Topological and functional aspects of the proton conductor, F₀, of the Escherichia coli ATP-synthase

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The isolated H⁺ conductor, F₀, of the Escherichia coli ATP-synthase consists of three subunits, a, b, and c. H⁺-permeable liposomes can be reconstituted with F₀ and lipids; addition of F₁-ATPase reconstitutes a functional ATP-synthase. Mutants with altered or missing F₀ subunits are defective in H⁺ conduction. Thus, all three subunits are necessary for the expression of H⁺ conduction. The subunits a and b contain binding sites for F₁. Computer calculations, cross-links, membrane-permeating photo-reactive labels, and proteases were used to develop tentative structural models for the individual F₀ subunits.

ATP-synthases catalyze the proton-motive-force-dependent phosphorylation of ADP in oxidative phosphorylation and photophosphorylation. Inversely, hydrolysis of ATP via the ATP-synthase is coupled to the formation of a proton-motive force. Both reactions are inhibited by dicyclohexylcarbodiimide (DCCD). The enzymes occur in remarkably conserved form in prokaryotic and eukaryotic cells. They are composed of two parts, a membrane-integrated part F₀, rendering the membrane proton-permeable, and a membrane-associated part F₁, bearing ATPase activity. The functions of F₀ and of F₀ subunits were examined by a variety of different methods: isolation of F₀, reconstitution of biological activities, isolation of F₀-mutants, elucidation of their structural alterations, and characterization of the functional implications. The structural features of individual F₀ subunits were investigated by determination of the primary structure, computer calculations of the secondary structure, cross-links, proteases, and photo-reactive labels.

Methods

The preparation of F₁, F₁F₀, F₀, membranes, F₁-depleted membranes, and antibodies against subunits was performed as described previously (Vogel & Steinhart, 1976; Friedl et al., 1979; Friedl & Schairer, 1981; Friedl et al., 1981). The constitution of strains and construction of plasmids are described elsewhere (Hansen et al., 1981; Hansen & von Meyenburg, 1980; Gunsalus et al., 1982; Friedl et al., 1981; Friedl et al., 1980); plasmid pOM 11-1 contains the structural gene of subunit a on a HindIII-Aval fragment cloned in pBR322 (Michelsen O, unpublished).
Assays of $F_1$ binding, $H^+$ conduction, and ATP-dependent $H^+$ translocation were performed as described (Sebald et al., 1982; Friedl et al., 1980), as well as polyacrylamide-gel electrophoresis (Friedl et al., 1979) and silver-staining of gels (Merril et al., 1980). Predictions of secondary structure and membrane-permeating segments were done as described previously (Chou & Fasman, 1978; Maxfield & Scheraga, 1976; Robson & Suzuki, 1976; Nagano, 1977; von Heijne, 1981).

Proteolytic treatment of $F_0$-subunits in $F_1$-depleted membranes

$F_1$-depleted membranes were prepared from an *Escherichia coli* overproducer (obtained from K. von Meyenburg). Membranes (5 mg/ml) were incubated in buffer (100 mM Tris/HCl, pH 7.8, 5 mM MgCl$_2$) for 60 min at 37°C with proteases at ratios (w/w) of 1:1000, 1:100, and 1:10. After addition of protease-inhibitor phenylmethylsulfonylfluoride (PMSF) to 1 mM, membranes were washed twice with buffer and submitted to dodecylsulfate-gel electrophoresis. The protein bands were visualized by the silver-staining procedure.

Cross-linking of $F_0$ subunit $b$ from *Escherichia coli*

Isolated ATP-synthase from *Escherichia coli* dissolved 1 mg per 0.2 ml in buffer (50 mM N-methylmorpholine, pH 8, 100 mM KCl, 2 mM MgCl$_2$, 0.2 mM EGTA, 0.2 mM PMSF, 25 mM Aminoxid WS35, 20% methanol) was incubated in the dark with 5 mM fluoronoitrophenylazide for 60 min at room temperature. Thereafter the reactive nitrene was generated by illumination at 360 nm for 10 min at 0°C. Proteins were separated electrophoretically on 0.7-mm-thick dodecylsulfate gels, and were then transferred electrophoretically onto nitrocellulose sheets (Towbin et al., 1979). The sheets were incubated with specific rabbit IgG directed against subunit $b$. Bound IgG was visualized after reaction with fluorescein-conjugated goat antirabbit IgG under u.v. light.

Results and Discussion

$F_1F_0$ and $F_0$ preparations

The *Escherichia coli* ATP-synthase has been purified in large quantities by extracting membranes with the zwitterionic detergent Aminoxid WS35 and subsequent chromatography of the extract on DEAE-Sepharose (Friedl et al., 1979). SDS/gel electrophoresis of the purified enzyme revealed 8 subunits: in addition to the five subunits of $F_1$, $\alpha$, $\beta$, $\gamma$, and $\delta$, 3 polypeptides, $a$, $b$, and $c$, are found (Fig. 1), with the apparent mol. wts. of 24 000, 19 000, and 8300. After reintegration into liposomes the enzyme is active in ATP-dependent proton translocation to more than 90% (Table 1). $F_0$ was isolated from the purified ATP-synthase (Friedl & Schairer, 1981). Fig. 1 shows that $F_0$ consists of the three subunits $a$, $b$, and $c$. The catalytic activities of $F_0$ could be determined after integration into liposomes (Table 1). $F_0$ catalyzed an electro-impelled proton flux which can be inhibited by DCCD or by binding of $F_1$. Binding of $F_1$ restores the ATP-dependent proton-translocation.
Fig. 1. Subunits and genes of the Escherichia coli ATP-synthase. SDS/polyacrylamide-gel electrophoresis was performed as described in methods. 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. The numbers below the genes indicate the number of amino acid residues in the subunit polypeptides.

**Fo-mutants**

Genetic studies of the E. coli ATP-synthase started with the isolation and characterization of mutants defective in oxidative phosphorylation (for review see Downie et al., 1979). The genes coding for the polypeptides of the ATP-synthase are organized in the unc operon (Fig. 1). The complete DNA sequence of the operon has been determined (Gay & Walker, 1981; Nielsen et al., 1981; Kanazawa et al., 1981). Mutants with an altered Fo-subunit a, b, or c are defective in proton conduction and ATP-dependent proton translocation. This indicates that in vivo all three subunits are necessary for these functions. In order to correlate certain functions to the different Fo subunits, mutant strains were constructed lacking one or two of the Fo subunits.
Table 1. Activities of F₁F₀ and F₀ from *Escherichia coli*

Membranes, F₁-depleted membranes, F₁F₀, F₀, and proteoliposomes were prepared and the assays were performed as described in 'Materials and Methods'.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Electro-impelled H⁺ conduction (nmol·min⁻¹·mg⁻¹)</th>
<th>ATP-dependent H⁺ translocation (Uf₁·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH-electrode</td>
<td>Fluorescence test</td>
<td>Fluorescence test</td>
</tr>
<tr>
<td>Membranes</td>
<td>-</td>
<td>130</td>
</tr>
<tr>
<td>F₁-depleted membranes</td>
<td>44</td>
<td>-</td>
</tr>
<tr>
<td>Liposomes with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F₁F₀</td>
<td>5</td>
<td>2330</td>
</tr>
<tr>
<td>F₀ + F₁</td>
<td>41</td>
<td>1910</td>
</tr>
<tr>
<td>F₀</td>
<td>870</td>
<td>1</td>
</tr>
<tr>
<td>F₀ + DCCD</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2 shows that for the expression of proton conduction all three subunits of F₀ are necessary. On the other hand subunit a or subunit b alone can bind F₁, whereas subunit c cannot. Binding of F₁ by these subunits does not reconstitute DCCD-sensitivity of the ATPase activity or ATP-dependent proton translocation.

Table 2. Activities of F₀ in unc-mutants lacking individual F₀ subunits

<table>
<thead>
<tr>
<th>Strain</th>
<th>F₀ subunits</th>
<th>H⁺ conduction</th>
<th>F₁ binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>a b c</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DG10/6</td>
<td>a b</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CM2080 (log)</td>
<td>b c</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AM12</td>
<td>a c</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CM1470 + pOM11-1</td>
<td>a</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CM1470 + pRPG51</td>
<td>b</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CM2080 (stat)</td>
<td>c</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CM 1470</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Strains: Al wild type; CM1470 and CM2080 deletion mutants; the presence of subunit b depends on growth phase (log = logarithmic, stat = stationary phase); AM12 and DG10/6, polar mutants; pOM11-1, plasmid with structural gene for subunit a; pRPG51, plasmid with structural gene for subunits b and c. Tests were performed as described in 'Materials and Methods'.
The stoichiometry of F₀ seems to be unusual. Homogeneous labelling of the proteins with radioactive precursors revealed a ratio for $a:b:c$ of 1:2:10 (Foster & Fillingame, 1982). The high number for subunit $c$ explains the observed strong negative complementation in $c^+/c^-$ heterozygotes (Friedl et al., 1980). But it is difficult to propose a reasonable model for F₀. The DNA sequence of the unc-operon combined with partial amino acid sequencing provides the primary structure of the F₀ subunits. Fig. 2 shows a computer printout for the F₀ subunits with predictions for the secondary structure (Chou & Fasman, 1978; Maxfield & Scheraga, 1976; Robson & Suzuki, 1976; Nagano, 1977) and for the membrane-permeating segments (von Heijne, 1981). The proposal for the DCCD-binding subunit $c$ is the 'hairpin' model (Sebald & Hoppe, 1981) with two hydrophobic segments spanning the membrane and a central polar loop at the membrane surface. The DCCD-binding aspartyl-residue 61 is located in the lipid phase. The replacement of this amino acid by glycine or asparagine leads to an F₀ defective in H⁺ conduction (Hoppe et al., 1980b; J. Hoppe, unpublished). DCCD-resistant mutants originate from substitutions of isoleucine 28 by threonine or valine (Hoppe et al., 1980a). In the hairpin model this isoleucine is located in the vicinity of the DCCD-reactive aspartyl residue. Circular-dichroism measurements support the high content of alpha-helical secondary structures of this subunit (Sebald & Hoppe, 1981).

Subunit $b$ consists of 156 amino acids and is mainly hydrophilic. Only the 33 amino acids of the $N$ terminus form a hydrophobic domain sticking in the membrane, the greatly alpha-helical polar moiety being exposed to the cytoplasm.

Subunit $a$ is the largest F₀ subunit. The sequence of 271 amino acids shows seven hydrophobic sequences of about 20–25 residues. According to the model, they are spanning the membrane, and the $N$-terminal region of 40 amino acids could be exposed to the cytoplasm. Due to its highly hydrophobic character no protein-chemical characterization has been performed so far.

Lipophilic membrane-permeating photoreactive labels were used to modify specifically amino acid residues located in the membrane. All three subunits get labelled with trifluoromethyliodophenyldiazirine. The characteristic labelling patterns of subunits $c$ and $b$ support the above-presented models for these subunits (J. Hoppe, unpublished). The characterization of water-exposed segments was achieved by protease treatment of F₁-depleted membranes from an ATP-synthase-overproducing strain (obtained from K. von Meyenburg). Fig. 3 shows that subunit $c$ was only marginally degraded even at high protease concentrations, and subunit $a$ was only partially affected at high concentrations of subtilisin and V8 protease. In contrast subunit $b$ was very protease-sensitive, indicating its accessibility from the water phase. Remarkably, chymotrypsin produced a membrane-bound cleavage product lacking 20 residues of the polar $C$ terminus. Proton conduction of F₀ and binding of F₁ were not affected, but ATP-dependent proton conduction was abolished (J. Hoppe, unpublished).
Fig. 2. Prediction of secondary structures and membrane-permeating segments in F₀ subunits of *Escherichia coli*. Alpha-helical segments (α) and β-turns (T) were consistently calculated applying four different prediction methods. The free-energy gains during a transition from a random coil in water to an alpha-helix in the membranes were calculated for all amino acid sequence positions. The area below the dotted lines indicates increased probability for a location in the lipid bilayer, and the numbers on the dotted lines give the sequence position of the amino acids. Plots for subunits a (A), b (B), and c (C).
Fig. 3. Proteolytic treatment of F₀ subunits in F₁-depleted membranes. F₁-depleted membranes were prepared from an Escherichia coli overproducer (obtained from K. von Meyenburg). Lane A shows separated F₁F₀, and lane B, separated F₁-depleted membranes. The protease 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 arrow indicates the digestion product obtained with chymotrypsin.

Cross-links of ATP-synthase subunits were obtained using the bivalent electrophilic and photoreactive reagent azidonitrophenylfluoride. The results of the subsequent analysis by SDS/gel electrophoresis, blotting, and immunochemical staining are shown in Fig. 4. The most prominent cross-link product is b-b, indicating a b-dimer in the ATP-synthase. Small amounts of additional subunit b cross links were observed which are tentatively assigned to cross-links with the subunits a, c, α, and β. This suggests that subunit b has multiple contacts with other subunits, indicating its location in the center of the ATP-synthase.

The results presented are in accordance with the prediction made for the structure of the F₀ subunits. Further elucidation of the structure of F₀ as well as of F₁ will be brought about by the use of monoclonal antibodies, peptide chemistry, and electron microscopy.
Fig. 4. Cross-linking of F0-subunit b from Escherichia coli. Isolated F1F0 was cross-linked with fluoro-nitrophenylazide and subjected to SDS/polyacrylamide-gel electrophoresis, and the separated products were transferred to nitrocellulose and stained for antigenic material as described in 'Materials and Methods'. Lane A shows the identification of subunit b in the absence of cross-linking. Lane B demonstrates the appearance of cross-links containing subunit b. The positions of subunits β, γ, and δ, which were separated in parallel, are indicated.

References