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THE PROTON CONDUCTING F_0-PART OF BACTERIAL ATP SYNTHASES

JÜRGEN HOPPE and WALTER SEBALD

GBF – Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-3300 Braunschweig (F.R.G.)

Contents

Abbreviations: DCCD, N,N'-dicyclohexylcarbodiimide; PTH, phenylthiohydantoin; TID, 3-trifluormethyl-m-(iodophenyl)-diazirine; NCCD, N-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-N'(cyclohexyl)carbodiimide.

I. Introduction

The membrane-integrated part F_0 of the ATP synthase translocates protons across the mem-

brane. In the whole ATP synthase (F_1F_0) the F_0-mediated proton transport is tightly coupled to the synthesis or hydrolysis of ATP catalysed by the F_1-ATPase part. By the removal of F_1, the proton pathway in F_0 is opened, and can be studied independently. F_0 represents, therefore, an interesting and up to now unique system for analysis of (i) the structure and function of a proton conductor, and (ii) the coupling of the F_0-mediated proton transport to the hydrolysis or synthesis of ATP occurring in the F_1-ATPase.

Several recent reviews covered the genetics of the ATP synthase [1,2], the nucleotide sequence of the ATP synthase genes of *E. coli* (Walker, J.E., Saraste, M. and Gay, N.J., unpublished results), the proton-ATPase from bacteria and mitochondria [4], the structural basis of proton-translocating protein function [5], and the structure and genetics of the proteolipid subunit of the ATP synthase [6]. This review tries to collect new proteinchemical data on F_0 and to combine them with genetical and functional data in order to give the present-day knowledge on the structure-function relationships in this proton conductor. Although detailed and definite information has been obtained on several structural and functional aspects of F_0, we are still far from an understanding of the proton transport at a molecular level.
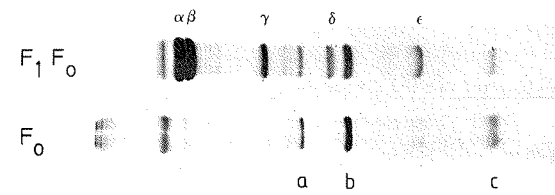
II. Background, problems, and approaches

The ATP synthase occurs in remarkably similar form in procaryotic and eucaryotic cells. Accord-
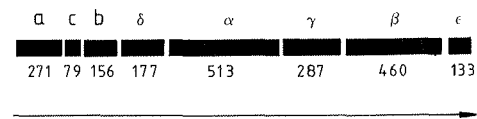
Subunits

Genes (atp operon)

Fig. 1. Subunits and genes of the *E. coli* ATP-synthase. The ATP synthase F_1F_0 and the F_0 were isolated from *E. coli* according to Friedl et al. [8,10] and analyzed by SDS/polyacrylamide gel electrophoresis. The order of the genes in the *unc* operon and the correlation of genes with ATP-synthase subunits was established genetically and by nucleotide sequence analysis [1,12–23]. The numbers below the genes indicate the number of amino acid residues in the subunit polypeptide.

ingly, our present knowledge on the proton-conducting F_0 part is derived from studies of the bacterial, mitochondrial or chloroplast protein, each offering special experimental possibilities. In recent time, research has focused on the F_0 from *Escherichia coli*. The ATP synthase of this organism can now be prepared in large quantities with a purity of at least 90% due to the work of Foster and Fillingame [7] and Friedl et al. [8]. From these preparations the F_0 part has been isolated by various procedures [9–11]. It contains three subunits *a*, *b* and *c* (χ, ψ and ω, or proteolipid or DCCD-binding protein) as shown in Fig. 1. The whole ATP synthase contains, in addition, the five F_1 subunits α, β, γ, δ and ϵ. These eight subunit polypeptides are coded for by the eight genes that form the *atp* (*unc*) operon (see section IV). The *atp* operon has been well defined genetically, especially by Downie, et al. [1,12], and by Von Meyenburg and co-workers [13], and its nucleotide sequence has been determined [14–23]. Therefore, the subunit composition of the ATP synthase and F_0 from *E. coli* is proven. The amount of protein of F_0 subunits *a*, *b* and *c* corresponds to molar ratios of $1:2:10$ according to Foster and Fillingame [24], or $1:2:12$ according to Von Meyenburg and co-workers [25]. For the mitochondrial [26] and chloroplast [27] ATP synthase, six proteolipid (subunit *c*) molecules have been determined. These different stoichiometries are difficult to explain for the time being. It is quite clear, however, that subunit *c* exists at least in six copies per F_0. The dimeric form of subunit *b* is also indicated by cross-linking experiments (see subsection IIID).

The isolated F_0 from *E. coli* can be reinserted into artificial membranes, and proton conductance can be measured in response to a valinomycin-induced potassium diffusion potential [9–11] (Table I). The proton conductance is inhibited strongly by DCCD and by added F_1. Reconstituted F_0 rebinds F_1, and, thereby, ATP-dependent proton translocation is restored. The data obtained with isolated F_0 are compiled in Table I together with corresponding data measured with F_0 in whole membranes. Accordingly, both proton conductance and functional rebinding of F_1 can be used to analyse F_0.

The nucleotide sequence of the genes of all three F_0 subunits of *E. coli* was determined independently by three groups in 1981 [15,17,21]. Amino-terminal amino acid sequences of the isolated subunits *a*, *b*, and *c* were established [17] in order to prove the start of the individual subunit
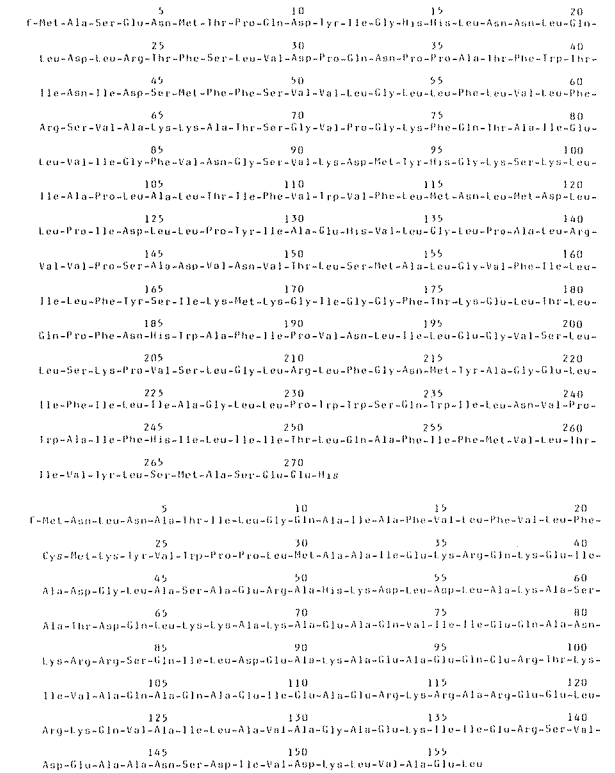
Fig. 2. Amino acid sequences of the F_0-subunits *a*, *b*, and *c* of the ATP synthase from *E. coli*.

genes. The derived amino acid sequences of the three F_0 subunits are presented in Fig. 2. They have been extensively evaluated by means of computer programs with respect to secondary structure predictions, polarity profiles and homologies with corresponding subunits from other organisms [3,4,28,29]. A summary of such evaluation is also shown in Figs. 12, 13 and 15 in the last section of this review.

The established subunit composition and covalent structure of the *E. coli* F_0 reflect a major achievement in the characterization of the ATP synthase proton channel. However, for the elucidation of F_0-mediated proton conductance, additional information is needed (i) on the three-dimensional structure of the protein, and (ii) on the

Fraction	Electro-impelled H$^+$ conduction		ATP-dependent H$^+$ translocation Fluorescence test
	pH-electrode (nmol·min^{-1}·mg^{-1})	Fluorescence test (U_{F1}·mg^{-1})	
Membranes	–	–	130
F_1-depleted membranes	44	65	–
Liposomes with			
F_1F_0	5	16	2330
$F_0 + F_1$	41	151	1910
F_0	870	2565	1
$F_0 + DCCD$	2	24	–

functional amino acid residues and domains constituting the proton pathway and participating in the coupling of F_0 and F_1 functions.

A high resolution of the three-dimensional structure of F_0 will be achieved finally only by X-ray analysis after crystallization of the protein. With other integral membrane proteins, two-dimensional crystals [30–34] and, more recently, also three-dimensional crystals [35–37] have been produced. Crystals of the F_0 protein have not yet been obtained. Anyway, X-ray analysis of complex proteins, as F_0, will be exceedingly complicated, and will probably always depend on auxiliary information from other structural studies.

As described in subsections IIIA–C, the surface of F_0 has been mapped by probes which have access either only from the polar water phase or from the hydrophobic lipid phase. Proteases and antibodies are valuable tools, not only for identification of polar domains but also for the determination of the orientation of the polypeptide chains in the membrane. The surface of F_0 accessible from the lipid bilayer has been studied with photoreactive membrane-permeating probes. Especially, carbene labels of broad specificity offer promising possibilities, since they should act like a razor blade and modify all accessible residues at the protein/lipid interphase. Not only would this allow an identification of membrane-embedded polypeptide segments, but, in addition, the pattern of accessible residues might yield information on secondary structures of the polypeptide chain.

The study of subunit contacts in F_0 by cross-linking experiments is handicapped (i) by the low content of reactive side-chains and (ii) by the complex subunit composition. These difficulties could be overcome partially by using photo-activated bifunctional reagents for cross-linking and specific antibodies for the identification of cross-linked subunits.

Further information on the structure of F_0 may be derived from the analysis of F_0 mutants. Amino acid residues altered in DCCD- or oligomycin-resistant mutants might be involved in the binding of the inhibitors, and might therefore be located in proximity to each other at the surface of the molecule. Subunits from assembly-deficient mutants might be altered in residues involved in intermolecular or intramolecular protein contacts.

Several approaches were applied to identify the functional amino acid residues and domains in the F_0. Due to the ubiquitous occurrence of F_0, it is feasible to compare the amino acid sequences of F_0 subunits from very distantly related organisms. Based on the assumption that the mechanism of proton conductance remained unchanged during evolution, it can be expected that the group of invariant amino acids also comprises the functional residues.

Chemical modification may lead to the identification of functional residues, provided that the reaction of a defined residue can be correlated with an inhibition of F_0 functions.

Molecular genetics and genetic engineering offer further promising possibilities to study functional properties of F_0. Especially in *E. coli*, but also in yeast, a variety of mutants which contain a defective F_0 have been isolated. In some of these strains, F_0 appears to be normally assembled, and, therefore, functional residues might have been modified by the mutation. The altered residues might be identified by protein or DNA sequence analysis. Furthermore, the function of the different F_0 subunits has been studied by constructing strains where one or two of the F_0 subunits are missing.

III. Topographical studies

IIIA. Studies with proteinases

The assymmetry of membrane proteins has been investigated in many instances by proteinase treatment of whole membranes and analysis of the cleavage product [38–41]. The sidedness of the C- and N-terminal segments as well as the location of some internal polar loops have been determined, for example, in the bacteriorhodopsin, by treatment of the purple membrane with papain, trypsin and chymotrypsin [42–44]. These studies, together with crystallographic data [30,31] and the primary structure [43,45], provided information on the folding and orientation of this polypeptide in the membrane [46,47].

First experiments on proteinase treatment of isolated F_0 from PS-3 were described by Sone et al. [48]. An F_0 subunit of M_r 13 000 was readily digested with trypsin and nagarse, but the proton permeability of the F_0 was not affected by pro-
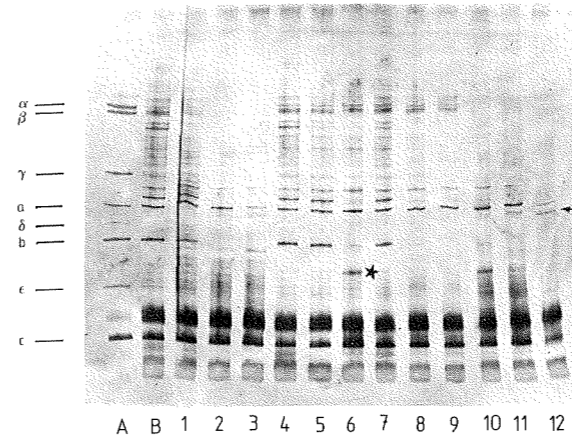
Fig. 3. Proteolytic treatment of F_0 subunits in F_1-depleted membranes. F_1-depleted membranes of *E. coli* were prepared from an ATP synthase-overproducing strain [49]. Lane A shows separated F_1F_0-, and lane B, separated F_1-depleted membranes. The proteinase concentrations, increase in the samples from left to right for V8 (lanes 1–3), chymotrypsin [4–6], trypsin [7–9] and subtilisin [10–12]. The star indicates the digestion product obtained with chymotrypsin. The arrow indicates the possible cleavage product from subunit *a*.

teinases. F_1 binding and ATP-dependent H^+ translocation were lost after digestion, indicating a binding activity of the extramembranous part of the 13.5 kDa protein. Corresponding studies on the proteinase sensitivity of the F_0-subunits from *E. coli* [49] gave similar results. Experiments were carried out using F_1-depleted everted membrane vesicles from strain CM 2786, which overproduces ATP synthase about 5-fold (see also subsections IV A and B). Thus, the three F_0-subunits could be easily identified in whole membranes by SDS-polyacrylamide gel electrophoresis in combination with a silver stain method (Fig. 3). Subunit *b* was most sensitive towards proteinase treatment. Trypsin, subtilisin and proteinase V8 produced small fragments, indicating that large portions of the protein were accessible to proteinases. These proteinases digested subunit *b* completely, demonstrating that the polar domain of subunit *b* extends to the cytoplasm in *E. coli*, since everted membrane vesicles were used. With chymotrypsin, however, a defined cleavage product of M_r 15 000 was

observed, which remained firmly bound to the membrane, as treatment with high salt, urea or guanidine hydrochloride failed to remove this fragment from the membrane. By proteinchemical analyses it was shown that chymotrypsin did not cleave at the primary cleavage sites phenylalanine, tyrosine, tryptophan or methionine, which are all located in the hydrophobic N-terminal segment of the subunit *b*. Thus, cleavage had occurred about 25 residues away from the C-terminus.

Under conditions where only subunit *b* was degraded by proteinases (see below), electro-impelled H^+-conduction (passive proton permeability) was not affected. Similar results were reported by Sone et al. [48] after degradation of the 13 kDa subunit of the PS-3 F_0. But in contrast to PS-3, in *E. coli* F_1 could be still bound to proteinase-treated membrane. The rebound ATPase activity was not sensitive towards DCCD and ATP-dependent proton translocating was not reconstituted. These results indicate that the polar part of subunit *b* plays an essential role in coupling the functions of F_1 with the H^+ translocation across the membrane. It is a distinct possibility that the proteinase-sensitive component at the interphase of F_0 and F_1 from rat liver [50] also corresponds to the *E. coli* subunit *b*.

Subunit *a* was not degraded by chymotrypsin and trypsin, while proteinase V8 and subtilisin at high concentrations were effective (Fig. 3). Subtilisin, at lower concentrations, digested all membrane proteins except subunit *a* to products with a M_r of less than 15 000. The appearance of the polypeptide with a M_r of 22 000 at higher subtilisin concentrations was thus indicative for a cleavage product of subunit *a*. Possibly, the quite polar N-terminal segment was attacked by the proteinase and, thus, might be located at the cytoplasmic side of the membrane.

Subunit *c* was not or only slightly affected by any of the proteinases. Even prolonged incubation times and high concentrations of proteinase did not lead to any extensive degradation (incubation time 5 days, at a ratio of proteinase to membrane protein of 1:5) [49]. Since subunit *c*, and especially the polar domain, is very resistant to proteolysis even in the denatured state, this result does not necessarily imply that the functional protein is completely inaccessible to proteinases.

IIIB. Studies with antibodies

Loo and Bragg studied the effect of antiserum raised against subunit c on proton conductance and F_1 binding in everted and right-side-out membrane vesicles of *E. coli* [51]. Antibodies added to everted membrane vesicles inhibited the proton conductance catalyzed by F_0. There was no effect when these antibodies were added to right-side-out vesicles.

At present, the antigenic site(s) of subunit c are not determined. Computer analysis of the primary structure led to the prediction that the antigenic sites are located at the hydrophilic N-terminus and the hydrophilic segment in the middle of the polypeptide chain [52]. In principle, it should be possible to identify the accessible fragments of subunit c employing antibodies against defined peptide fragments.

IIIC. Surface label

In recent years, the chemistry of photoreactive labels has rapidly advanced [for review see Refs. 53–56]. One type of these reagents are lipophilic compounds, which distribute in the membrane and upon illumination form a highly reactive nitrene or carbene group. Ideally, such a group should react unspecifically with a broad range of amino acid side-chains. This requires that the half-life of the reactive intermediate be shorter than its rotation correlation time [55]. Only if labelling occurs randomly will all amino acid residues at the protein/lipid interface be detected, thus allowing a detailed mapping of the protein surface. So far, none of the available photoreactive probes has been found to have an equal reactivity for all side-chains. Two types of hydrophobic label have been devised. The first group comprises small hydrophobic molecules which might randomly distribute in the lipid bilayer and thus can reach all residues which are in contact with the hydrophobic core of the bilayer. Among a series of reagents iodonaphthylazide, iodophenylazide, adamantyldiazirine and 3-trifluormethyl-*m*-(iodophenyl)diazirine (TID) have been used most frequently [57–60]. Labelling was so far analyzed on the level of intact subunits of whole proteins, or large fragments [57–61]. The identification of
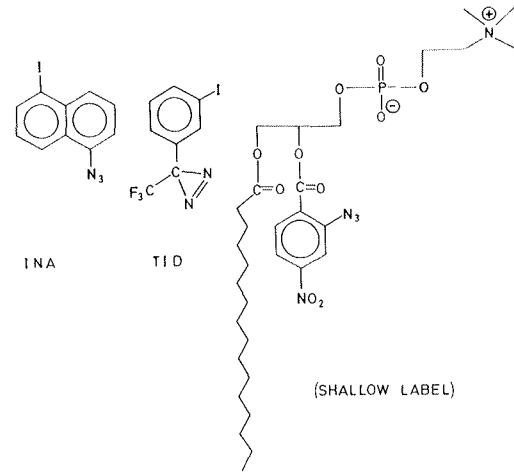

Fig. 4. Structure of the hydrophobic photoreactive labels used for the labelling of F_0. INA, iodonaphtylazide.

modified residues has not been reported so far.

The second group comprises phospholipid derivatives, bearing a phenylazidoprecursor, a phenyldiazirine [62–67], or a trifluoromethylphenyldiazirine [68]. In these compounds, the photoreactive group is fixed and is expected to cross-link with defined regions of the protein. The 'shallow label', shown in Fig. 4, will only label residues which are in proximity to the headgroup of the lipids. There are a few examples with phospholipid analogues where the attached label has been traced back to individual amino acids. The 'shallow label' has been used by Bisson et al. [69] to label the membrane surface of the cytochrome c oxidase. The label has been traced back to amino acid residues, marking the beginning of one stretch of hydrophobic residues in subunit II of the complex. Ross et al. [70] described the labelling of the transmembrane domain of glycophorin A with phospholipids, containing carbene precursors (aryldiazirine). They found that Glu-70 was the predominant site of cross-linking with phospholipid derivatives. Brunner and Richards [68] found a tryptophan as the major site of carbene and nitrene-generating phospholipid analogue in contact with gramicidin.

For the *E. coli* F_0 labelling was studied with a photoreactive phospholipid analogue ('shallow probe') and the two small lipophilic reagents (^{125}I)iodonaphthylazide and (^{125}I)TID (Fig. 4) [71–73]. The probes bearing a nitrene precursor
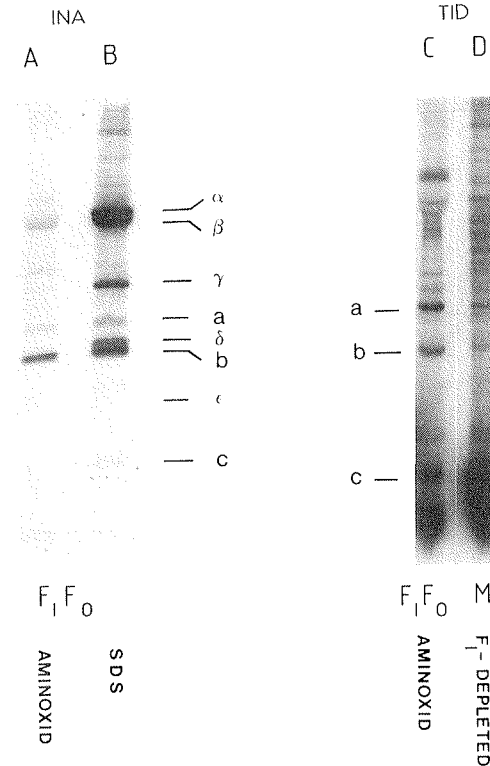

Fig. 5. Reactivity of F_0-subunit from *E. coli* towards membrane permeation photoreactive probes (Ref. 72; see also Hoppe, J., Brunner, J. and Jørgensen, B.B., unpublished data) (A) Isolated F_1F_0 dissolved in Aminoxid WS 35 buffer reacted with (^{125}I)iodonaphthylazide. (B) Isolated F_1F_0 dissolved in 2% SDS buffer reacted with [^{125}I]iodonaphthylazide. (C) Isolated F_1F_0 dissolved in Aminoxid WS35 buffer reacted with (^{125}I)TID. (D) F_1-depleted membranes from strain CM2786 reacted with [^{125}I]TID. After reaction with the photolabel, subunits were separated on SDS-polyacrylamide gel electrophoresis. Bound label was visualized by autoradiography. The position of the individual subunits as determined by staining with Coomassie Blue is indicated. INA, iodonaphthylazide.

Fig. 6. Identification of amino acids of subunit b labelled with [^{125}I]iodonaphthylazide and the 'shallow probe'. Labelled subunit b was isolated from F_1F_0 by HPLC gel permeation chromatography, immobilized on phenylenediisothiocyanate-activated porous glass beads and sequenced [71,72]. Released PTH amino acids were analyzed for radioactivity. O———O, ^{3}H-radioactivity from the phospholipid derivative; ●———●, ^{125}I-radioactivity from [^{125}I]iodonaphthylazide.

(^{125}I)iodonaphthylazide and the 'shallow probe' exhibited a high selectivity, both on the level of whole subunits and on the level of individual amino acid residues.

In the functional enzyme, solubilized in the detergent Aminoxid WS 35, (^{125}I)iodonaphthylazide almost exclusively labelled subunit b, but not the other two hydrophobic subunits a and c (Fig. 5). With the subunits dissociated in sodium dodecyl sulfate, all subunits including the F_1 subunits
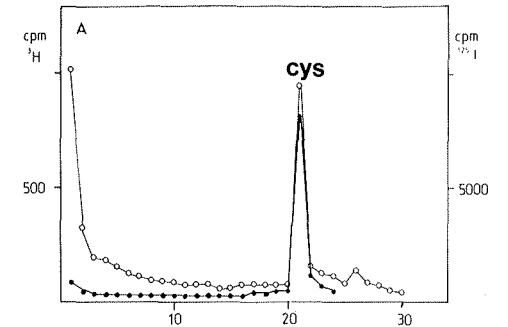
were labelled with the exception of subunit c. Similarly, the 'shallow probe', also a precursor of a nitrene, reacted only with subunit b in the F_1F_0 complex reconstituted into proteoliposomes. Modified amino acid residues were identified by isolation of labelled peptides and amino acid sequencing. Both probes reacted only with the hydrophobic N-terminal segment of subunit b. The distribution of radioactive label among the sequenced amino acid residues is shown in Fig. 6. (^{125}I)iodonaphthylazide labelled almost exclusively Cys-21. Also the 'shallow probe' reacted predominantly with this residue. In addition, Trp-26 which is close to Cys-21, received a label, and possibly the first three residues including Asn$_2$ at the N-terminus were attacked. But with the latter, a clear interpretation was not possible due to the high radioactive background in the first step of Edman degradation. In conclusion, nitrene-generating hydrophobic precursors seem to be insufficient for a detailed map of the surface of membrane domains of proteins which are mainly composed of aliphatic residues.

The insufficiency may result from the rather long lifetime of the nitrene. Furthermore, arylnitrenes may undergo intramolecular rearrangements leading to long-lived electrophilic species [74]. Cysteines, as the most potent nucleophiles

among the amino acid residues, may act as a sink for such intermediates. The situation appears to be slightly better with the phospholipid, where diffusion of the reactive species is restricted.

Carbenes are much more reactive than nitrenes. They even insert into CH bonds of the aliphatic chain of phospholipids [67]. The aryldiazirines represent one type of carbene precursor. As a side-reaction of photolysis, these compounds undergo a rearrangement to the corresponding diazoisomer, which specifically will esterify carboxy groups. The problems with this type of precursor have been discussed recently [70].

To circumvent this problem, trifluoromethyldiazirines have been introduced by Brunner [68]. The undesired diazoisomer is stabilized by the strong electron-withdrawing trifluoromethyl group, and esterification by the diazoisomer is thus negligible. Fig. 5 C and D shows that all three F_0 subunits in the isolated complex F_1F_0 are labelled by the carbene, roughly according to their protein mass. In F_1-depleted membranes, the same labelling pattern of subunits a and b is observed, the labelling of subunit c is obscured by the large amount of labelled phospholipids.

Labelling patterns were recorded with (i) isolated ATP synthase (F_1F_0), (ii) F_1 depleted membranes from strain CM 2786 which overproduces the ATP synthase, and (iii) subunits denatured in a 2% SDS-solution.

$[^{125}I]TID$ labelled subunits b and c could be isolated and the modified residues could be determined. Sequence analysis was performed by automated solid phase Edman degradation. Released PTH amino acids were identified by thin-layer chromatography and associated ^{125}I-radioactivity was measured as shown in Fig. 7A–C. The reaction product with $[^{125}I]TID$ was further analyzed by autoradiography of the thin-layer plates. This allowed the identification of labelled amino acid residues even over a high background and an overlap from the preceding amino acid residue.

Proteinchemical analyses of TID-labelled subunit b by CNBr cleavage showed that more than 98% of the label was attached to the N-terminal segment. Fig. 7A, B depict the labelling of individual amino acid residues after reaction of (^{125}I)TID with the isolated complex F_1F_0- or F_1-depleted

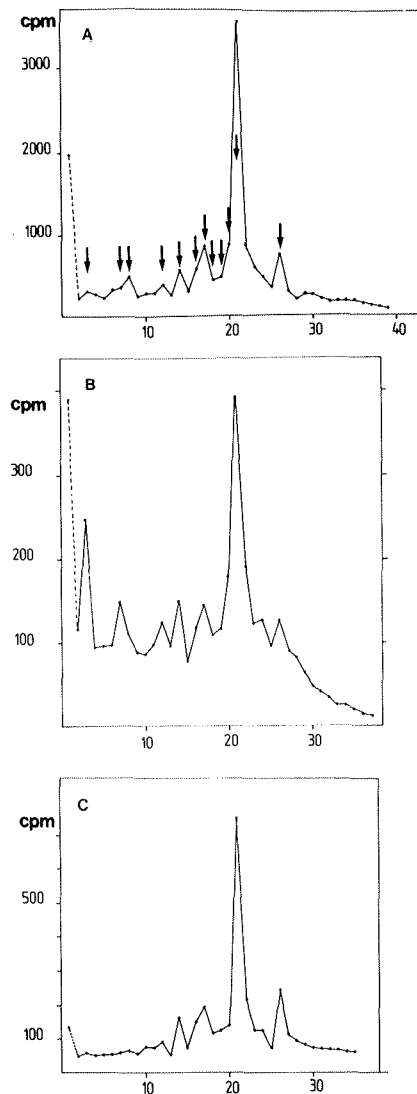

Fig. 7. Identification of amino acids of subunit b, labelled with $[^{125}I]TID$ (Hoppe, J., Bronner, J. and Jørgensen, B.B., unpublished data). After labelling, subunit b was isolated from F_1F_0 by HPLC gel permeation chromatography. For F_1-depleted membranes, proteins were separated on a SDS polyacrylamide gel and the band corresponding to subunit b was eluted from the gel strip. Subunit b from (A) F_1F_0 in Aminoxid WS35 buffer, (B) F_1-depleted membranes, (C) F_1F_0 in SDS-buffer... Arrows indicate labelled amino acid residues, identified by autoradiography of the PTH amino acids after thin-layer chromatography.

membranes. In the N-terminus of subunit b, again Cys-21 received the bulk label. But other amino acids are significantly labelled. Most interestingly,
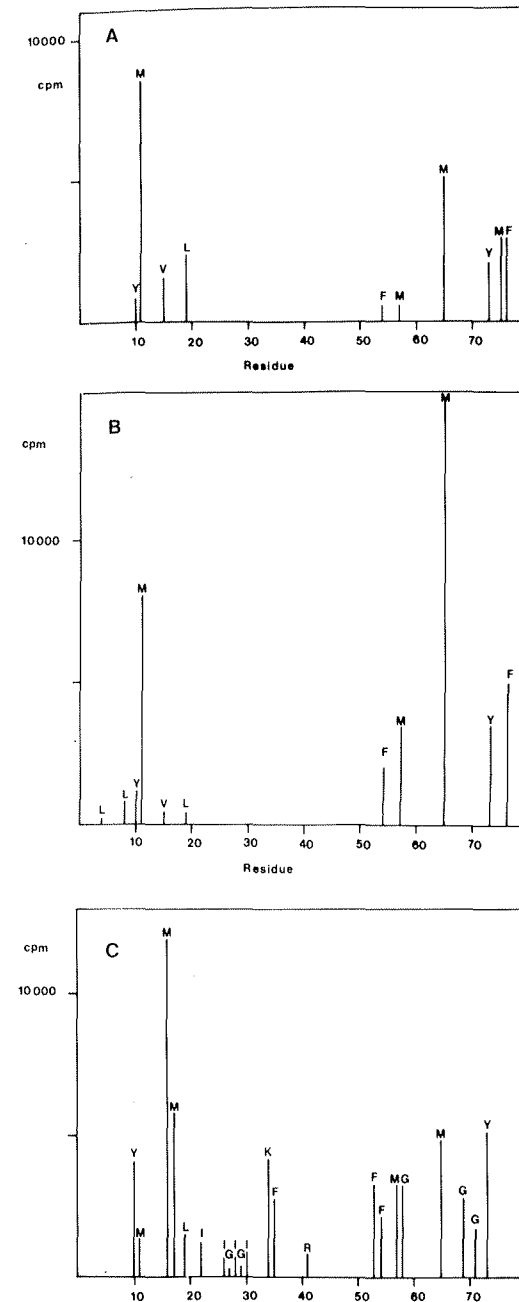
Fig. 8. Labelling of individual amino acids of subunit c by (^{125}I)TID (Hoppe, J., Brunner, J. and Jørgensen, B.B., unpublished data). Labelling was determined by sequencing the whole protein, attached to glass beads. Samples were analyzed for ^{125}I-radioactivity and labelled amino acids were detected by autoradiography of the released PTH amino acids after thin-layer chromatography. The amount of label bound to an amino acid side-chain was then calculated, assuming a repetitive yield of 95% for each cycle of Edman degradation. (A) Subunit c

Leu-3, Ile-7, Leu-8, Trp-26 and all residues from Phe-14 to Cys-21 were labelled as confirmed by thin-layer chromatography. A very similar labelling pattern of the N-terminal segment was obtained after reaction of $[^{125}I]TID$ with isolated subunit b in SDS-solution (Fig. 7C). Thus, most of the amino acid residues of the membrane-integrated N-terminus of subunit b are freely accessible from the lipid phase.

After reaction of $[^{125}I]TID$ with a functional F_0 (F_1-depleted or F_1F_0), only a few residues of subunit c were attacked (Fig. 8A–B) in contrast to the multiple labelling in the N-terminal segment of subunit b. Labelling is restricted to four to six residues in the N-terminal segment and to five to six residues in the C-terminal segment. No labelling occurred in the middle of the polypeptide chain from positions 20–52, despite the fact that the sequence from residues 20–33 is highly enriched in hydrophobic residues, and thus is a possible candidate for a membrane-embedded segment. The labelling pattern changes dramatically if $[^{125}I]TID$ is reacted with the SDS-solubilized isolated subunit c (Fig. 8C). Labelled residues are distributed over the whole length of the polypeptide, including the hydrophobic segment 20–30, which was not labelled in the functional subunit c. Apparently, these residues are shielded by other protein segments in functionally assembled subunit c.

In the functional subunit c, the $[^{125}I]TID$ modified residue at the N- and C-terminals exhibit a remarkable repeat. They occur at positions 4, 8, 10, 11, 15, 19 and 53, 57, 65, 73, 76 [74]. Such $n + 3$ or $n + 4$ patterns are typical for residues located on the same side of an α-helix.

IIID. Cross-linking experiments

The subunit contacts in the *E. coli* F_0 were studied with the isolated complex F_1F_0 by cross-linking experiments. Fluoronitroazidophenyl was used, which in the first step binds to the side-chains of cysteine, lysine, tyrosine or histidine. Subsequently,

from F_1F_0 in Aminoxid WS 35 buffer; (B) subunit c in F_1-depleted membranes; (C) subunit c in an SDS solution.
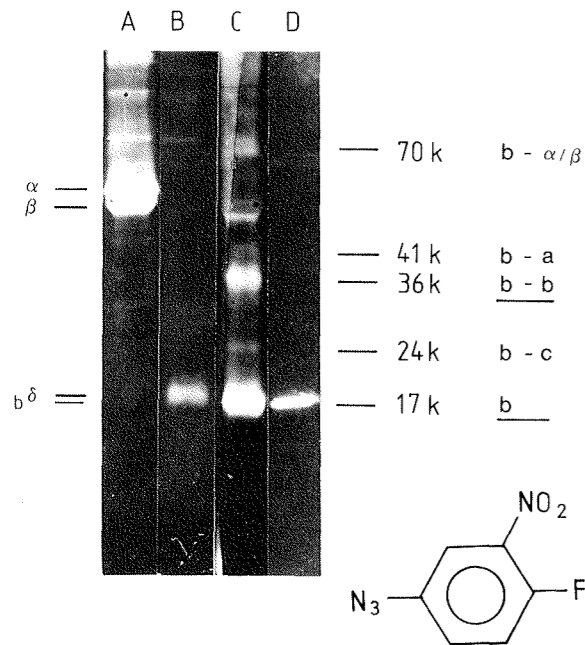
Fig. 9. Cross-linking of F_1F_0 subunits from *E. coli* with a bifunctional photoreactive probe [74]. Isolated ATP synthase from *E. coli* dissoved 5 mg/ml in 50 mM *N*-methylmorpholine (pH 8.0)/25 mM Aminoxid WS 35/100 mM KCl/2 mM $MgCl_2$/0.2 mM EGTA/0.2 mM phenylmethylsulfonylfluoride/20% methanol was incubated in the dark with 5 mM fluoronitrophenylazide for 60 min at room temperature. Unreacted reagent was quenched by a 30 min incubation with 0.1 M Tris-HCl (pH 8.0). Thereafter, a reactive nitrene was generated by illumination at 360 nm for 10 min at 0°C. Proteins were separated electrophoretically on SDS-gels, and were then blotted on nitrocellulose sheets. The sheets were incubated with specific rabbit immunglobulin, directed against the indicated subunits. Bound IgG was then visualized after reaction with fluorescein-conjugated goat-anti-rabbit IgG under ultraviolet light. Lane A: IgG against subunits α and β; lane B: IgG against subunit δ; lane C: IgG against subunit *b*. In a control experiment (lane D), the cross-linking reagent was omitted. Immunostaining was performed with IgG against subunit *b*.

the azido group can be converted into a reactive nitrene by illumination. Proteins were separated by SDS-gel electrophoresis. After blotting the proteins from the gel onto nitrocellulose, cross-link products were detected by specific antibodies directed against the individual subunits [74]. As shown in Fig. 9, after staining with subunit *b* antibodies, the most prominent cross-link product exhibits an M_r of 36 000. This is clearly a *b*-*b* dimer, since no reaction product with a similar M_r was found with antibodies directed against the

δ-subunits or any of the other subunits (Fig. 9). In addition, cross-linking products containing subunit *b* with an apparent M_r of 24 000 and 41 000 were found which might be dimers of subunit *b* with subunit *a* and subunit *c*. Two products with an M_r of about 70 000 were found. Most likely, subunit *b* was crosslinked with the F_1 subunits α and β.

Apparently, the *b*-subunit has multiple contact with other subunits, and, therefore, should be located in the center of the complex as also suggested by analysis of the protein sequence [29]. Futhermore, the appearance of a cross-linked subunit *b* dimer substantiates findings that two subunits *b* are present in one F_0-complex.

IIIE. Circular dichroism measurements

The secondary structure composition *of the proteolipid subunits isolated from E. coli and Neurospora crassa* (Table II) has been studied by circular dichroism measurements [6,75,76]. The spectra were recorded after dissolution of the protein in trifluoroethanol, SDS or octylglucoside, as well as after reconstitution with different preparations of small phospholipid vesicles. By means of a computer program, the CD spectra of pure α-helices of different length, of pure β-sheet, and of β-turn structures were fitted to the proteolipid spectra. A very high content of α-helix was calculated from all measurements. For the *N. crassa* protein, this high α-helical fraction seemed to be almost unaffected by the different environments. In the subunit *c* from *E. coli*, however, there was a more significant decrease in the α-helical content, especially in the length of the α-helical segments upon transition from organic solvents to the lipid phase. The major difference between the two sets of data was the calculated content of β-sheet. For the *N. crassa* protein incorporated into lipid vesicles, no β-sheet was calculated. In contrast to the *E. coli* subunit *c*, about 20% β-sheet was reported. It is questionable whether these differences are significant, since the accuracy in the estimation of β-sheet content in the presence of a high fraction of α-helix is quite low.

Most remarkably, the α-helical content in the large cyanogen bromide fragment from *E. coli* subunit *c* was considerably reduced. This suggests

TABLE II

SECONDARY STRUCTURE COMPOSITION (FIGURES IN %) OF THE PROTEOLIPID SUBUNIT FROM *E. COLI* (A) AND *N. CRASSA* (B) IN VARIOUS SOLUTIONS [6,75,76]

Solution	Content in				
	α-Helix	β-Sheet	Turn	Random	$\bar{n}$
A Trifluoroethanol	76.6	15.2	8.2	0	24.6
10% 2-Chloroethanol	66.4	18.2	15.4	0	8.5
Phospholipid vesicles 1	67.6	32.4	0	0	5.3
Phospholipid vesicles 2	71.8	24.4	0		11.9
Sodiumdodecylsulfate	44.6	35.3	0	20.1	25.0
Octylglucoside	53.6	20.1	0	25.8	25.0
B6 in 10% 2-chloroethanol	38.5	34.3	6.9	20.3	5.6
B Vesicle I	69.0	0	15.0	16.0	
Vesicle I	69.0	0	12.0	19.0	
Vesicle II	83.0	0	17.0	0	
Vesicle III	73.0	0	16.0	10.0	
Trifluoroethanol	72.0	0	0	28.0	
Octylglucoside	83.0	13.0	4.0	0	
Solubilized sodium dodecyl sulfate	60.0	18.0	0	22.0	

that non-helical segments of the protein reside in this fragment, which contains the conserved hydrophilic stretch, as well as the cluster of conserved glycine residues. It is uncertain at this time in how far the isolated proteolipid retains the secondary structure elements of the functional F_1F_0-integrated protein. It has to be noted, however, that the secondary structure derived from CD measurements roughly compares with secondary structure predictions [6,77–80].

IV. Genetics of F_0

IVA. General aspects

The arrangement of the genes coding for the subunits of F_0 and F_1 is completely different for the enzyme from the plasma membrane of *E. coli* and from mitochondria or chloroplasts of eukaryotic cells. As described in section II (Fig. 1) and reviewed in detail elsewhere, the *E. coli* F_0F_1 is coded for by an operon of eight genes [1,12,13]. The genes of F_0 preceding the F_1 genes are transcribed in the order *a, c, b*. The nucleotide sequence has been determined [14–23]. A few subunits of mitochondrial F_0 are coded for on mitochondrial DNA. In all analysed organisms, subunit *a* has been located on mitochondrial DNA

and the sequence has been determined [81–84]. In yeast, subunit *c* (proteolipid subunit 9) is encoded on mitochondrial DNA [85], in other organisms in the nucleus [82–86]. The other genes have not been located, so far. With one possible exception they appear to be localized in the nucleus, as deduced from biosynthetic studies [89]. The chloroplast enzyme is coded for to a large part on chloroplast DNA. Genes for F_1 subunits α, β, γ and ϵ, and for F_0 subunits *a* and *c* have been localized [87] in the organelle, and for some of them the sequence has been determined [90,91]. From biosynthetic studies, it can be concluded that only the genes for the F_1 subunit δ and F_0 subunit *b* are located in the nucleus [92].

Mutants defective in ATP synthase can be detected only in facultative anaerobic organisms which in the absence of the ATP synthase, survive by substrate-level phosphorylation. For all the F_1F_0 genes of the *atp* operon in *E. coli* mutations are known which result in a defective ATP synthase, This provides an excellent proof that the eight genes code for vital subunits of the enzyme. The genes have been designated *unc* A to *unc* H or *atp* A to *atp* H by different laboratories. In addition, inhibitor-resistant strains of *E. coli* are known which all are affected in the *atp* E (*unc* E) gene, coding for subunit *c*. In yeast, the mitochondrial
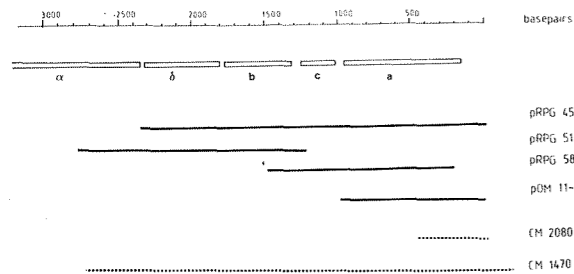
Fig. 10. Plasmids expressing selected genes of the *atp* operon in *E. coli* and strains carrying defined deletion in the *atp* operon. ▭, coding region for the subunits *a*, *c*, *b*; ——, indicates the cloned region of the *atp* operon in the respective plasmid [101,102]; -----, indicates the deleted region in the two strains [13].

genes encoding subunit *a* and *c* are covered by mutations leading to defective ATP synthase (Pho I, Pho II), or resistance towards inhibitors (Oli I, Oli II, Oli III, Oli IV) [93–96]. The corresponding nuclear subunit *c* gene in *N. crassa* can also be mutated to an allele leading to oligomycin resistance [97].

In *E. coli*, phages and plasmids have been isolated containing all F_0F_1 subunit genes, i.e., the whole *atp* operon [98–101]. This allows, for example, the construction of strains where the level of F_0F_1 in the membrane is increased several-fold [48]. The membranes of such overproducing strains offer advantages for structural studies. Furthermore, plasmids are now available containing only segments of the *atp* operon [101,102]. Thus, a subset of subunits or in certain cases even a single subunit can be expressed at an increased level in the cell (Fig. 10). The latter plasmids are useful, especially when introduced into mutant strains where large parts of the *atp* operon are deleted or not expressed.

In principle, a mutation in a subunit gene can affect the properties and function of F_0 at several levels. (i) The mutation may prevent the biosynthesis of a subunit or its integration into the membrane. (ii) A mutation may affect the assembly of F_0, even if the mutated subunit is integrated into the membrane. (iii) A mutation may affect the function of F_0, even if the altered subunit is assembled into F_0.

In the following, mutant strains will be de-

scribed, where both the genotypes and the phenotypic alterations have been studied in some detail.

IVB. Deletion mutants and plasmids

The deletion mutants of *E.coli*, compiled in Fig. 10, allow the study of membranes in which selected F_0 subunits are missing. Strain CM 2080, characterized in some detail, has a deletion of about 500 basepairs at the start of the subunit *a* gene [102]. Membranes of this deletion mutant contain F_0 subunits *b* and *c*, but only if isolated from exponentially growing cells. If membranes are prepared from mutant cells in the stationary growth phase, only subunit *c* is detected. Most likely, subunit *b* was proteolytically degraded in stationary phase cells [49]. As described in subsection IIIA, the large polar domain of subunit *b* is easily attacked by proteinases in vitro [49]. Another deletion mutant CM 1470 has a large deletion including genes of subunits *a*, *c*, *b*, δ and part of α [13]. The membrane of this strain is devoid of all F_0 subunits.

In other types of mutant, a *Mu* phage has been inserted at various points of the *atp* operon. The inserted phage DNA prevents the efficient transcription of all genes located downstream. These polar mutations, therefore, result in membranes which are devoid of certain subunits. One example is the strain AS 12 (Table IV) [103,104], where the *Mu* phage is inserted in the *atp* A gene coding for F_1 subunit α. Accordingly, F_1 subunits α, γ, β, and ε are not expressed. In addition, F_0 subunit *b* is not present in the membrane. Possibly, the defect in F_1 prevents either the assembly of subunit *b* or facilitates a proteolytic degradation.

Finally, several segments of the *atp* operon have been cloned on plasmids. One plasmid, pOM 11, contains the intact coding sequence for subunit *a* [13]. A functional subunit *a* is expressed from the cloned gene, since it complements the deletion of the subunit *a* gene in strain CM 2080. Another plasmid, pRPG 51 [101], contains the sequences coding for subunits *b* and δ. The cloned genes are expressed. The plasmids have been introduced into the deletion mutant CM 1470, where all F_0 genes are missing [13]. The strains obtained contain in the membrane either only F_0 subunit *a* or only subunit *b*.

TABLE III

ACTIVITIES OF F_0 IN *unc*-MUTANTS LACKING INDIVIDUAL F_0 SUBUNITS [102]

Strains: A 1 wild-type; CM 1470 and CM 2080 deletion mutants; the presence of subunit *b* depends on growth phase (log = logarithmic, stat = stationary phase); AM 12 and DG 10/6, polar mutants; pOM 11-1, plasmid with structural gene for subunit *a*; pRPG 51, plasmid with structural gene for subunits *b* and δ [102].

Strain	F_0 subunits	H^+ conduction	F_1 binding
A 1	a b c	+	+
DG 10/6	a b	−	+
CM 2080 (log)	b c	−	+
AM 12	a c	−	+
CM 1470 + pOM 11-1	a	−	+
CM 1470 + pRPG 51	b	−	+
CM 2080 (stat)	c	−	−
CM 1470		−	−

The membranes of all these strains, in which single or multiple F_0 subunits are genetically deleted, have been analysed for their capability to

rebind F_1 and to conduct H^+ [102] (Table III). Also included in these studies was a mutant membrane which is devoid of subunit *c* due to a point mutation in the *atp* E (*unc* E) gene (see below). The results are summarized in Table III. The membranes of these strains comprise all possible subsets of F_0 subunits. None of the F_0 subunits alone and none of the combinations of two F_0 subunits allows a H^+ conductance across the membrane. Subunit *a*, as well as *b*, each present individually in the membrane, promote the binding of F_1, while subunit *c* alone does not. Accordingly, each pairwise combination of two F_0 subunits allowed a rebinding of F_1 to the membrane. The observed physical binding of F_1 to incomplete F_0 must be different from the binding to intact F_0. The rebound F_1-ATPase is active, although the proton conductance is abolished in the incomplete F_0.

IVC. Point mutations

IVC-1. Mutants with defective F_0

All mutant strains compiled in Table IV revert

TABLE IV

E. COLI MUTANT STRAINS WITH DEFECTIVE OR DCCD-RESISTANT F_0

n.d., not determined.

Genotype or strain	Affected subunit	Amino acid exchange	Complementation: dominant(+), recessive(−)	Presence in the membrane	H^+ conduction	ATPase activity	Ref.
DG 25/3	a	n.d.	−	n.d.	−	+	105
DG 25/9	b	n.d.	−	+	+	+	105
unc F 469	b	n.d.	−	−	−	−	12
DG 7/1	c	Asp$_{61}$–Gly	+	+	−	+	108
DG 18/3	c	Asp$_{61}$–Asn	+	+	−	+	109
DG 27/10	c	Ala$_{21}$–Val	−	+	−	+	(Hoppe, J., Schairer, H.U. and Von Meyenburg, K.)
unc E 463 (= unc E 408)	c	Leu$_{31}$–Phe	−	−(+)[a]	−(+)[a]	−(+)[a]	2, 111
unc E 410	c	Pro$_{64}$–Leu	+	+	−	+	12, 2
unc E 429	c	Gly$_{23}$–Asp	−	−	−	−	12, 111
DC 13	c	Ile$_{28}$–Val	n.d.	+	+	+	115
DC 25	c	Ile$_{28}$–Thr	n.d.	+	+	+	115

[a] At high gene dosage.

readily, and consequently originated from single point mutations [12,105–112]. The mutational events in genes for subunit a and b have not yet been identified. Several amino acid substitution in the subunit c gene have been determined by amino acid sequence analysis of the mutant protein or nucleotide sequence analysis of the mutated gene [107–111]. Remarkably, most of the subunit c mutations are dominant over the wild-type allele [106,113]. A few subunit c mutations, and all analysed mutations in genes for subunit a and b, are recessive [12]. The dominant character of many subunit c mutants is explained most probably by negative complementation [113]. F_0 contains 6–10 subunit c polypeptides which function in an interdependent way. Thus, in a diploid situation a mutated subunit c, which is still assembled into F_0, will inactivate most part of the available wild type subunit c. Accordingly, such dominant mutations in subunit c do not interfere with the assembly process, whereas the recessive mutations apparently are defective in assembly. Negative complementation should occur either not at all or to a lesser extent with mutations in genes for subunits a and b, which exist in F_0 as single or dimeric copies, respectively.

In some of the compiled mutant strains, the affected subunit is not present in the membrane. No direct information is available on the subunit a mutant. This extremely hydrophobic protein could not be analyzed by two-dimensional gel electrophoresis, and antibodies are not yet available. Effects in subunit b mutants are difficult to interpret at the moment. The membrane of atp F mutant DG 25/9 contains subunit b, as determined by immunological techniques (Friedl, P., unpublished data). This mutant has a leaky phenotype, since ATP-dependent protontranslocation is partially retained [105]. A similar phenotype has been described recently for another atp F mutant [113]. In strain unc F 469 subunit b was not detected when whole membranes were analysed by two-dimensional gel electrophoresis [12]. Since subunit b is extremely prone to proteolytic degradation, as described above, it is uncertain whether the mutation prevents integration into the membrane or whether a defective assembly results in a proteolytic degradation. Tentatively, it may be concluded that the latter strain is assembly-defec-

tive, whereas in mutant DG 25/9 subunit b is assembled and thus protected from degradation.

The situation is better defined in subunit c mutants. In two strains, the mutation interferes with the integration of the polypeptide into the membrane. This is due to a substitution of Leu-31 by phenylanaline (unc E 463) and Gly-23 by aspartic acid (unc E429). [111]. The mutant subunit c containing Phe-31 is forced into the membrane, and a functional F_0F_1 originates, if the mutant gene is present at high copy number. This is not true for mutant subunit c containing Asp-23, i.e., a highly polar carboxyl group [111].

Of particular interest is the amino acid substitution of Ala-21 by valine (DG 27/10) (Hoppe, J., Schairer, H.U. and Von Meyenburg, K., unpublished data). This mutant subunit c is integrated into the membrane. The mutation is recessive, thus the mutant subunit c is apparently not assembled into F_0 (see above). In another mutant, the subunit c, exhibiting an exchange Pro-64 by leucine is present in the membrane [2]. The mutant allele shows a partial dominance over the wild-type allele. This suggests that this mutation does allow the assembly into F_0 [106,111].

Mutants DG 7/1 and DG 18/3 are both affected in the invariant DCCD-reactive aspartic residue 61 [107–110]. These mutations are dominant, and both mutant proteolipids are present in the membrane [113]. Thus, these alterations allow an assembly of the mutant protein into F_0.

The membranes of all F_0 mutants compiled in Table IV are tight for protons with the exception of unc F mutant DG 25/9. Thus, these mutations interfere with the H^+ conduction catalysed by F_0. This applies for mutants where the assembly of F_0 is defective. The phenotype of these point mutants corresponds to that of deletion mutants, which have been discussed in detail above (Table III). But it applies also to those mutants where the mutated subunit apparently is assembled into F_0.

Of particular interest is the question of whether the binding of the F_1-ATPase is influenced by mutations in F_0 subunits. The binding affinity of F_1 has not yet been quantitatively analysed. This may explain discrepancies reported for the rebinding of F_1 to F_0 from certain mutants by different groups [102,106]. Despite these uncertainties, the most surprising result is that in mutant strains

with apparently assembled F_0, the binding of F_1 is altered [109]. The bound ATPase activity of F_1 is not inhibited by the mutant F_0, as one would expect in analogy to the inhibition by DCCD-modified F_0 [114]. This is particularly puzzling in the case of mutant strains DG 18/3 exhibiting an exchange of the DCCD-reactive Asp-61 by an asparagine [109]. This minimal alteration leads to the impaired binding of an F_1 whose ATPase activity remains fully active. In contrast, if F_1 is rebound to wild-type F_0 whose Asp-61 has been modified by DCCD, the ATPase activity is inhibited [114]. Thus, even subtle changes in 'functional' residues lead to changes in the architecture of the whole complex. This structural or conformational effects are further indicated by the observation (Friedl, P., unpublished data) that subunit b is more easily extracted from F_0 of mutant strains DG 18/3 and DG 7/1 than from wild-type F_0.

IVC-2. Inhibitor-resistant mutants

Dicyclohexylcarbodiimide-resistant mutants have been isolated from $E.$ $coli$. Six mutant strains have been described in detail [107,115]. Measurement of the inhibition of ATPase activity as well as ATP-dependent H^+-translocation at various concentrations of DCCD revealed two classes of mutant strains [115]. Whereas the wild-type enzyme is inhibited half-maximally at 3–5 nmol DCCD per mg of membrane protein, the enzyme from the class-one and class-two mutants are inhibited half-maximally only at 30 and 200 nmol/mg, respectively. DCCD at higher concentration still binds specifically to subunit c as revealed by sequence analyses. For type one, Ile-28 was substituted by valine. In type two mutant protein, a threonine was found at position 28. Most likely, Ile-28 affected in all resistant mutants is part of the DCCD-binding site. Enzymic activities, such as ATPase activity and ATP-dependent H^+ translocation, of these mutants are comparable to the wild-type protein. These results provide indirect evidence that the protein folds back, bringing Ile-28 and Asp-61 into proximity.

The analysis of oligomycin-resistant mutant from $Saccharomyces$ $cerevisiae$ and $N.$ $crassa$ revealed that two proteins may be involved in the inhibitor binding. Besides the subunit c (proteo-

lipid) [97], in yeast subunit 6, a 259-residue protein has been found to determine oligomycin sensitivity of the ATP synthase complex [81] (Fig. 12). This protein is homologous to subunit a of the $E.$ $coli$ ATP synthase. Interestingly, the mutations in subunit 6 are located close to the small conserved region of the protein.

Four different amino acid exchanges leading to oligomycin resistance have been described for the yeast and three for $N.$ $crassa$ (proteolipid) [97,116]. All amino acid exchanges reside in the C-terminal hydrophobic stretch and are clustered around the invariant DCCD reactive glutamic (aspartic) residue. It is a distinct possibility, that the amino acid residues altered in the resistant mutants are involved in the binding of oligomycin. Thus, these residues might be located at the lipid/protein interphase. Some of them result in a hypersensitivity towards DCCD. Both facts suggest that the binding sites for DCCD and oligomycin are overlapping.

V. Functional aspects

VA. Evolutionary aspects

In recent years, numerous proteolipid subunits from various organisms have been sequenced, as extensively reviewed elsewhere [6]. The proteins contain 70–82 residues and all may be purified by chloroform/methanol extraction. They are extremely hydrophobic, containing only 16–25% polar residues, which explains their solubility in organic solvents.

Long hydrophobic stretches of about 25 amino acid residues are found both in the N-terminal as well as in the C-terminal part of the sequence. This is reminiscent of the most thoroughly investigated membrane protein, the bacteriorhodopsin from $Halobacterium$ $halobium$, where stretches of 25 hydrophobic amino acids are found to traverse the lipid bilayer most probably in an α-helical conformation (Fig. 11).

A comparison of the sequences of the homologous proteolipid subunits revealed numerous conserved amino acid residues. These residues might be indispensible for function or the maintainance of a certain structure of the protein. In the middle of the N-terminal hydrophobic stretch four glycine

residues are conserved (Gly-27, Gly-29, Gly-31, and Gly-33). Gly-42, Arg-45, Asn/Gln-46 and Pro-47 localized in the middle hydrophilic segment were also found to be invariant. In this segment the single conserved basic amino acid residue (Arg-45) occurs. The single invariant acidic amino acid residue is observed in the middle of the C-terminal hydrophobic stretch (position 65). With one exception (aspartic acid in *E. coli*), this amino acid is a glutamic acid. This acidic residue is flanked by two conserved alanine residues (position 62 and 66). At the C-terminus (position 80), a conserved phenylalanine was found.

There seem to be some specifically conserved residues either for the mitochondrial type or for the bacterial type of proteolipid. Lys-14 is typical for the mitochondrial proteins plus *Rhodospirillum rubrum*. For the same family also the following positions are occupied by an identical or isofunctional residue: Leu-16, Gly-17, Gly-19, Gly-24, Leu-39, Arg/Lys-50, Gly-60, Phe-61, Gly/Ala-68, Leu-69, Phe-70, Met/Val-73, Val-74 and Ala/Ser-75. For the bacterial family the following residues are invariant or isofunctional: Ala-16, Ala-17, Ala/Gly-18, Gly-22, Leu-23, Ala/Gly-24, Ala/Ser-25, Lys-38, Glu-41, Glu-48, Phe-58, Ile/Leu-59, Val/Met-61, Leu-63, Val-64, Pro-68 and Ile/Leu-70.

The two analyzed 'chloroplast' proteins resemble more the bacterial ones. It is noteworthy that the proteolipids from spinach and wheat chloroplasts have the same amino acid sequence [90,91].

These clear differences between the 'oligomycin-sensitive' proteolipids and the 'bacterial' proteolipids could indicate a different environment and different interactions of the protein in the assembled F_1F_0. Reports on the higher complexity in the subunit composition of F_0 in mitochondria support this view [4,123].

Amino acid sequences of proteins from other organisms homologous to subunit *b* from *E. coli* ATP synthase have not been reported so far.

For subunit *a*, homologous proteins have been sequenced. DNA sequences of the respective gene have been described for *S. cerevisiae*, human, bovine and mouse [81–84]. Conserved residues are indicated in Fig. 12.

VB. Chemical modification

Group specific reagents are versatile tools for detecting essential residues in enzymes. Ideally, these reagents modify single amino residues.

The interpretation and validity of chemical modification studies thus heavily depend on the determination of the modified amino acid(s). Furthermore, direct correlation between the extent of the modification of a specific amino acid residue with function must be established.

The binding of [14C]DCCD to subunit *c* (proteolipid) in all analyzed organisms was rather specific and occurred at low concentration (1–10 nmol/mg membrane protein) [6,118]. Maximum inhibition was observed when only a fraction of the subunits was labelled, indicating a cooperative interaction of the subunits [26,27,118–123]. For the beef-heart ATP synthase, the existence of a dimer of two proteolipids binding [14C]DCCD with a different affinity [123,124] compared with the monomeric subunits has been demonstrated.

Sequence analyses revealed an acidic residue (glutamic acid, with the exception of *E. coli* where aspartic acid was found) as the target of DCCD. This residue is located in a homologous position in all ten analyzed proteolipids [110,125].

The hydrophobicity of DCCD indicates that the acidic residue should be located in the lipid bilayer. This observation was confirmed by the use of the paramagnetic carbodiimide NCCD (*N*-2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N'*-(cyclohexyl) carbodiimide [126]. Ascorbate, a water-soluble agent, did not reduce the signal-generating nitroxide group. From spin-spin interactions between the NCCD molecules bound to the protein, it was suggested that the proteins exist in a polymeric form, in which some of the monomers are located at a maximal distance of 15–20 Å from each other. Bound NCCD was firmly immobilized as rotation correlation of 3–4 μs where determined. Reconstitution of the purified protein yielded a high mobility of 2–3 ns. Apparently, in this experiment the polymeric structure was not reconstituted.

The high immobilization of NCCD in native membrane may be caused by a rather tight fitting of the DCCD into a 'binding site' at the proteo-
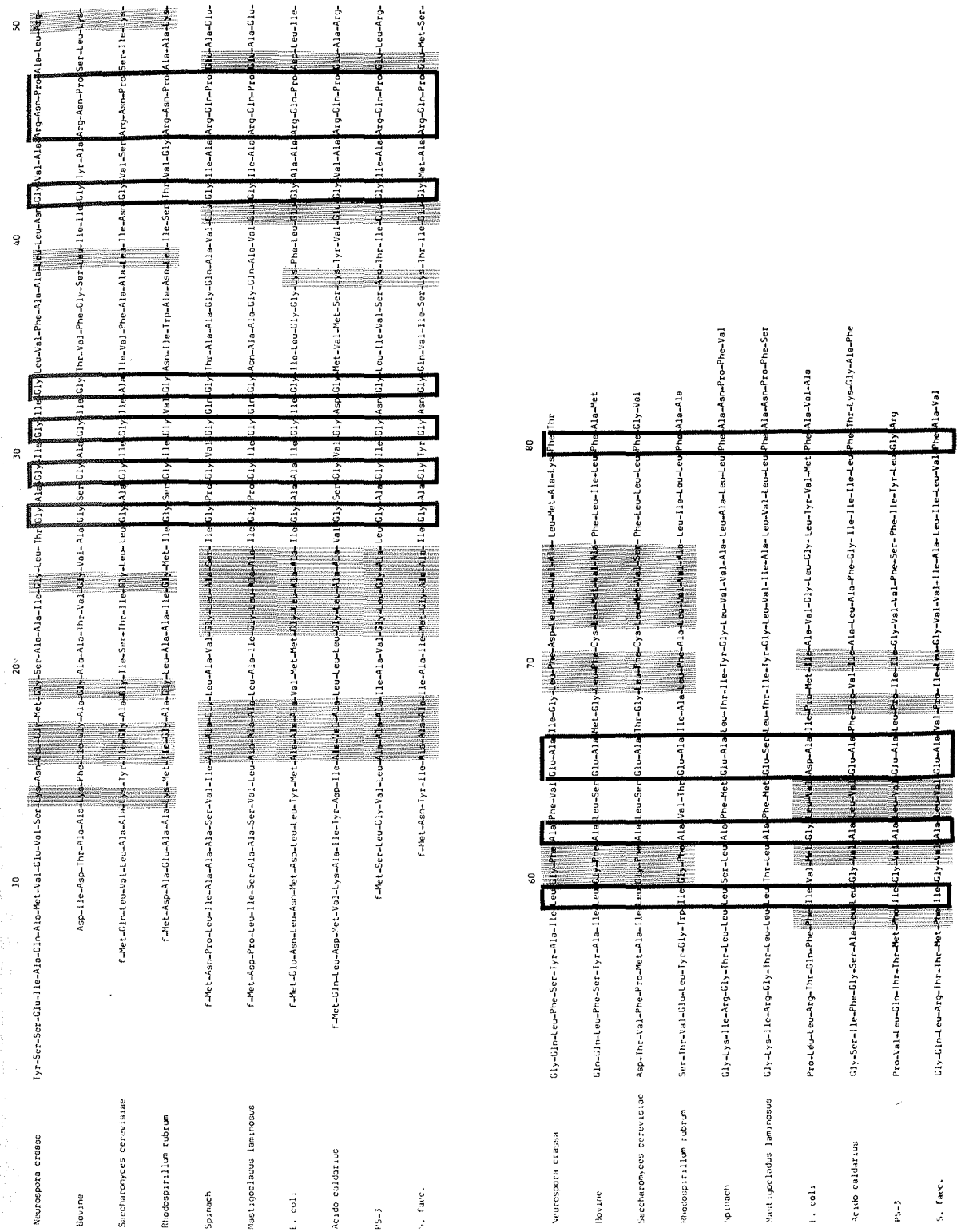
TABLE V

I_{50} VALUES FOR SOME DCCD ANALOGUES

$R - N = C = N - R'$, R = cyclohexyl. I_{50} values for the analogues were obtained using incubation for 16 at 0°C at various inhibitor concentrations by measuring either ATPase activity or ATP-dependent H^+ translocation.

Compound R	I_{50} at R' (nmol/mg membrane protein)
Phenylethyl	2
Cyclohexyl	5
2,2,6,6-Tetramethylpiperidyl-1-oxyl	5
Phenyl	20
Adamantyl	> 200

lipid subunit. To obtain further information on the steric requirements, a small set of DCCD analogues was synthesized (Table V) (Hoppe, J., unpublished data). Apparently, it is difficult to assign the different effect of these reagents exclusively to the size of the ligand, as the reactivity of the carbodiimide might be different. But, interestingly, the grade of substitution at the carbon atom in proximity of the carbodiimide seems to be crucial, as N-phenylethyl, N'-cyclohexyl carbodiimide which contains a primary carbon atom was more reactive than DCCD itself. N-adamantyl-N'-cyclohexyl carbodiimide, containing a tertiary carbon atom, was not an inhibitor. Further hints for a tight fitting of the shape of DCCD to the surface of the proteolipid came from the analyses of DCCD-resistant mutants from E. coli [115].

There are data on the modification of tyrosine with tetranitromethane and iodine and arginines with phenylglyoxal in a TF_0 preparation from PS-3 [127,128]. Modification of either of these residues inhibited the proton conductance. Though modification of these residues has been demonstrated in the proteolipid, it is not possible to correlate the modification of these residues with the inhibition of functions, since the fate of corresponding residues in the other membrane subunits has not been studied.

VC. H^+ conduction in reconstituted subunits

So far, reconstitution experiments with isolated subunits have only been performed with the pro-

teolipid subunit (subunit c). For the proteolipids extracted with butanol from lettuce chloroplasts or yeast mitochondria, a proton permeability of reconstituted lipid-proteolipid bilayers has been demonstrated [129–132].

Recently, single-channel conductance has been measured after reconstitution of the proteolipid from yeast into planar lipid bilayers [133]. Thus, in contrast to earlier studies, proton conductivities could be measured at a high-time resolution as a function of voltage, protein concentration, H^+ concentration and lipid composition. At the high H^+ concentration of about 10 mM (corresponding to a pH of 2.2), single-channel conductivity has been established.

Discrete current steps were measured, indicating the opening and closing of single channels. The observed ion path represents the operation of a single type of pathway with a mean conductance of 20 ps and a life-time of 4 ms. The single channels operate independently, since their occurrence obeys a Poisson distribution. About 10^7 protons pass each channel per s at 100 mV potential and pH 2.2. At pH 7 and 100 mV, about 100 protons/s are transported by one channel. The channel was highly selective for protons as the conductivity for potassium, sodium and chloride ions was at least 1000-fold lower.

Interestingly, most of the proteolipid molecules were not in an active state, explaining the low overall activity measured in previous systems [129–132]. The dependence of proton conductance on proteolipid concentration at pH 2.2 had a molecularity of about 2. The formation of channels from a large pool of proteolipid molecules thus involved a bimolecular reaction. In a most simple assumption, the associating species might be the monomeric proteolipid. In principle, however, the reacting species might already be a dimer or trimer.

Between the bimolecular dependence of the proton conductance of the proteolipid concentration at pH 2.2 and the linear dependence at pH 5.5, there was a transition point at pH 4.5 where the molecularities were much larger than 2. This suggests that the stabilization of the proton pathway at higher pH values involves the formation of structures larger than dimers. These associates were considered as oligomerized dimers.

But it could not be excluded that the increased conductivity at higher pH results from an increased proton permeability of a single channel.

In the reported experiments, the pH induced conductance transition was only found in the presence of cholesterol and sufficiently high proteolipid concentrations. The interaction between proteolipid subunits therefore may not be sufficiently strong to stabilize proton-conducting proteolipid oligomers in a biological membrane.

VI. Summary and Models

VIA. Subunit a

If the sequence of 271 amino acid residues of subunit a from E. coli is analyzed, seven segments can be discriminated where predominantly lipophilic residues are clustered (Fig. 12). In segments 1, 3, 4, 6 and 7, the hydrophobic character is most pronounced. With the possible exception of segment 1, no charged residues are present. The hydrophobic character of segments 2 and 5 is less pronounced. A charged residue occurs just in the middle of the segments. The hydrophobic profile of subunit a is similar to the respective profiles of the mitochondrial proteins [3] if a deletion from residue 125 to residue 150 in the E. coli protein is assumed. It is noteworthy that the homology between the E. coli and the mitochondrial subunits is restricted to a short segment near the C-terminus corresponding to residues 189–219 of subunit a from E. coli, whereas the rest of the polypeptide chains are completely unrelated. The similarity in the polarity profile suggests, however, that the general folding of the subunit has been conserved. The length of subunit a as well as the occurrence of six or perhaps seven lipophilic segments are reminiscent to the amino acid sequence of bacteriorhodopsin. It has to be mentioned that in bacteriorhodopsin, segments 3, 4, 6, 7 contain several basic and acidic residues and exhibit only a weak hydrophobicity [43,46]. Thus, in the F_0 subunit a, segment 2, despite its relatively high polarity, might traverse the membrane. In yeast subunit 6, two amino acid substitutions leading to oligomycin resistance have been identified [81]. The mutated residues would correspond to positions 195 and 256 in subunit a of E. coli, located in

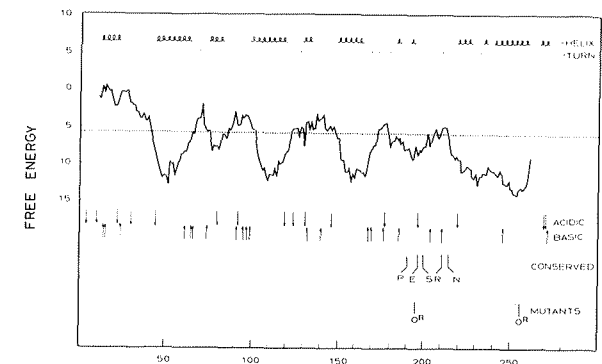Fig. 12. Prediction of membrane-permeating segments and secondary structures in F_0 subunit a from E. coli. α-helical regions (ℓℓ) and β-turns (T) were consistently predicted by four different prediction methods [77–80]. The free energy gains (KJ/mol) during a transition from a random coil in water to an α-helix in the membrane were calculated for all amino acid sequence positions using the parameters given by Von Heijne [134]. The locations of acidic (↓) and basic (↑) residues are indicated by arrows. A proline residue and four polar residues conserved in the amino acid sequences of E. coli F_0 subunit a and mitochondrially coded F_0 subunit 6 from yeast [81] and mammals [82–84] are indicated by the one-letter code. Two amino acid exchanges leading to oligomycin-resistance (O^R) were identified in F_0 subunit 6 from yeast [81].

the middle of the hydrophobic segments 5 and 7. These residues might be directly involved in the binding of oligomycin, and thus be located in proximity to each other at the lipid phase. But this straightforward conclusion is not possible if these mutated residues affect allosterically the oligomycin binding. This alternative will be discussed in detail with subunit c.

Subunit a is heavily labelled by the membrane-soluble carbene-generating label [^{125}I]TID [73] (cf. Fig. 5). Unfortunately, the modified amino acid residues could not yet be identified in this large hydrophobic protein. A rough quantitative evaluation of [^{125}I]TID radioactivity bound to each of the three F_0 subunits (see Fig. 5) indicates that subunit a is labelled twice as much as subunit b (Hoppe, J., Brunner, J. and Jørgensen, B.B. (1983), unpublished data). Accordingly, large parts of subunit a are accessible from the lipid phase.

About 20 amino acid residues of subunit a can be removed proteolytically from the cytoplasmic side with subtilisin (see Fig. 3) [49]. Since the C-terminus is very hydrophobic, it is most likely that the N-terminal end is susceptible to pro-

teinases, and thus located at the cytoplasmic surface [49]. Taken that seven transmembrane segments exist, this would indicate that the C-terminal end would be located on the periplasmic side. The proteolytic breakdown products of subunit a are difficult to analyze, even with membranes from an ATP synthase overproducing strain, due to the presence of many additional proteins. But at raised concentrations of proteinases the amount intact of subunit a in the membrane decreases, indicating that other parts of the polypeptide chain are accessible to proteinases. It is conceivable that polar segment domains of subunits exposed at the cytoplasmic surface of the membrane are involved in the binding of F_1 as was observed in mutant strains containing only this F_0 subunit.

VIB. Subunit b

The polarity profile of the sequence of 151 residues of subunit b is striking in that about 30 hydrophobic amino acids are clustered at the N-terminus (Fig. 13), whereas the rest of the polypeptide chain is very polar similar to a water-soluble protein. This immediately suggests that the N-terminus of subunit b is integrated in the membrane. In fact, all applied hydrophobic photoreactive probes reacted exclusively with this hydrophobic segment (Refs. 71 and 72, see also Hoppe,
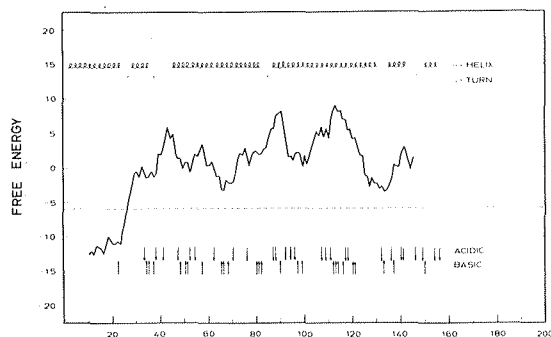

Fig. 13. Prediction of membrane-permeating segment and secondary structures in the F_0 subunit b from E. coli. α-helical regions (ℓℓ) and β-turns (T) were consistently predicted by four different prediction methods [77–80]. The free-energy gains for a transition from a random coil in water to an α-helix in the membrane was calculated for all amino acid sequence positions using the parameters given by Von Heijne [134]. The location of acidic (↓) and basic (↑) residues is indicated by arrows.

J., Brunner, J. and Jørgensen, B.B. (1983), unpublished data).

Labelling with the freely mobile carbene-generating probe [125I]TID started very closely to the N-terminus at Leu-3 and ceased at Trp-26. With a nitrene-generating probe fixed to the polar headgroups of a phospholipid ('shallow probe'. Fig. 14), residues Asn-2 as well as Cys-21 and Trp-26 were modified. Thus, the entire N-terminus up to Trp-26 is embedded in the membrane and the boundaries are defined by the residues affected by the 'shallow probe'. Most likely, the N-terminal segment traverses the whole phospholipid bilayer in an α-helical conformation [134,135]. But the other possibility, that the chain folds back and that Asn-2 and Trp-26 are located at the same boundary, is not excluded by the available experimental results [136]. At positions 27 and 28, two proline residues are located which will not fit into an α-helix, and, consequently, their peptide bonds can generate a rather large hydrophilic surface area. It is thus reasonable that the polypeptide chain leaves the membrane at this point.

Surprisingly, most of the N-terminal residues were accessible to the small diffusables probe [125I]TID. This demonstrates that this segment is not buried in a core of F_0 but rather is located at the periphery. As two subunits b exist in one F_0, it
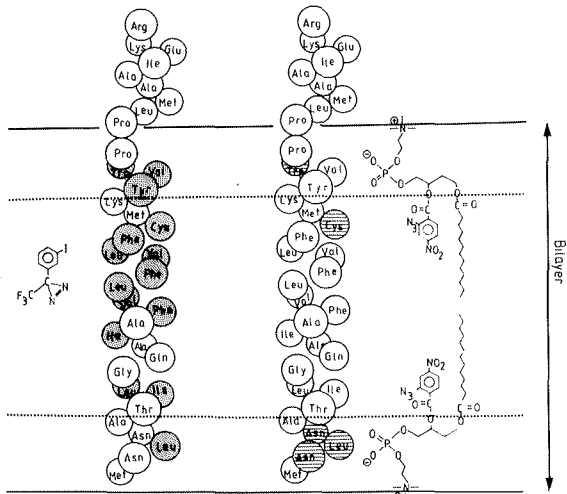

Fig. 14. A possible arrangement of the N-terminus from subunit b in the lipid bilayer. Shaded amino acid symbols indicate labelling with TID. Hatched areas indicate labelling with the 'shallow probe'.

remains uncertain whether the observed [125I]TID labelling pattern represents the same modification of each of the subunits or the sum of two different labelling patterns. Several of the residues not attacked by [125I]TID have hydrogen-bonding capacity (Asn-2, Thr-6, Gln-10, Lys-23, Tyr-24). These residues might be involved in contacts with other subunits. But it could well be that a microenvironment created by these side-chains does not allow the accumulation of [125I]TID at these positions and thus prevents an efficient modification.

The large polar domain of subunit b is clearly exposed at the cytoplasmic side of the membrane, since it can be completely degraded with proteinases [49]. The two molecules of subunit b in F_0 can be efficiently cross-linked (see Fig. 9). They exist, therefore, as a dimer. Most likely, the contact is formed by the large polar domain. This large polar domain binds F_1 as demonstrated in mutant membrane where the other F_0 subunits a and c are deleted [102]. This is not the only contact side between F_0 and F_1, since – as already mentioned – subunit a also is involved in the binding of F_1 [102]. In cross-linking experiment, dimers of subunit b with F_1 subunits α and β are observed. A close interaction of the F_1 subunits α and β with subunit b is also indicated by the observation that in certain mutant strains subunit b is protected against proteolytic degradation in vivo by the F_1-subunits α and/or β, even in the absence of the subunits γ, δ, and ε [49]. Interestingly enough, the polar domain of subunit b contains internal repeats [29]. Residues 53–82 are clearly homologous with residues 85–105. Furthermore, a shorter repeat is found, residues 84–98 are homologous to residues 101–113 [29]. Thus it can be visualized that the subunit b dimer contains four roughly equivalent domains which might interact with the multiple copies of subunits α and β present in F_1. Point mutations in subunit b (see Table III) [105,112] result in a weakened binding of F_1. Unfortunately, the mutated amino acid residue has not yet been identified.

VIC. Subunit c

The polarity profile of the amino acid sequence of the E. coli subunit c (see Fig. 15), i.e., two hydrophobic segments interrupted by a hydro-
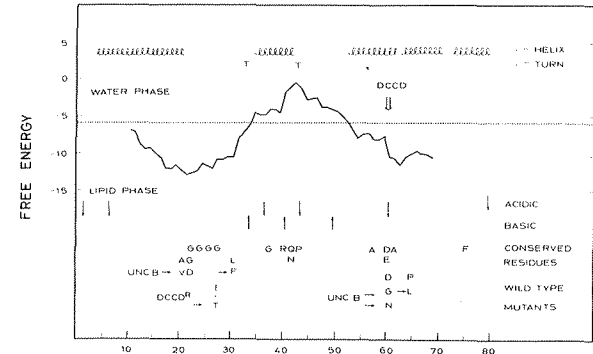

Fig. 15. Prediction of secondary structures and membrane-permeating segments in F_0 subunit c of E. coli. α-helical segments (ℓℓ) and β-turns (T) were consistently calculated applying four different prediction methods [77–80]. The free-energy gains during a transition from a random coil in water to an α-helix in the membranes were calculated for all amino acid sequence positions using the parameters given by Von Heijne [134].

philic segment, is found to be conserved in the homologous subunits from other bacteria, as well as from mitochondria and chloroplasts. This clustering of hydrophobic and hydrophilic residues immediately suggested that the protein might traverse the membrane twice in a hairpin-like structure. Indeed, labelling experiments with [125I]TID indicate both hydrophobic segments being located in the lipid bilayer. Especially intriguing is the location of the acidic residue Asp-61 in the membrane, as demonstrated by its reactivity towards only hydrophobic carbodiimides. Remarkably, an acidic group is not tolerated in the N-terminal segment at position 23 of the E. coli protein, as seen with subunit c from a mutant unc E 429, (see Table IV) which is not integrated into the membrane. Only distinct residues of the subunit c are accessible to the label [125I]TID in functional F_0. In certain special cases, the nonlabelling by TID might be explained by a low reactivity of the side chain (e.g., alanine; see Hoppe, J., Brunner, J. and Jørgensen, B.B. (1983), unpublished data) or by the instability of the reaction product (e.g., aspartyl ester). But certainly, in most cases residues are not labelled, since they are buried in the interior of the quarternary structure of F_0. It may be relevant in this connection that a mutant proteolipid from E. coli, which is unable to assemble (see Table IV), has an amino acid substitution

of Ala-21 by valine in a segment not accessible to TID.

Possibly, the small side-chain of the alanine at position 21 is part of a contact site in the tertiary structure of the proteolipid or the quarterly structure of F_0, and, therefore, no larger side-chain is tolerated at this position. The pattern of [125I]TID-labelled residues cannot be unambiguously interpreted at the moment, since the labelling of individual subunit c molecules in the F_0 might be different. It is conspicuous, however, that at the N-terminal segment the labelled residues would mark one side of a continuous α-helix. Also in the C-terminal part the labelled residues could be located on α-helical segments.

It has been discussed previously that certain residues of the proteolipid altered in oligomycin-resistant mutants from yeast and *Neurospora* are accessible from the lipid phase. It was therefore interesting to examine whether these residues correspond to the TID accessible residues in the *E. coli* proteolipid. Three amino acids altered in the proteolipid from inhibitor-resistant mutants occur at positions coinciding or juxtaposed to TID-labelled residues from the *E. coli* proteolipid (Fig. 16). In two instances, however, the mutated residue is located two positions apart.

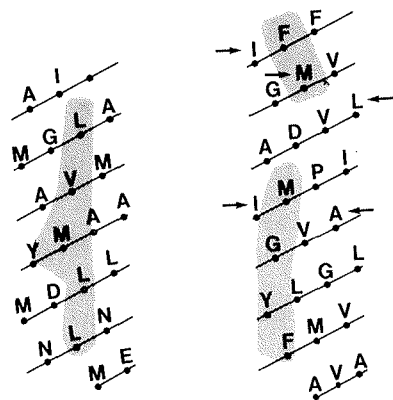Unfortunately, [125I]TID labelling experiments

Fig. 16. Areas of subunit c from the *E. coli* ATP synthase which are exposed at the lipid phase and positions of amino acid exchanges in oligomycin-resistant mutants from yeast and *N. crassa*. A planar representation of the two putative α-helices at the N-terminus and the C-terminus is shown. Shaded areas indicate residues possibly in contact with lipids as revealed by labelling with [125I]TID. Arrows indicate positions where amino acid substitution leading to oligomycin resistance was found.

have not been performed with the mitochondrial proteolipids. Thus, it is uncertain whether corresponding positions in the bacterial or mitochondrial proteolipids are equallly accessible from the lipid phase. It has to be stated, however, that in an α-helical confirmation, some mutated residues would point in opposite directions.

The various positions determining the oligomycin resistance all are located in the vicinity of the DCCD-reactive acidic residue in the C-terminal segment [97]. In contrast, only one position determining DCCD resistance in the *E. coli* proteolipid, i.e., Ile-28, was found in the N-terminal segment [115]. In a hairpin-like structure of the proteolipid, this residue would be located in proximity to the carbodiimide reactive aspartic residue. It is likely that Ile-28 is involved in noncovalent binding of e.g., the cyclohexyl moiety of DCCD. Surprisingly enough, Ile-28 is not accessible to TID in the functional F_0. Differences in sterical factors between TID and DCCD are difficult to evaluate, but are not a likely explanation for the different accessibility of Ile-28 for both compounds. It is a distinct possibility that either DCCD is bound via an 'induced fit' or that DCCD binds only to a certain conformation of the F_0 occurring with a low probability.

The experimental data discussed above are relevant with respect to the structural properties of the proteolipid. In order to elucidate the mechanism of the proton conduction, it is also important to identify functional residues – i.e., residues directly involved in proton conductance. It has been suggested that Asp-61 in the *E. coli* proteolipid is such a functional residue. (All structural evidence indicates this residue being located in the interior of the membrane.) Its selective modification with DCCD abolishes proton conductance. The importance of this residue was further indicated by the observation that this acidic residue is strictly conserved in the proteolipids from mitochondria, bacterial and chloroplasts. Futhermore, proton conduction is lost if this residue is genetically altered.

Some observations, however, are difficult to reconcile with a purely functional role of this acidic residue. This residue is accessible from the lipid phase and is therefore most likely not located in the interior of a pore. A minimal genetic altera-

tion of this residue (Asp → Asn) leads to impaired binding of F_1 (see chapter IVC-1) and the F_0 subunit b. Apparently, large conformational changes are induced in the whole F_0 by this mutation.

VID. Models for proton conductance

All available evidence points to the proteolipid subunit as the protonophoric entity of the F_0. Several lines of evidence argue against a direct participation of subunits a and b in transmembrane protontranslocation. The large hydrophilic part of subunit b might be involved in the coupling between the proton translocation across the membrane and ATP synthesis/hydrolysis on the F_1, but it is unlikely, that the short uncharged NH_2-terminal segment, which has only loose contact with the other subunits, provides residues participating in the mechanism of H^+ conduction. If subunit a contains the proton pathway, then only the few conserved residues may be considered which are clustered in a short segment. For subunit c Schindler and Nelson [133] provided convincing evidence that the isolated protein functions as a protonophore, when reconstituted in black lipid membranes. These in vitro experiments seem to contradict results obtained with mutant membranes from *E. coli*, which contain only the F_0-subunit c and which are unable to conduct protons [102]. This discrepancy might be resolved by the observation of Schindler and Nelson that the active proton channel formed in black lipid membranes is at least a dimer and probably a higher oligomer of the subunit c. *In vivo* this functional oligomer might be stabilized by the F_0 subunit a and/or b as originally proposed by Nelson and Schatz [92].

Therefore, at present any discussion on the mechanism of proton conductance will concentrate on a subunit c oligomer. However, based on the presently available structural data, the mechanism of proton conductance is completely obscure. The membrane spanning segments of subunit c contain only a few polar residues and only one charged residue – the invariant DCCD-reactive aspartic acid. Therefore, it appears difficult to construct a network of hydrogen bonds across the membrane from amino acid side-chains

[137]. An alternative would be that the oligomer of subunit c binds water either by the amino acid side-chains or by the polar groups of peptide bonds. In many instances, buried water clusters were found in the interior of globular proteins. For example, in studies with chymotrypsin, it was discussed that each region associated with buried charged groups contained an extensive hydrogen-bonded network including several buried water molecules [138]. Recent X-ray studies on alamethicin, which forms an oligomeric voltage-gated ion channel, shed light on the possible involvement of bound water molecules in cation transport [139]. The alamethicin monomer forms an helix which has a bend in the middle due to the occurrence of a proline residue. This proline-induced bend results in two free carbonyls and one free amide group of the peptide backbone, thus providing a hydrophilic surface (Fig. 17). It was visualized that this area, together with a buried glutamine residue, is able to bind a large number of water molecules. A corresponding water-binding structural element might be present in subunit c. In the middle of the C-terminal hydrophobic segment a proline residue is found in all bacterial proteins. In the mitochondrial and chloroplast proteolipids generally a helix-breaking residue (threonine, glycine) exists at the corresponding position. This bend is located four residues apart from the membrane buried acidic residue Asp-61.
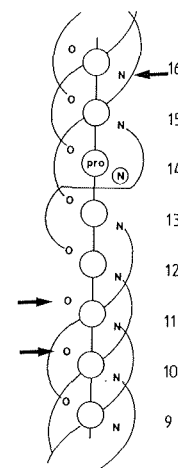

Fig. 17. Backbone hydrogen bonding in alamethicin around proline-14 [139]. The arrows indicate free carbonyl or amide groups of peptide bonds.

However, the participation of this acidic residue in the formation of a water cluster inside a pore is difficult to reconcile with its reactivity to DCCD.

The central question concerning all studies directed to structure and function is whether F_0 can exist in different conformations. In the F_1-ATPase, extensive conformational changes occur upon energization of the membrane and during ATP hydrolysis or synthesis [4]. It could well be that conformational changes in F_1 are accompanied or even coupled to conformational changes in F_0. Papa and co-workers [140] provided experimental evidence for two different conformation states of the proton conductor in dependency of the applied $\Delta\mu_{H^+}$ in beef-heart mitochondria. Also, the inhibitory action of oligomycin can be readily explained if this macrocyclic compound induces or stabilizes one structural state. The same inhibitory mechanism may apply for DCCD. Pleiotropic effects of amino acid substitutions in subunit c (see subsection IVC) might be caused by allosteric interaction. A mechanism of proton translocation by a reorientating basic or acidic group has been developed by Boyer [141]. Obviously, such a mechanism requires conformational changes in the F_0. It should be possible with the techniques now available to define these putative conformational states more precisely and to correlate such states with the function of the enzyme.

VII Perspectives

ATP synthase and the proton conducting component F_0 from the bacterial PS-3 [48] and *E. coli* [9–11] have been purified only a few years ago. Since then, our understanding of structure and function of F_0 has rapidly advanced, as detailed in this review. For the studies on F_0, the experimental possibilities now available have not been fully exploited. The availability of isolated subunit F_0-genes will allow the contruction of defined mutants by site-directed mutagenesis. Hopefully, mutants will be obtained which exhibit specific defects, e.g., only in proton conductance or only in F_1-binding. Furthermore, the assembly of F_0 might now be studied, and an in vivo reconstitution of F_0 seems to be feasible after recombination of the isolated subunit genes with appropriate expression vectors.

The topology of all three F_0-subunits might be elucidated in more detail by means of antibodies, directed against defined polar segments of the polypeptide chains. The amino acid sequences will furthermore give hint for possible chemical modification studies directed towards identification of functional residues or towards the accessibility of certain segments.

Conformational changes might be defined by membrane permeating photoreactive probes and by the covalent attachment of reporter groups, i.e., spin labels or fluorescence label. Finally, it might be possible to obtain F_0-crystals sufficient for high resolution X-ray analysis. Hopefully, the results obtained by proteinchemical, physiochemical and genetic methods then can be integrated into a consistent picture.

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