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Topological studies suggest that the pathway of the protons through F_0 is provided by amino acid residues accessible from the lipid phase

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Summary – The structure of the F_0 part of ATP synthases from *E. coli* and *Neurospora crassa* was analyzed by hydrophobic surface labeling with [^{125}I]TID. In the *E. coli* F_0 all three subunits were freely accessible to the reagent, suggesting that these subunits are independently integrated in the membrane. Labeled amino acid residues were identified by Edman degradation of the dicyclohexylcarbodiimide binding (DCCD) proteins from *E. coli* and *Neurospora crassa*. The very similar patterns obtained with the two homologous proteins suggested the existence of tightly packed α-helices. The oligomeric structure of the DCCD binding protein appeared to be very rigid since little, if any, change in the labeling pattern was observed upon addition of oligomycin or DCCD to membranes from *Neurospora crassa*. When membranes were pretreated with DCCD prior to the reaction with [^{125}I]TID an additionally labeled amino acid appeared at the position of Glu-65 which binds DCCD covalently, indicating the location of this inhibitor on the outside of the oligomer. It is suggested that proton conduction occurs at the surface of the oligomer of the DCCD binding protein. Possibly this oligomer rotates against the subunit *a* or *b* and thus enables proton translocation. Conserved residues in subunit *a*, probably located in the lipid bilayer, might participate in the proton translocation mechanism.

proton conduction / membrane proteins / carbenes

Résumé – Etudes topologiques qui suggèrent que l'itinéraire des protons à travers F_0 passe par les résidus d'amino-acides accessibles à partir de la phase lipide. *La structure de la partie F_0 de l'ATP synthase a été analysée au moyen de marquage par le réactif hydrophobe TID[^{125}I]. Les trois sous-unités de* E. coli *F_0 sont accessibles au réactif ce qui semble indiquer que ces sous-unités sont intégrées dans la membrane de façon indépendante. Les amino-acides marqués ont été identifiés par la dégradation d'Edman des protéines d'*E. coli *et de* Neurospora *associées au dicyclohexylcarbodiimide (DCCD). L'analogie des courbes obtenues pour les deux protéines homologues suggère l'existence d'α-hélices rangées de façon serrée. La structure oligomérique de la protéine associée au DCCD semble être très rigide puisque pratiquement aucun changement dans le marquage n'a été observé par addition d'oligomycine ou de DCCD aux membranes de* Neurospora crassa. *Quand les membranes sont traitées avec le DCCD avant la réaction avec TID[^{125}I], un amino-acide additionnellement marqué apparaît à la position Glu-65 et forme avec le DCCD une liaison covalente. Ce dernier résultat indique la localisation de cet inhibiteur à l'extérieur de l'oligomère. Il semble donc que la conduction des protons ait lieu à la surface de l'oligomère de la protéine associée au DCCD. Il serait possible que l'oligomère se retourne contre la sous-unité* a *ou* b, *permettant de ce fait la translocation des protons. Les résidus conservés de la sous-unité* a, *probablement localisés dans la double couche lipidique, pourraient participer au mécanisme de translocation des protons.*

conduction de protons / protéines membranaires / carbènes

Introduction

The membrane-integrated part F_0 of the ATP synthase translocates protons across the membrane. In whole ATP synthase (F_1F_0) the F_0-mediated proton transport is tightly coupled to the synthesis or hydrolysis of ATP catalyzed by the F_1 part. By removal of F_1, the proton pathway in F_0 is opened, and can be studied independently [1–4]. Recently, a functional F_0 was reconstituted from its isolated components [5]. The availability of the complete primary structure of the eight subunits of the F_1F_0 from *E. coli* and an increasing number of protein chemistry data, obtained with crosslinking reagents, proteolytic digestions and hydrophobic labeling techniques, has enabled us to propose models for the arrangement of the three F_0 subunits in the membrane [1–4,6–18].

Based on this mainly structural information we propose a rotational motion model involved in the proton translocation across the membrane which might find its counterpart in rotations postulated in F_1 during ATP synthesis or hydrolysis [19].

Secondary, tertiary and quarternary structure of the subunits

Subunit a

When the sequence of 271 amino acid residues of subunit *a* from *E. coli* is analyzed, seven sequences can be distinguished where lipophilic residues are predominantly clustered (Fig. 1). In segments 1, 3, 4, 6 and 7, the hydrophobic character is most pronounced. There is some discussion about the membrane integration of segment 2 [1–3]. The hydrophobic profile of subunit *a* is similar to the respective profiles of the mitochondrial proteins [20–23] if a deletion is assumed from residue 125 to residue 150 in the *E. coli* protein. It is noteworthy that the homology between the *E. coli* and the mitochondrial subunit 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 profiles suggests, however, that the general folding of the subunit has

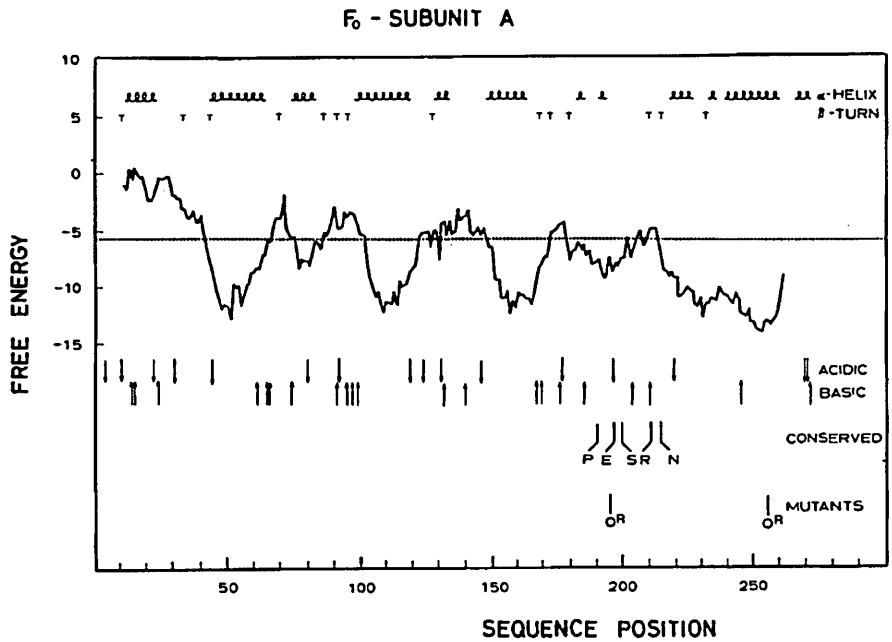

Fig. 1. Prediction of membrane-permeating segments and secondary structures in F_0 subunit *a* from *E. coli*. The α-helical region (⌇⌇⌇) and β-turns (T) were consistently predicted by our different methods [3,24]. The free energy gains (kJ/mol) during a transition from a random coil in water to an α-helix in the membrane were calculated for all amino acid sequence positions using the parameter given by van Heijne [3]. Values were averaged over a segment of 20 consecutive residues. The location of acidic (↓) and basic (↑) residues are indicated by arrows. A conserved proline residue and four conserved polar residues are indicated by the one-letter code. The positions of two amino acid exchanges in the yeast protein leading to oligomycin resistance are indicated.

been conserved. In yeast subunit 6, two amino acid substitutions leading to oligomycin resistance have been identified [20]. The mutated residues would correspond to positions 195 and 256 in subunit *a* of *E. coli*, located in 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 and subunit *c* in the lipid phase. But this straightforward conclusion is not possible if these mutated residues affect the oligomycin binding allosterically.

Subunit *a* is heavily labeled by the membrane-soluble carbene-generating label [^{125}I]TID [14]. So far, the modified amino acid residues could not 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 from *E. coli* indicates that subunit *a* is labeled twice as much as subunit *b* [14]. Accordingly, large parts of subunit *a* are accessible from the lipid phase. In *Neurospora crassa* mitochondria there is also TID labeling of subunit 6 corresponding to subunit *a* of *E. coli*, indicating that this subunit 6 is in a similar environment [15].

Subunit b

The polarity profile of the sequence of 151 residues of subunit *b* is striking in that about 30 hydrophobic residues are clustered at the N-terminus, whereas the rest of the polypeptide chain is very polar, similar to water-soluble proteins. In fact, all the hydrophobic photoreactive probes applied reacted exclusively with this hydrophobic segment [12–14].

Labeling with the freely mobile carbene-generating probe [^{125}I]TID started very close to the N-terminus at Leu-3 and ceased at Trp-26. With a nitrene-generating probe fixed to the polar head group of a phospholipid, 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. Most likely, the N-terminal segment traverses the whole phospholipid bilayer in an α-helical conformation.

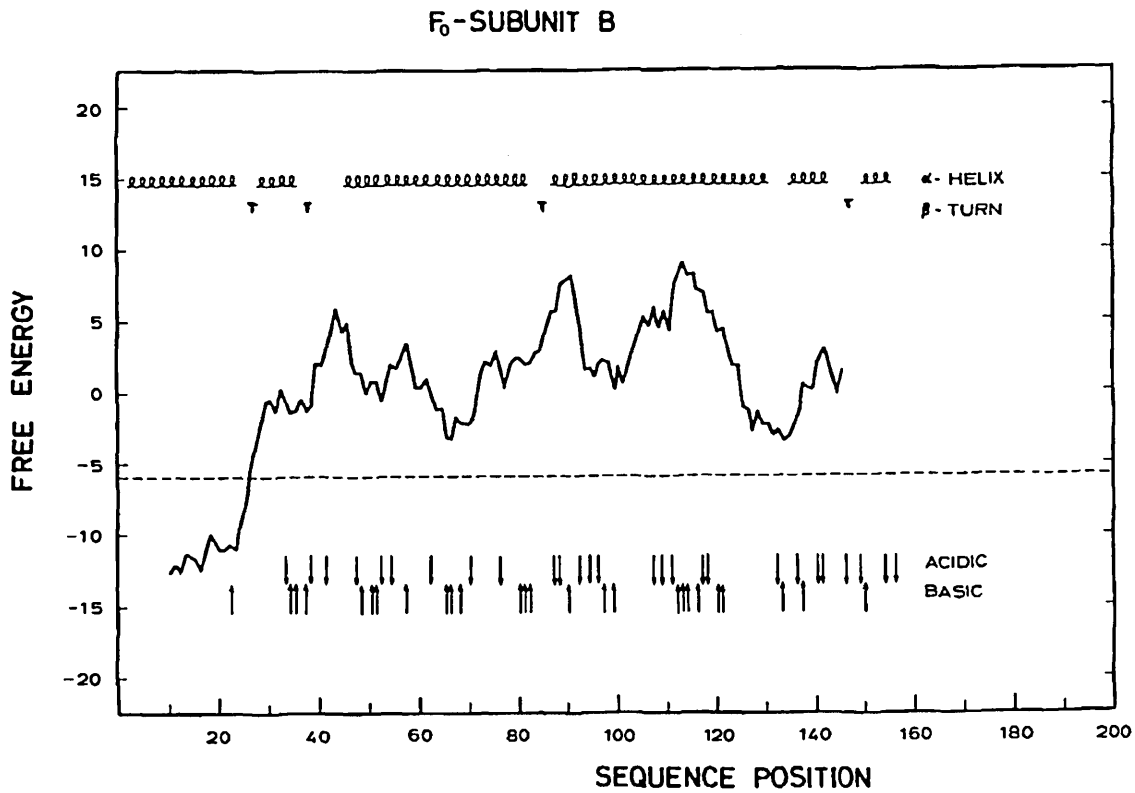

Fig. 2. Prediction of membrane-permeating segments and secondary structures in the F_0 subunit from *E. coli* (*cf.* Fig. 1).

Surprisingly, most of the N-terminal residues were accessible to the small diffusable probe. This demonstrates that this segment is not buried in a core of F_0 (oligomer of subunit *c*) but rather is located at the periphery. Several of the residues not attacked by [^{125}I]TID have hydrogen-bonding capacities (Asn-2, Thr-6, Gln-10, Lys-23, Tyr-24). These residues might be involved in contacts with other subunits.

The experiments with a photoreactive lipid [12] show that the N-terminus of subunit *b* must have some contact with lipid. This is especially evident from the experimental procedure when purified F_1F_0 was added to preformed liposomes containing the photoreactive phospholipid.

The large polar domain is clearly exposed at the cytoplasmic side of the membrane, since it can be completely removed by proteinases. Removal of the polar domain had no effect on the proton permeability of F_0. The two molecules of subunit *b* can be efficiently cross-linked. They exist therefore as a dimer [3, 11, 16–18].

Remarkably, in *Neurospora crassa* mitochondria there was no labeling by [^{125}I]TID of a corresponding protein [16]. Unfortunately the subunit composition of F_0 from *Neurospora crassa* has not yet been established, but from other eukaryotic ATP synthases it is clear that a corresponding protein is missing.

Subunit c

The polarity profile of the amino acid sequence of the *E. coli* subunit *c* (Fig. 3), *i.e.*, two hydrophobic segments interrupted by a hydrophilic segment, is found to be conserved in the homologous subunits from other bacteria, as well as from mitochondria and chloroplasts [4, 24]. This clustering of hydrophobic and hydrophilic residues immediately suggests that the protein might traverse the membrane twice in a hairpin-like structure. Indeed, labeling experiments with [^{125}I]TID indicate both

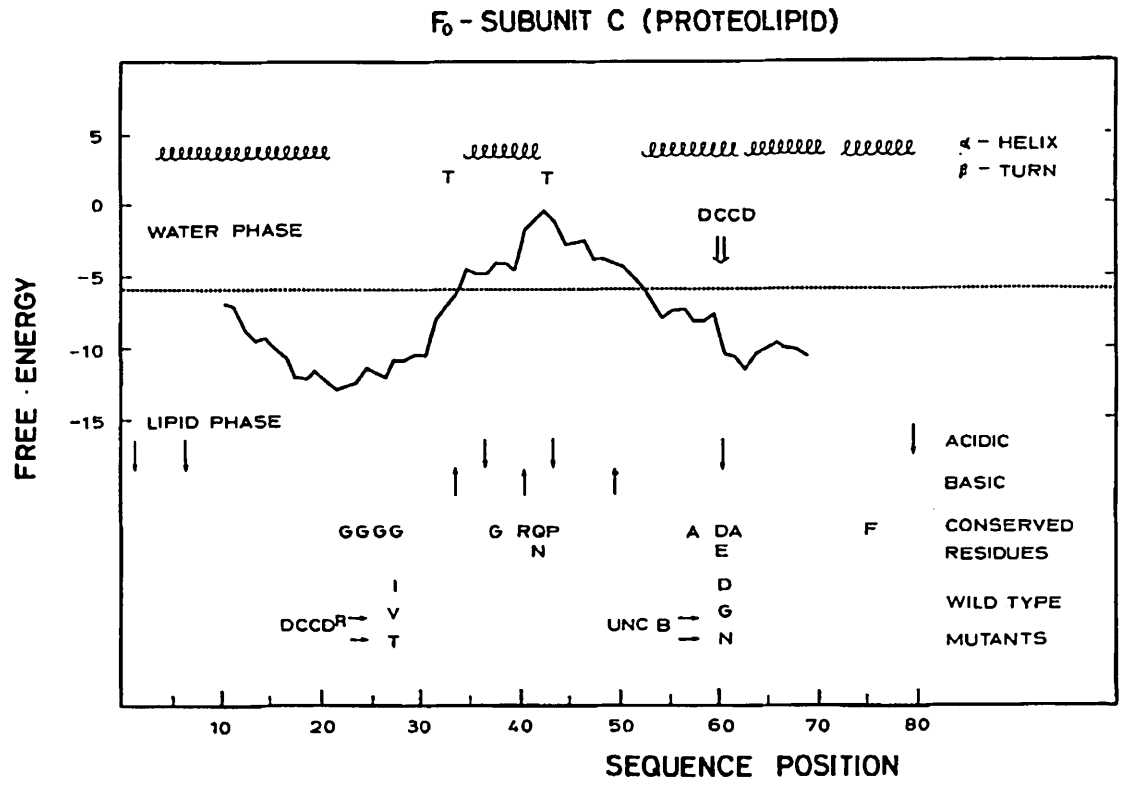

Fig. 3. Prediction of secondary structures and membrane-permeating segments in F_0 subunit *c* of *E. coli*. The location of conserved residues and of mutated residues are indicated by the one-letter code.

hydrophobic segments as being located in the lipid bilayer [14]. But labeling of subunit *c* is strikingly different from that of subunit *b* since only discrete amino acids were labeled. Strikingly similar labeling patterns were obtained in the homologous proteolipid subunits (subunit *c*) from *E. coli* and *Neurospora crassa* [15] (Fig. 4). Labeled residues occupy identical or juxtaposed positions. These findings are even more remarkable since labeling occurs in segments which are not homologous. These results suggest that both proteins have very similar secondary structures and environments.

YSSEJAQAMVEVSKNLGMGSAAJGLTGAGJGJGLVFAALLNGVARNP
MENLNMDLLYMAAAVMMGLAAJGAAJGJGJLGGKFLEGAARQP

ALRGQLFSYAJLGFAFVEAJGLFDLMVALMAKFT
DLJPLLRTQFFJVMGLVDAJFMJAVGLGLYVMFAVA

Fig. 4. Comparison of [^{125}I]TID-labeled residues in the sequence of the proteolipid subunit (subunit *c*) from *Neurospora crassa* (first line) and from *E. coli* (second line). Labeled residues are indicated by bold letters.

Interestingly, those residues of segments that were labeled are distributed in such a way that they would lie on the same side of an α-helix [14, 15]. Radioactive patterns is which consecutively labeled residues appear in a sequence with an average periodicity of 3 to 4 residues may therefore be indicative of: (1) an α-helical conformation of the respective polypeptide segment, and (2) tight packing exposing only a fraction of the helix surface to the lipid phase.

Based on these findings the following conclusions regarding the topology of the proteolipid subunits may be drawn: The N-terminal segment from residues 9–25 is membrane-integrated and coiled up in an α-helical conformation. The entire C-terminal moiety starting from residue 55 is membrane-embedded and exists in two α-helical segments. The absence of label within the segment ranging from residue 40 to residue 52 provides supporting experimental evidence that this part of the polypeptide chain extends from the lipid phase into the cytoplasm [3, 25]. Although, as inferred from its hydrophobicity, the glycine-rich, conserved segment from residue 26 to about 37 would be predicted to be embedded in the lipid bilayer, no labeling was obtained in ATP synthases from either organism. In *E. coli*, however, this stretch was labeled in an SDS solution. The absence of label in the native protein *c* (proteolipid) was thus not due to a low reactivity of the amino acids in this segment. These observations are interpreted to suggest that this region is located within the membrane but shielded by other polypeptide segments from the lipid bilayer. In this case the lipid phase would not exert an α-helix promoting force on this segment, which, as predicted (Fig. 3), may adopt a β-sheet conformation [14, 24]. It is possible that in the oligomeric complex these sheets are assembled into a β-barrel, which is a common structural element of many proteins (Fig. 6).

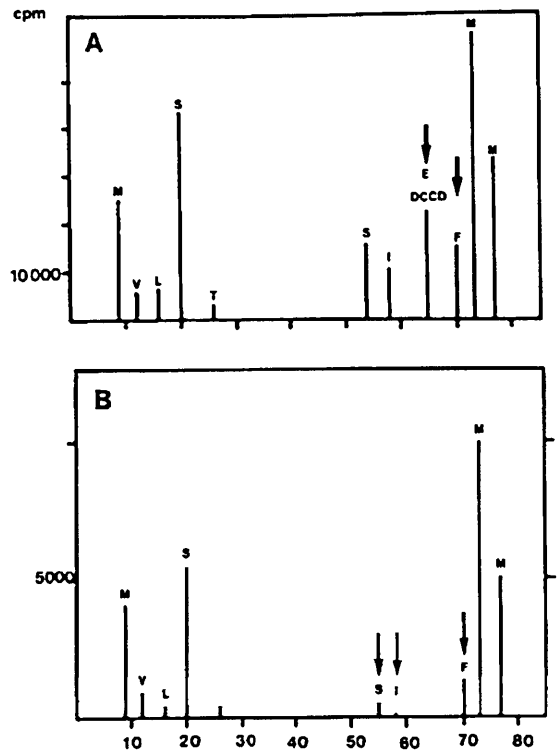

Fig. 5. Histogram of labeled amino acid residues in the sequence of the proteolipid subunit from *Neurospora crassa*. Labeling was performed after incubation with DCCD (A) or in the presence of oligomycin (B).

A mutation has been found remarkably close to this segment with a valine at position 25 (Fig. 4) instead of an alanine [3]. The mutant protein integrates into the membrane but does not assemble in the F_0 complex. Possibly, the small side-chain of the alanine at this position is part of a contact site in the tertiary structure of the proteolipid or the quarternary structure of the F_0.

Oligomycin and DCCD specifically inhibit F_0-mediated H^+ conduction. The inhibitory

mechanism is still uncertain. One possibility is that the inhibitors induce or stabilize a nonfunctional conformation. Therefore, it was interesting to see whether bound oligomycin or DCCD change the TID-reactive residues in F_0. The labeling profile in Fig. 5B was obtained after reaction with *Neurospora crassa* mitochondria in the presence of an oligomycin concentration 10-fold higher than necessary for maximal inhibition. The identical group of residues was TID-reactive in the presence and in the absence of oligomycin. Only quantitative differences were observed; the labeling of Ser-55, Ile-58 and Phe-70 was reduced by more than 50%.

Fig. 5A presents the histogram of TID-reactive residues in DCCD-modified proteolipids from mitochondrial F_0. Although a large excess of DCCD had been applied (300 nmol/ng protein) the accessibility of residues towards TID remained essentially unaltered with the exception of a possibly slightly reduced labeling of Phe-70.

Apparently, the conformation of F_0 in nonenergized membranes is not altered by the binding of oligomycin and DCCD to an extent which would result in changes of the lipid–protein interphase detectable by TID-accessible residues. This supports the above notion that the proteolipid oligomer forms a compact and rigid core in the F_0.

An intriguing feature of all analyzed proteolipid subunits is an acidic group located in the middle of the C-terminal segment (Glu-65) [24,26]. This residue is the target of the hydrophobic inhibitor dicyclohexylcarbodiimide (DCCD) which binds covalently to this residue. Furthermore, the importance of this acidic group has been demonstrated by mutations at this position [27–29] leading to a nonfunctional F_0. Therefore, it is likely that this acidic residue plays a functional role in proton conductance. Unfortunately, the reaction products between [^{125}I]TID and carboxygroups are not stable and no information about the location of this important group could be derived from the [^{125}I]-TID labeling patterns [14]. This drawback could be circumvented when Glu-65 in the *Neurospora crassa* proteolipid was first labeled with DCCD. Fig. 5A shows that a considerable amount of ^{125}I radioactivity was recovered at step 65 corresponding to the modified Glu-65. This result indicates that at least the DCCD covalently bound to Glu-65 is in contact with lipids and thus located at the outside of the oligomer. Based on the labeling of the proteolipid with DCCD it had already been speculated that Glu-65 is located at the surface of F_0, possibly at the protein–lipid interphase, rather than in the interior of a channel or pore [24].

Rotational motion of subunit *c* as a possible mechanism in the proton translocation

If we construct models for proton translocation we have to consider the following observations:

(1) All three subunits are necessary for proton conduction. This was shown by two independent approaches. Friedl *et al.* [30,31] used *E. coli* strains which expressed all possible combinations of the individual subunits of the F_0 part. Only when all three subunits were expressed was proton translocation detected. Schneider and Altendorf [5] dissociated isolated F_0 into its subunits. Only when all three subunits were reconstituted in stoichiometric amounts was proton translocation restored.

(2) The high conservation of subunits and their sequences of ATP synthases implies that an identical mechanism is used for proton translocation. Thus, we have to focus our attention on the conserved residues.

(3) The individual subunits are seperately integrated into the membrane. Subunits *a* and *b* are not surrounded by a circle of subunit *c*, as was discussed in the previous section.

(4) The DCCD-reactive glutamic acid or aspartic acid which seems to be intimately involved in proton translocation is located on the outside of the oligomer of proteolipid subunits. It is therefore difficult to construct a conducting pore inside a protein structure (proteolipid subunits).

All available evidence indicates that the proteolipid subunit is directly involved in proton translocation. For the homologous protein from yeast Schindler and Nelson provided convincing evidence that the isolated subunit functions as a protonophore when reconstituted in black lipid membranes [34]. These *in vitro* experiments seem to contradict results obtained with *E. coli* F_0 as discussed above. This discrepancy might be resolved by the observation that the active channel formed in black lipid membranes is at least a dimer and probably a higher oligomer of subunit *c*. *In vivo,* this functional oligomer might be stabilized by other F_0 subunits.

However, it seems difficult to construct a hydrogen bond network across the membrane using only the proteolipid subunit, since the membrane-spanning segments of this protein contain only a few polar residues and only one charged residue – the invariant Glu-65. But a proline occurs very close to this residue in the sequence of bacterial proteolipid subunits. In mitochondrial or chloroplast proteolipids this position is occupied by threonine or glycine which also have a tendency to act as α-helix breakers. A break in the α-helix would result in several free carbonyl and amide groups, thus gen-

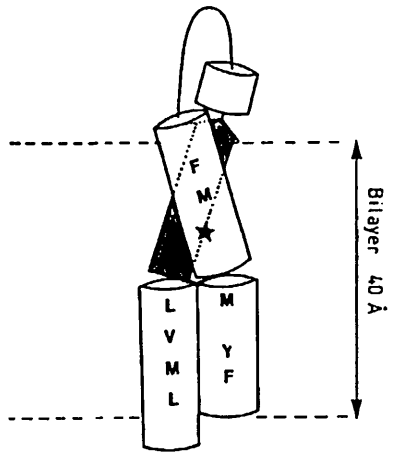

Fig. 6. A possible structure for the proteolipid subunit (subunit *c*) of *E. coli* . [^{125}I]TID-labeled residues are indicated by the one-letter code. The star indicates the position of the conserved amino acid. Cylinders represent α-helix, and the shaded arrow β-sheet.

erating a hydrophilic surface which is able to bind water molecules. A similar structure has been recently described for alamethicin which also contains a proline in the middle of a hydrophobic segment [35].

Nevertheless, the number of hydrogen-bonding residues would still be too small to form a network across the membrane.

Other mechanisms of proton translocation imply the migration of charged residues through the membrane, assuming large conformational changes in the F_0. The limitation of our method using [^{125}I]-TID as a monitor of conformational changes has not enabled us to detect large alterations in the conformation.

The most plausible model which attempts to integrate all discussed considerations seems to be as follows. Proton conductance may occur when a certain Glu-65 is in contact with residues of the other two F_0 subunits. It might be speculated that the conserved hydrophilic residues in subunit *a*, which are located on two membrane-embedded segments, directly participate in the proton translocation mechanisms. In any event it is certain that the membrane segment of subunit *b* also plays a decisive role proton translocation since it cannot be removed without loss of activity.

We have to recall our assumption that the three subunits are integrated separately in the membrane and that the oligomer of the proteolipid subunits forms a very rigid core. If we postulate that proton conductance occurs at the interphase between the F_0 subunits, only one out of the 6–10 Glu-65 residues would be involved at a given time in proton translocation.

It seems therefore most attractive to speculate that the core of proteolipids rotates against the other two F_0 subunits, thus bringing the other Glu-65 residues into contact with the residual proton wire on the other subunit(s). This model would combine the two basic mechanisms: proton translocation through a hydrogen bond network and by conformational changes.

In general this model is very similar to the flagellar rotor [32, 33] which also uses protons to drive the flagella of *E. coli*. It is also highly interesting that at the present time rotational models are emerging for the catalysis in the F_1 part of ATP synthase. A 'rolling well and turnstile' hypothesis has been recently presented by P. Mitchell [19].

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