amino acid derivatives cleaved during each sequencer step were analyzed for  $^{125}\text{I}$  radioactivity. The amino acid sequence determined in parallel is represented in the one-letter code.

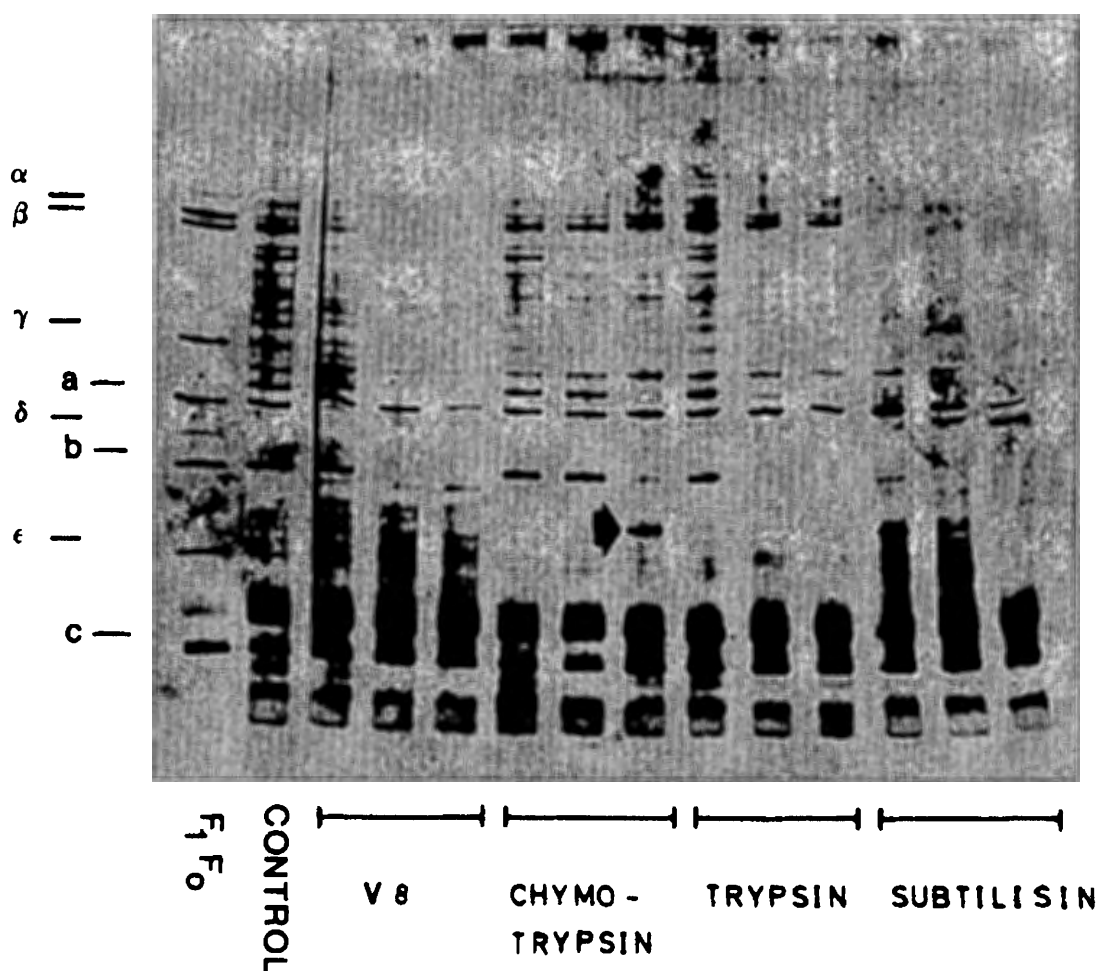


FIGURE 7. Proteolytic degradation of  $F_0$  subunit  $b$  in  $F_1$ -depleted membranes from *E. coli*.  $F_1$ -depleted membranes were prepared from an *E. coli* strain overproducing the ATP synthase five- to sixfold due to the presence of a pBR322 recombinant plasmid carrying the *atp* operon (obtained from K. von Meyenburg). Membranes (5 mg/ml) were incubated in buffer (100 mM tris-HCl, pH 7.8, 5 mM  $MgCl_2$ ) for 60 minutes at  $37^\circ C$  with proteases at ratios (w/w) of 1:1000, 1:100, and 1:10. After addition of protease inhibitor PMSF to 1 mM, membranes were washed twice with buffer and submitted to dodecylsulfate gel electrophoresis. The protein bands were visualized by the silver-staining procedure.<sup>36</sup> Lane A shows separated  $F_1F_0$ , lane B shows separated  $F_1$ -depleted membranes. The protease concentrations increase in the samples from the left to the right. The arrow indicates the digestion product obtained with chymotrypsin.

dodecylsulfate gel electrophoresis and visualized by a sensitive silver stain procedure<sup>36</sup> (FIGURE 7). Subunit  $c$  was only marginally degraded even at high protease concentrations and long incubation times. Subunit  $a$  was partially cleaved at high concentrations of V8 protease and subtilisin. In contrast, subunit  $b$  is degraded by all employed proteases at low concentrations. Remarkably, chymotrypsin produced a cleavage product that is still firmly bound to the membrane and that has lost about 20 residues. No significant breakdown of subunits  $a$  and  $c$  was caused by chymotrypsin. Protein-chemical analysis suggests that the cleavage had occurred at the C-terminus of subunit  $b$  between a pair of isoleucines at positions 134 and 135. These results indicate that the polar domain is accessible from the water phase and that it is oriented toward the  $F_1$  side of the membrane. Remarkably,  $H^+$  conductance of  $F_0$  as well as the rebinding of  $F_1$  is

not affected by the removal of the polar domain of subunit *b*. ATP-dependent H<sup>+</sup> translocation, however, is abolished. Apparently the F<sub>1</sub>-ATPase is no longer bound correctly. There is a distinct possibility that *E. coli* F<sub>0</sub> subunit *b* corresponds to the trypsin-sensitive interphase components identified by Pedersen et al. in rat liver F<sub>0</sub>.<sup>37</sup>

#### SUBUNIT INTERACTION AND STOICHIOMETRY

The cross-linking of ATP synthase subunits was studied by J. Hoppe using azidonitrophenylfluoride. This bifunctional reagent is first covalently coupled to

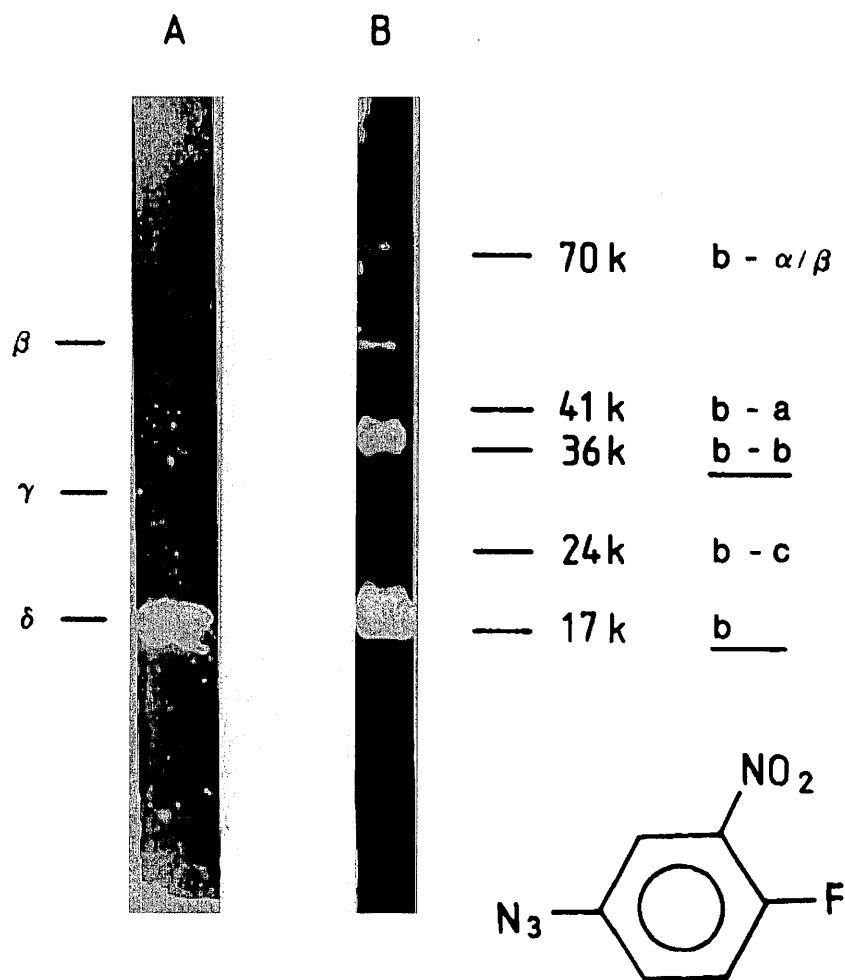


FIGURE 8. Cross-linking of F<sub>0</sub> subunit *b* from *E. coli* with a bifunctional photoreactive probe. Isolated ATP synthase from *E. coli* dissolved 1 mg per 0.2 ml 25 mM Aminoxide WS 35 in buffer (50 mM *N*-methylmorpholin, pH 8, 100 mM KCl, 2 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 0.2 mM phenylmethylsulfonylfluoride, 20% methanol) was incubated in the dark with 5 mM fluoronitrophenylazide for 60 minutes at room temperature. Thereafter the reactive nitrene was generated by illumination at 360 nm for 10 minutes at 0°C. Proteins were separated electrophoretically on 0.7-mm-thick dodecylsulfate gels, and were then transferred electrophoretically on nitrocellulose sheets.<sup>47</sup> The sheets were incubated with specific rabbit immunoglobulin G (IgG) directed against subunit *b*. Bound IgG was visualized after reaction with fluorescein-conjugated goat-antirabbit IgG under ultraviolet light. Lane A shows the identification of subunit *b* in the absence of cross-linking. Lane B demonstrates the appearance of cross-links containing subunit *b*. The positions of subunits  $\beta$ ,  $\gamma$ , and  $\delta$ , which were separated in parallel, are indicated.

various nucleophilic side chains via a displacement of the fluoro group. Subsequently, the azido group is activated by illumination, and cross-linking can occur. The cross-linked products were analyzed by a blotting technique using specific antibodies directed against individual subunits. The results obtained for subunit *b* are presented in FIGURE 8. Most notable is the occurrence of a *b-b* cross-linked product, which indicates the presence of a *b*-dimer. Small amounts of additional subunit *b* cross-links were observed that are tentatively assigned to cross-links with  $F_0$  subunits *c* and *a*, as well as with  $F_1$  subunits  $\beta$  and  $\alpha$ . Apparently the *b* subunit has multiple contacts with other subunits, and it is therefore located in the center of the complex.

The stoichiometry of the *E. coli*  $F_0$  subunits has been studied by methods based on a homogeneous labeling of the proteins with radioactive precursors, such as  $^{14}\text{C}$ -glucose and  $^{35}\text{S}$ -sulfate. For  $F_0$  subunits *a*, *b*, and *c*, molar ratios of 1:2:10 were determined by Foster and Fillingame.<sup>38</sup> For the mitochondrial ATP synthase, the occurrence of a hexameric subunit *c* has been determined employing similar techniques.<sup>39</sup> This discrepancy concerning the stoichiometry of subunit *c* cannot be explained at the moment. Although a radioactive mass labeling of the subunits appears to be the most reliable protein determination, there exist several possible pitfalls in the analysis of the stoichiometry of this complex enzyme; e.g., it is still uncertain as to how far the subunit stoichiometry of the isolated enzyme corresponds to that of the functional ATP synthase in the membrane.

#### PROTON CONDUCTANCE IN MUTANT MEMBRANES WITH DELETIONS OF $F_0$ SUBUNITS

One of the most intriguing questions concerning  $F_0$  is determining which of the subunits provides the pathway for the protons through the membrane. Early and pioneering experiments where isolated subunit *c* from yeast<sup>40</sup> and chloro-

TABLE 2  
MEMBRANES OF *E. coli* STRAINS LACKING SUBUNITS OF  $F_0$

| Strain*                    | $F_0$ Subunit<br>in the<br>Membrane† |   |   |
|----------------------------|--------------------------------------|---|---|
|                            | a                                    | b | c |
| A 1 (wild type)            | a                                    | b | c |
| AM 12                      | a                                    | — | c |
| DG 10/6                    | a                                    | b | — |
| CM 2080 (log phase)        | —                                    | b | c |
| CM 1470 plus pOM 11        | a                                    | — | — |
| CM 1470 plus pRPG 51       | —                                    | b | — |
| CM 2080 (stationary phase) | —                                    | — | c |
| CM 1470                    | —                                    | — | — |

\*The wild-type A 1 and mutant strains AM 12 and DG 10/6 have been described by Schairer *et al.*<sup>48</sup> Deletion mutants CM 2080<sup>45</sup> and CM 1470<sup>40</sup> were obtained from K. von Meyenburg. Plasmid pOM 11 contains the structural gene of subunit *a* in a Hind III-Ava I fragment cloned in pBR 322 (K. von Meyenburg, personal communication). The plasmid pRPG 51 containing structural genes for subunits *b* and  $\delta$  was obtained from R. D. Simoni.<sup>46</sup> The occurrence of subunit *b* in strain CM 2080 depends on the growth phase. Most likely it is degraded proteolytically in stationary cells.

†The occurrence of subunit *a* was determined by physical analysis of the DNA or by genetical analysis. The occurrence of subunits *b* and *c* was determined immunologically as shown in FIGURE 9.

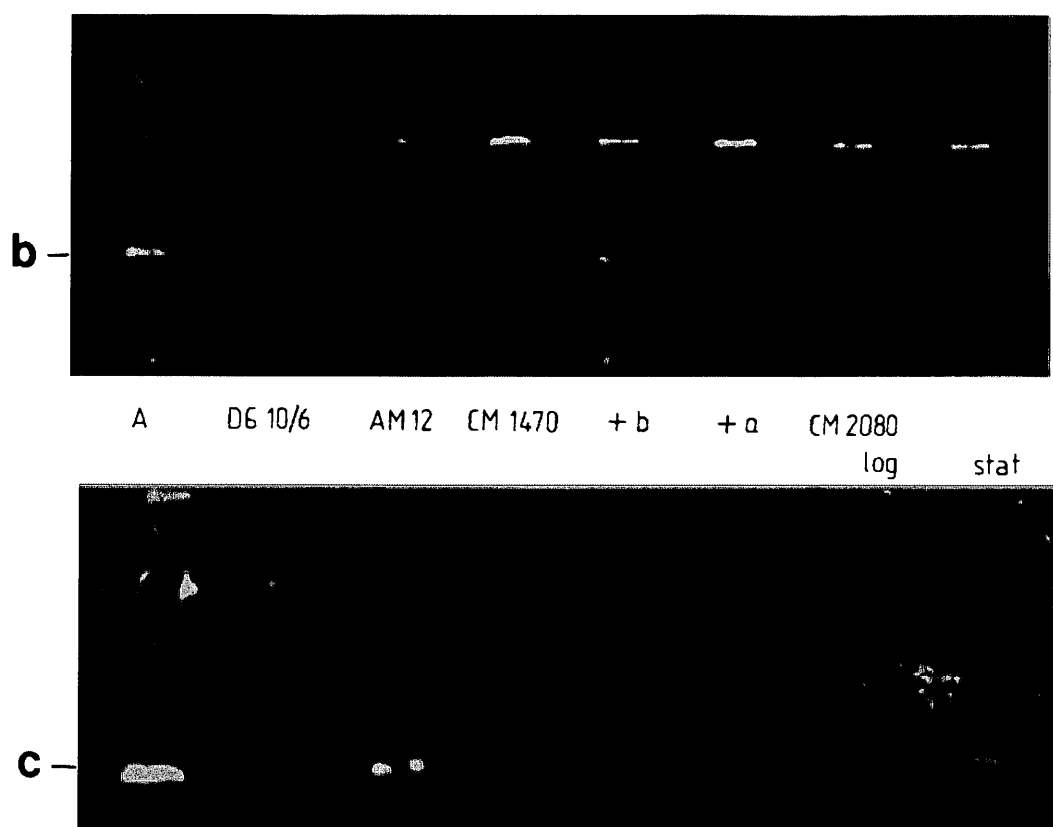


FIGURE 9. Immunological analysis of mutant membranes from *E. coli* lacking  $F_0$  subunit b or c. The membrane proteins of the mutant strains described in TABLE 2 were separated on dodecylsulfate gels, and thereafter the proteins were electrophoretically transferred to nitrocellulose sheets. Duplicates were incubated with rabbit IgG directed against either subunit b or c. Bound IgG was visualized after reaction with fluorescein-conjugated goat-antirabbit IgG under ultraviolet light.<sup>43</sup> The high-molecular-weight fluorescent bands represent nonspecific binding of IgG to unidentified membrane proteins. They are not observed when isolated  $F_1F_0$  is analyzed.

plasts<sup>41</sup> was reconstituted with artificial lipid membranes suggested that this subunit exhibits protonophoric activity. Recently, in studies with black lipid membranes, it was postulated that only an oligomeric subunit c acts as protonophore and that subunit a or b stabilizes the oligomer.<sup>42</sup> These results were challenged, however, since the purity of reconstituted subunits was not rigorously established and since the reconstituted activities were very low.<sup>43</sup> In thermophilic  $F_0$ , the largest subunit could be removed without loss of the  $H^+$  conductivity.<sup>44</sup>

Another approach to this problem is the construction of *E. coli* strains where one or two of the  $F_0$  subunits are deleted. TABLE 2 shows a collection of strains containing all possible combinations of  $F_0$  subunits in the membrane, which were analyzed by P. Friedl. The presence or absence of an  $F_0$  subunit was determined genetically (subunit a) or immunologically with specific antibodies after gel electrophoretic separations and blotting onto nitrocellulose filters (FIGURE 9). None of the  $F_0$  subunits alone—and no combination of two  $F_0$  subunits in the membrane—leads to the expression of  $H^+$  conductance in the membrane (data not shown). This suggests that in *E. coli*, at least *in vivo*, all three  $F_0$  subunits are necessary for an efficient proton transport. Remarkably, subunits a and b alone or together can bind  $F_1$  (FIGURE 10), but without reconstitution of DCCD-sensitive ATPase or ATP-dependent  $H^+$  translocation. In contrast, membranes containing only subunit c are unable to bind  $F_1$ .

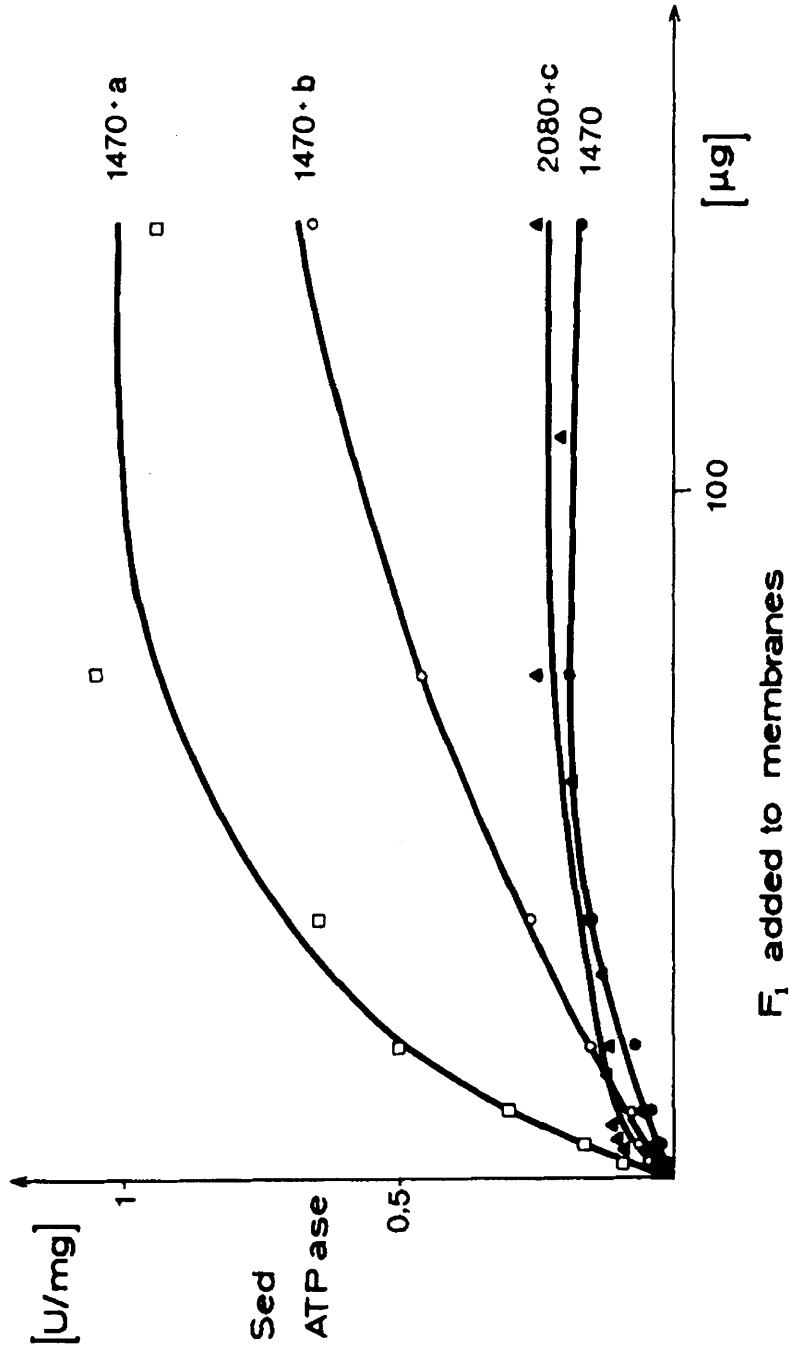


FIGURE 10. Binding of  $F_1$  to mutant membranes containing only one of the  $F_0$  subunits.  $F_1$ -depleted membranes of mutant strains described in TABLE 2 were incubated with the indicated amounts of  $F_1$  in 1 ml buffer (50 mM morpholinopropane sulfonic acid, pH 7.0, 10 mM  $MgCl_2$ , 175 mM KCl) for 20 minutes at room temperature. Then the samples were centrifuged at  $200,000 \times g$  for 20 minutes. The sedimented membranes were resuspended in the original volume of buffer, and ATPase activity was tested.<sup>48</sup>

The strains used in these experiments are not yet ideal. In some cases, residual amounts of the  $F_1$  subunits are still synthesized or the presence of fragments of deleted  $F_0$  subunits cannot be excluded. These might interfere with the assembly of the expressed subunits. It should be possible to exclude these uncertainties in future experiments with cloned defined structural genes of individual  $F_0$  subunits.

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#### DISCUSSION

B. MCKEEVER (*State University of New York, Stony Brook, N.Y.*): Is the messenger RNA of the ATP operon from *E. coli* polycistronic?

W. SEBALD: All the evidence points to this.

P. L. PEDERSEN (*Johns Hopkins University, Baltimore, Md.*): Is the DCCD-binding protein alone sufficient to translocate protons in reconstituted bilayers?

W. SEBALD: I don't know. The latest evidence for this is presented in Reference 42. Single-channel conduction of yeast mitochondria proteolipid was measured at pH 2.2 in black lipid membranes.

B. J. BOWMAN (*University of California, Santa Cruz, Calif.*): There are several correlations that indicate that DCCD and oligomycin bind at the same site. However, we don't really know where oligomycin binds. Do you have any information on this?

W. SEBALD: In the first approximation, the amino acid residues altered in oligomycin-resistant mutants from yeast and *Neurospora* may be considered as side chains providing the binding sites for oligomycin. Such substitutions have been identified in subunit *c* (proteolipid) and in subunit *a* (subunit 6). In some instances, these amino acid substitutions are quite conservative, e.g., isoleucine for methionine or leucine for valine. Thus, allosteric effects on oligomycin binding in the mutants appear to be unlikely.

P. L. PEDERSEN: Is the  $F_0$  channel sensitive to SH reagents?

W. SEBALD: There exists only one option, the cysteine in subunit *b* at position 21. Experiments are in progress to investigate the results of a modification of this SH group on  $H^+$  conduction and  $F_1$  binding.