THE F₀ COMPLEX OF THE PROTON-TRANSLOCATING F-TYPE ATPase OF ESCHERICHIA COLI

BY GABRIELE DECKERS-HEBESTREIT AND KARLHEINZ ALTENDORF

Universität Osnabrück, Fachbereich Biologie/Chemie, Arbeitsgruppe Mikrobiologie,
Postfach 4469, D-4500 Osnabrück, FRG

Summary

The ATP synthase (F₁F₀) of Escherichia coli consists of two structurally and functionally distinct entities. The F₁ part is composed of five subunits α, β, γ, δ and ε (3:3:1:1:1) and carries the catalytic centres of the enzyme. The membrane-bound F₀ complex functions as a proton channel and consists of the three subunits a, b and c (1:2:10±1).

Subunit c (8288 Mᵋ) exhibits a hairpin-like structure within the membrane. A conserved acidic residue (Asp-61) in the C-terminal hydrophobic segment is absolutely required for proton translocation through F₀, whereas the hydrophilic loop region is necessary for F₁ binding. Expression of the chloroplast proteolipid together with subunits a and b of E. coli did not produce an active F₀ hybrid complex. Therefore, the construction of hybrid c subunits consisting of parts of the proteolipid from both organisms is in progress to determine those parts of subunit c that are essential for a functional interplay with subunits a and b.

Subunit a (30276 Mᵋ), which is also involved in proton translocation, is an extremely hydrophobic protein with 5–8 membrane-spanning helices. Studies with alkaline phosphatase fusion proteins resulted in controversial conclusions about the localization of the N and C termini of the protein. A foreign epitope (13 amino acids) has been inserted into the N- or C-terminal region of subunit a without affecting the function of F₀. Binding studies with a monoclonal antibody against this epitope are now under investigation to determine the orientation of subunit a.

Subunit b (17265 Mᵋ) is anchored in the membrane by its apolar N-terminal region, whereas the hydrophilic part protrudes into the cytoplasm. Studies with proteases and truncated b' subunits revealed that the C-terminal part of subunit b is involved in binding of F₁ to F₀ and is necessary for correct assembly of F₀.

ATP synthase

The membrane-bound ATP synthase (F-type ATPase) of Escherichia coli (EF₁F₀) catalyzes the synthesis of ATP in a reaction that is driven by an electrochemical gradient of protons generated by respiration. The enzyme can also function in the reverse direction, generating an electrochemical proton gradient by ATP hydrolysis. Other bacteria, mitochondria (MF₁F₀) and chloroplasts (CF₁F₀) contain similar enzyme complexes (Schneider and Altendorf, 1987; Fillingame, 1990; Senior, 1990). However,
in *Propionigenium modestum* an F-type ATPase (PF$_1$F$_0$) is present, which utilizes an
electrochemical gradient of sodium ions for the synthesis of ATP from ADP and
inorganic phosphate (Laubinger and Dimroth, 1988). The enzyme complexes are
composed of two structurally and functionally distinct entities, designated F$_1$ and F$_0$. The
peripheral F$_1$ part carries the catalytic centres of the enzyme and can be easily dissociated
from the membrane. It consists of five different subunits with a stoichiometry of $\alpha_3$, $\beta_3$,
$\gamma_1$, $\delta_1$, $\epsilon_1$. The membrane-integrated F$_0$ complex functions as a proton channel. In *E. coli*
the F$_0$ complex (EF$_0$) is composed of the three subunits $a$, $b$ and $c$, with relative molecular
masses of 30276, 17265 and 8288, respectively, and a stoichiometry of 1:2:10±1 for
$a:b:c$. The EF$_0$ complex can be isolated and reconstituted into liposomes in a functional
form. In addition, reconstitution is also possible starting from the three individual
subunits. Furthermore, analyses of deletion strains and reconstitution experiments with
purified EF$_0$ subunits have revealed that all three subunits are necessary to obtain an EF$_0$
complex functional in proton translocation and F$_1$ binding (Schneider and Altendorf,
1987; and references therein).

Previous studies with mutant strains (Cox *et al.* 1981) led to the proposal that subunits
$\alpha$ and $\beta$ of EF$_1$ are necessary for the assembly of EF$_0$. However, more recent experiments
have revealed that plasmid-encoded EF$_0$ subunits form a functional EF$_0$ complex in the
absence of coordinated synthesis of EF$_1$ (Fillingame *et al.* 1986), while a ‘non-
conducting’ EF$_0$ complex is assembled in the absence of any EF$_1$ subunit (Pati and
Brusilow, 1989; Pati *et al.* 1991). In the latter case, addition of EF$_1$ brings about the
‘education’ of EF$_0$ to translocate protons. These results indicate that, after binding of EF$_1$
to membranes and the subsequent depletion of those EF$_1$ complexes, the EF$_0$ complex has
been transformed from the closed state to an open proton-translocating channel (Pati and
Brusilow, 1989). In addition, although reconstitution of EF$_0$ sectors expressed in the
presence or absence of EF$_1$ subunits revealed major differences in their ability to
translocate protons, the rebinding of EF$_1$ is similar in both cases (Pati *et al.* 1991).

**Subunit c**

Subunit c, also referred to as proteolipid, is the most extensively studied subunit of the
EF$_0$ complex (Schneider and Altendorf, 1987; Fillingame, 1990; Senior, 1990). The
protein has been sequenced from various sources and found to possess a number of
common features. Subunit c consists of two hydrophobic stretches separated by a central
polar loop (Fig. 1). Within the C-terminal hydrophobic region, a conserved acidic
residue, Asp-61, exists; this reacts with dicyclohexylcarbodiimide (DCCD), resulting in
an inhibition of H$^+$ translocation through F$_0$. The view that this carboxyl group plays an
important role is supported by the observation that proton translocation is abolished by
substitution of Asp-61 with glycine or asparagine (Schneider and Altendorf, 1987;
Fillingame, 1990; Senior, 1990; and references therein). Furthermore, studies with a
slow-growing revertant strain, in which in addition to the original Asp-61→Gly mutation
Ala-24 has been replaced by aspartic acid, indicated that a carboxyl group in the middle
of the membrane is absolutely required for H$^+$ translocation, but that it can be moved from
the C-terminal hydrophobic helix to the N-terminal one (Miller *et al.* 1990).
Observations supporting a hairpin-like structure of the *E. coli* subunit c within the membrane (Fig. 1) are the following: (i) analyses of DCCD-resistant mutants indicate that Ile-28 and Ala-24 are close to Asp-61; (ii) analyses of revertant strains, which show partial suppression of the original mutations affecting proton translocation, indicate that the amino acid residues Asp-61/Ala-24 and Pro-64/Ala-20 are close to each other; (iii) the hydrophobic regions Leu-4 to Leu-19 and Phe-53 to Phe-76 are labelled with 3-trifluoromethyl-3-(m-[125I]iodophenyl)diazirine (TID), suggesting that these regions are membrane-integrated; (iv) the C-terminal region is located at the periplasmic side of the membrane, as shown by chemical modification studies; and (v) studies with peptide-specific antibodies reveal that the polar loop region is exposed to the cytoplasm (Schneider and Altendorf, 1987; Fillingame, 1990; Senior, 1990; and references therein; Fillingame *et al.* 1991; Hensel *et al.* 1990; Miller *et al.* 1990).

Analyses of mutant strains revealed that point mutations in the hydrophobic regions of subunit c produced effects on the assembly of EF₀ or affected proton translocation, whereas the hydrophilic loop region seemed to function in the coupling of proton translocation through EF₀ to ATP synthesis in EF₁ (Schneider and Altendorf, 1987; Fillingame, 1990; Senior, 1990; and references therein). Although the amino acid residues Ala-40, Arg-41, Gln/Asn-42 and Pro-43 of the polar loop region are highly conserved in bacteria, mitochondria and chloroplasts, it can be concluded from oligodeoxyribonucleotide-directed mutagenesis studies that Arg-41 is the only residue in this region that is absolutely required for function (Fraga and Fillingame, 1991).}

Expression of chloroplast subunit III (homologous to subunit c of *E. coli*) together with subunits a and b of *E. coli* did not produce an active Fo hybrid complex (Burkovski *et al.* 1990), although immunoprecipitations with antibodies against CF₀ III indicate that an Fo complex is assembled (A. Burkovski, G. Deckers-Hebestreit and K. Altendorf, ...
unpublished results). We are therefore constructing hybrid c subunits, consisting of parts of both proteolipids, to determine those parts of subunit c that are essential for a functional interplay with subunits a and b.

Subunit a

It has been proposed that subunit a, which is an extremely hydrophobic protein, is involved in proton translocation together with subunit c. The analyses of missense mutants, which exhibit a reduction in H⁺ translocation without a concomitant effect on the assembly of EF₀, revealed that the amino acid residues Arg-210, Glu-219 and His-245 are critical for proton translocation, whereas other residues, such as Asp-119, Glu-196, Gly-197, Ser-206 and Asn-214, are thought to affect proton translocation indirectly (Schneider and Altendorf, 1987; Fillingame, 1990; Senior, 1990; and references therein). In addition, characterization of the double mutant Glu-219→His/His-245→Glu, in which the function is less impaired than in each of the single mutants, has suggested a linked functioning of these two residues in subunit a (Cain and Simoni, 1988). Furthermore, the suggested involvement of Arg-210, Glu-219 and His-245 in proton translocation is strikingly similar to the ‘proton relay’ system proposed for the lac permease (Lee et al. 1989).

Subunit a exhibits structural homology to its counterpart in other F₁F₀ complexes (Fig. 1) with strong amino acid homology in two regions towards the C-terminal part (Pro-190 to Leu-220 and Phe-244 to Tyr-263). Models containing 5–8 membrane-spanning helices have been predicted, but convincing evidence favouring one of those is still lacking (Fillingame, 1990; and references therein; Lewis et al. 1990; Bjorbaek et al. 1990). In addition, studies with alkaline phosphatase (PhoA) fusion proteins resulted in controversial conclusions about the localization of the N and the C termini of the protein with regard to the membrane (Lewis et al. 1990; Bjorbaek et al. 1990).

However, the following experimental evidence is useful in evaluating the different models: (i) Arg-210, Glu-219 and His-245, which are proposed to be critical for H⁺ translocation, are located in transmembrane helices; (ii) analysis of the double mutant Glu-219→His/His-245→Glu indicated that both residues are close to each other in the native protein; (iii) a second-site suppressor mutation in subunit a (Pro-240→Ala or Leu) partially corrects defects of the Gly-9→Asp mutation in subunit b; (iv) analyses of subunit a/PhoA and subunit a/β-galactosidase fusion proteins revealed that the N terminus of subunit a is located in the periplasm; (v) subunit a/PhoA fusion proteins exhibiting high phosphatase activities have their fusion joints located in two regions of subunit a: between Val-110 and Leu-136 and between Leu-229 and Ile-246. Both regions are predicted by hydropathy analyses to be intramembranous. Therefore, from the theory of alkaline phosphatase fusions, both regions should be membrane-spanning, coming from the periplasm and going to the cytoplasm (Schneider and Altendorf, 1987; Fillingame, 1990; Senior, 1990; and references therein; Bjorbaek et al. 1990; Lewis et al. 1990; Lewis and Simoni, 1992).

To determine the localization of both termini of subunit a, a small DNA fragment has now been inserted into the AcylI site at the 5′ or the 3′ end of the uncB gene producing
subunit $a$ proteins, which carry at Thr-7 or at Ala-267 of the polypeptide chain an additional epitope of 11 amino acids (Asp-Asn-Pro-Ala-Ser-Thr-Thr-Asn-Lys-Asp-Lys; Charbit et al. 1986). These modified $uncB$ genes are still able to complement an $uncB$ deletion strain (H. Jäger, G. Deckers-Hebestreit and K. Altendorf, unpublished results). Binding studies with a monoclonal antibody against this epitope and with membrane vesicles of different orientation are now under way to disclose the location of this epitope.

**Subunit $b$**

The primary sequence of subunit $b$ suggests that it is a hydrophilic protein, which is anchored in the cytoplasmic membrane by its apolar N-terminal region (about 25 amino acids), whereas the hydrophilic, highly charged part protrudes from the membrane in a predominantly $\alpha$-helical structure. Labelling experiments with TID, hydrophobic nitrenes and a hydrophobic maleimide support this view. The TID labelling pattern and the analyses of mutants in which Cys-21 or Lys-23 have been substituted indicate that the region around Cys-21 is exposed to the lipid phase with little or no contact with other polypeptide chains. In contrast, the characterization of revertant strains, which partially suppress the Gly-9$\rightarrow$Asp mutation of subunit $b$, as described above, supports the notion that the region around Gly-9 is in close contact with subunits $a$ and $c$. In addition, the absence of TID labelling in the region Asn-2 to Gln-10 also suggests that these residues are part of a protein contact area. Studies with proteases and subunit-$b$-specific antibodies revealed that the hydrophilic part is exposed to the cytoplasm (Fig. 1). However, although subunit $b$ is protected from proteolysis when EF$_1$ is bound to EF$_0$, polyclonal anti-$b$ antibodies are able to bind to subunit $b$ even in the presence of EF$_1$. The binding produced a partial removal of EF$_1$ from EF$_0$, whereas the residual membrane-bound ATPase activity is less DCCD-sensitive (Schneider and Altendorf, 1987; Fillingame, 1990; Senior, 1990; and references therein; Kauffer et al. 1991; Deckers-Hebestreit et al. 1992).

Treatment of F$_1$-stripped everted membrane vesicles or F$_0$ liposomes with trypsin, which cleaves subunit $b$ from the C terminus, resulted in impairment of F$_1$ binding, whereas the proton translocation remained unaffected. In contrast, if the degradation of subunit $b$ with trypsin occurs prior to the isolation and reconstitution of F$_0$, H$^+$ translocation is also impaired. However, both functions can be restored by addition of intact subunit $b$ (Steffens et al. 1987). In addition, cells carrying amber mutations within the $uncF$ gene, which cause premature termination of subunit $b$ biosynthesis, are not able to assemble a functional F$_0$ complex even when only Glu-155 and Leu-156 are missing at the C terminus (Porter et al. 1985; Takeyama et al. 1988). These studies support the view that an intact subunit $b$ is necessary for the correct assembly of F$_0$, but that, once F$_0$ is formed, the C-terminal region is dispensable for proton translocation.

**Arrangement of EF$_0$ subunits**

Very little information exists about the interplay of all three subunits (stoichiometry of $a_1b_2c_{10\pm1}$) in the formation of an F$_0$ complex functional in proton translocation and F$_1$
binding. However, subunit \( a \) has a strong cooperative effect on the structure of the 10±1 copies of subunit \( c \) in the \( F_o \) complex, since the TID labelling pattern of subunit \( c \) is largely reduced when subunit \( c \) is reconstituted into liposomes together with subunit \( a \) or when it is present in the \( F_o \) complex compared to only subunit \( c \) in the membrane (Steffens et al. 1988). Two different models have been proposed for the arrangement of all three subunits in the \( EF_0 \) complex. The first model (Fig. 2B,D) exhibits a ring of \( c \) subunits surrounding subunits \( a \) and \( b \) (Cox et al. 1986). However, this model does not fit very well with the TID labelling data. In contrast, Hoppe and Sebald (1986) and Schneider and Altendorf (1987) have suggested that the \( a_1b_2 \) complex is located outside a ring of \( c \) subunits and that it interacts only at one side with the subunit \( c \) oligomer (Fig. 2A,C). In addition, this model is much better at explaining the relative ease of extraction of subunit \( b \) from \( F_o \) (Schneider and Altendorf, 1984).

Application of the electron spectroscopic imaging mode in electron microscopy, which allows detection of small particles with optimal density and high resolution, made it possible to distinguish different regions of high density in \( EF_0 \) complexes dissolved in

Fig. 2. Models for the arrangement of \( EF_0 \) subunits. (A,B) Putative arrangements of subunits \( a, b \) and \( c \) of the \( EF_0 \) complex in the membrane. (C,D) Top view of the \( EF_0 \) complex. A and C are modified from Schneider and Altendorf (1987); D is modified from Cox et al. (1986).
cholate-containing buffer. Although single subunits of EF<sub>0</sub> could not be identified, the overall shape of the single enzyme complexes fits much better with the F<sub>0</sub> model proposed by Schneider and Altendorf (1987) and Sebald and Hoppe (1986) (Fig. 2A,C). In addition, electron micrographs of EF<sub>0</sub> complexes immunodecorated with monoclonal antibodies against subunits a, b and c indicate a peripheral arrangement of all three subunits in an EF<sub>0</sub> complex seen from the F<sub>i</sub> binding site (R. Birkenhager, M. Hoppert, G. Deckers-Hebestreit, F. Mayer and K. Altendorf, unpublished results).

**H<sup>+</sup> translocation through F<sub>0</sub>**

A ‘proton relay’ system or ‘proton wire’, in which protons cross the membrane by sequential interaction with a series of charged amino acids, has been proposed as a mechanism of proton translocation (Nagle and Tristam-Nagle, 1983; Cox et al. 1986). Amino acid residues involved include Arg-210, Glu-219 and His-245 of subunit a and Asp-61 of subunit c. However, one copy of subunit a must interact with an oligomer of 10±1 subunits c. Furthermore, one molecule of DCCD is able to abolish proton translocation through F<sub>0</sub> by reaction with just one copy of subunit c (Hermolin and Fillingame, 1989). Rotational models were therefore suggested to account for these features (Cox et al. 1986; Schneider and Altendorf, 1987). However, only protons can be moved by such a mechanism. Alternatively, a transmembrane water-filled channel formed by subunits a and c could be envisaged, which would allow specific H<sup>+</sup> translocation, when equipped with an H<sup>+</sup>-specific filter or gate. Studies with amphiphatic peptides, which have been constructed to mimic ion channels, indicate that differences in the number of polar groups have an influence on the number of water molecules bound in the channel and therefore also have an influence on the ion specificity (Lear et al. 1988). In addition, bacteriorhodopsin-bound water molecules are also involved in H<sup>+</sup> translocation (Henderson et al. 1990). For H<sup>+</sup> translocation through F<sub>0</sub> such an ion transport mechanism has to be favoured, especially in the light of the recently discovered F-type ATPase of *Propionigenium modestum*, which uses an electrochemical gradient of sodium ions for the synthesis of ATP instead of a proton gradient (Laubinger and Dimroth, 1988). Furthermore, in the presence of low sodium concentrations, reconstituted PF<sub>i</sub>F<sub>0</sub> switches from a Na<sup>+</sup>-translocating to a H<sup>+</sup>-pumping mode (Laubinger and Dimroth, 1989).

The translocation rate for protons by EF<sub>0</sub> reconstituted into liposomes was measured to be 20 H<sup>+</sub> per EF<sub>0</sub> per second, which is in agreement with values obtained using F<sub>i</sub>-stripped everted membrane vesicles from *E. coli*. In contrast, Schönknecht et al. (1986) and Lill et al. (1986) calculated the proton translocation rate through CF<sub>0</sub> in thylakoid membranes from chloroplasts to be between 6.2×10<sup>3</sup> and 2×10<sup>5</sup> H<sup>+</sup> per CF<sub>0</sub> per second measured by flash kinetic spectrophotometry. However, these high rates could only be observed for a fraction of the CF<sub>0</sub> complexes available and only for a limited period after removal of CF<sub>i</sub> (Lill et al. 1986). In the case of *E. coli* F<sub>0</sub>, the proton translocation rates could be increased up to 100–120 H<sup>+</sup> per EF<sub>0</sub> per second by using, for the purification of EF<sub>0</sub>, an *E. coli* strain that extensively overproduces the ATP synthase (Moriyama et al. 1991) and by applying the pH-indicating dye Phenol Red instead of using a pH electrode,
which allows proton translocation measurements on a very short time scale (in the millisecond range) (G. Deckers-Hebestreit, G. Althoff, W. Junge and K. Altendorf, unpublished results). Although the apparent proton translocation rate of EF₀ has been increased at least by a factor of five, the discrepancy between the values obtained for CF₀ and EF₀ still exists. Therefore, the question remains as to whether the differences depend only on the experimental conditions used for the measurement of H⁺ translocation or whether the translocation rate in everted membranes and in EF₀ liposomes represents only some residual activity due to unrecognized structural changes within EF₀ caused during preparation or due to structural changes upon removal of F₁ (Nelson, 1980). Alternatively, the enzyme in E. coli may work much slower than CF₁F₀.

References


HOPPE, J. AND SEBALD, W. (1986). Topological studies suggest that the pathway of the protons through F₀ is provided by amino acid residues accessible from the lipid phase. Biochimie 68, 427–434.


