Assembly of the *Escherichia coli* F₀F₁ ATP synthase involves distinct subcomplex formation

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Abstract

The ATP synthase (F₀F₁) of *Escherichia coli* couples the translocation of protons across the cytoplasmic membrane by F₀ to ATP synthesis or hydrolysis in F₁. Whereas good knowledge of the nanostructure and the rotary mechanism of the ATP synthase is at hand, the assembly pathway of the 22 polypeptide chains present in a stoichiometry of ab₂c₁₀α₃β₃γδε has so far not received sufficient attention. In our studies, mutants that synthesize different sets of F₀F₁ subunits allowed the characterization of individually formed stable subcomplexes. Furthermore, the development of a time-delayed *in vivo* assembly system enabled the subsequent synthesis of particular missing subunits to allow the formation of functional ATP synthase complexes. These observations form the basis for a model that describes the assembly pathway of the *E. coli* ATP synthase from pre-formed subcomplexes, thereby avoiding membrane proton permeability by a concomitant assembly of the open H⁺-translocating unit within a coupled F₀F₁ complex.

Introduction

ATP synthases (F₀F₁) utilize the energy of the proton/ion-motive force for the synthesis of ATP from ADP and inorganic phosphate. Dependent on the physiological demand of the cell, ATP synthases can operate in either directions, synthesizing ATP or hydrolysing ATP to drive transmembrane proton/ion flow. During ATP synthesis, these rotary nanomachines convert the electrochemical energy of the proton/ion-motive force via a mechanical mechanism into chemical energy. In most bacteria, the genes encoding the individual subunits are located within the *atp* operon (*atpB/E/F/H/A/G/D*) exhibiting separate clusters of genes coding for F₀ subunits and F₁ subunits respectively [1,2]. In addition, F₀F₁ ATP synthases can be divided into two different structural and functional units, namely, in *Escherichia coli*, the membrane-integrated subunits *ab*c₁₀ of F₀ and, with orientation to the cytoplasm, the peripherally associated F₁ subunits α₃β₃γδε as clearly visible in electron-density maps [3,4]. Furthermore, separation of both complexes generates an F₀ complex exhibiting passive H⁺ conduction, whereas F₁, which harbours the three catalytic centres, shows ATPase activity [5–7].

During ATP synthesis, the energy stored in the transmembrane electrochemical gradient of protons drives the rotation of the subunit c ring in F₀, as well as of the elongated γε central stalk in F₁ by a flow of H⁺ through two half channels within subunit α. Subunit γ rotates inside a molecular bearing composed of the alternately arranged α₃β₃ hexamer and causes conformational changes in the three catalytic binding sites leading to an alternate site mechanism. This binding change mechanism describes a conformational cycling of each of the three αβ protomers through three different states, termed open, loose and tight, with strong positive cooperativity for substrate binding as well as catalytic activity (multi-site catalysis). To counteract the tendency of the α₃β₃ hexamer to follow the rotation of the rotor, a peripheral stator stalk, composed of subunit δ and the b dimer closely interacting with subunit a, is essential to hold the α₃β₃ hexamer in position by balancing the force generated by the rotor [8,9].

Regarding evolutionary aspects, it has been proposed that ATP synthases have evolved from functionally unrelated ancestor proteins. Whereas the c ring has been proposed to be derived from ion pumps, possibly Na⁺ pumps [10,11], the α₃β₃ hexamer is quite similar to ATP-dependent ring helicases [12,13]. Furthermore, a structural relationship between ATP synthases and DNA/RNA/protein translocases has been described suggesting that the rotary central stalk occupies the same position as the translocated polymer [12,13].

All observations strengthen the point of view that the assembly pathway of the multimeric ATP synthase involves the formation of distinct subcomplexes in a specific pattern. However, one has to take into account that an independent formation of an F₀ complex would result in an increased membrane proton permeability due to passive H⁺ conduction followed by a breakdown of the electrochemical proton gradient across the membrane and would therefore possibly be lethal for the cell or at least produce a severe reduction in growth [14]. In general, one can assume that the formation of a H⁺-translocating unit from different subunits is a rather difficult highly regulated step during assembly of all kinds of H⁺-translocating enzymes. Accordingly, previous studies imply that the formation of functional F₀ in proton
Figure 1 | Model for the assembly of the E. coli ATP synthase

The c₁₀ ring and the b dimer are independently present in the cytoplasmic membrane and assemble independently of other F₀F₁ subunits. The presence of αβγε even in the cytoplasm of wild-type cells implies that pre-formed αβγε subcomplex binds to the c₁₀ ring present in the membrane. Details on the formation of αβγε are not known for E. coli ATP synthase, however, an assembly from αββ and γε has been proposed for yeast mitochondrial F₀F₁ [41,42] and a γε subcomplex has also been purified from E. coli cells [60]. A stable membrane insertion of subunit α is interdependent on the presence of subunits b and c. For integration of the b dimer into F₀F₁, subunit δ is essential, whereas the binding of subunits α and δ to b₂ has apparently no preferred sequence. Protein complexes involved in membrane insertion of F₀ subunits [19,20,32,33] as well as chaperones [25-28,31] known to participate in the assembly of bacterial ATP synthases are indicated in dark red. Atp12p, a molecular chaperone for subunit α, is present in Alphaproteobacteria and has been crystallized from Paracoccus denitrificans [61]; however, until now, no corresponding protein has been detected in E. coli. αAtp1 has been shown to be essential for formation of the oligomeric c ring in Na⁺ translocating ATP synthases, whereas it is not absolutely required for the assembly of H⁺ translocating F₀F₁ complexes [25-28,31].

Although type II signal anchor as topogenic sequences [19-21], studies on the oligomerization of the c₁₀ subcomplex reveal that YidC [22] as well as the other F₀F₁ subunits are not involved [23,24], whereas Atp1 participates in a chaperone-like manner [25-28] (Figure 1). Atp1, an extremely hydrophobic protein of 14 kDa that can be purified by chloroform/methanol extraction [29], has been shown to be present in substoichiometric amounts in purified F₀ and F₀F₁ preparations [30]. Whereas Atp1 has been shown to be essential for the oligomerization of the c ring in Na⁺ translocating ATP synthases and therefore for the assembly of F₀F₁ [26,27], it is not absolutely required for the function of H⁺ translocating ATP synthases [25,28,31], although in its presence the stability as well as the activity of the membrane-bound ATPase activity is modestly increased [28,31].

Membrane insertion of subunit b is a co-translational process and involves the SecYEG translocon as well as the SRP (signal recognition particle) pathway [32], whereas insertion of subunit a depends on Sec/SRP as well as YidC [20,33]. Subunits b and c are present in the membrane independently of other F₀F₁ subunits [23,18]. In contrast, subunit a shows interdependence on subunits b and c [18,34] (Figure 1). In the absence of one of these subunits, subunit a is rapidly degraded by the ATP-dependent metalloprotease PtsH [35]. Recent studies suggest that monomeric c subunit(s) might be sufficient for a stable membrane insertion of subunit a [36].

Assembly pathway of E. coli ATP synthase involves distinct subcomplex formation

Synthesizing different sets of F₀F₁ subunits, we were able to characterize individual subcomplexes stably present within the cytoplasmic membrane by nearest neighbour analyses using individually substituted cysteine pairs for disulfide cross-linking as well as His-tag affinity purification of subcomplexes [17,18,23], namely c₁₀, b₂, ab₂, c₁αβγε, c₁αβγε, c₁αβγε, and b₂c₁αβγεδ (Figure 2 and Table 1).

Whereas c₁₀ forms a highly stable subcomplex present in the membrane in amounts comparable with that of wild-type [23], b₂ is less stable and is known to be susceptible to protease degradation from its C-terminal region when present in F₁-stripped membranes [7]. However, both subunits do not interact with each other in the absence of other F₀F₁ subunits even when highly overexpressed as analysed by disulfide cross-linking in native membrane vesicles [18], thereby contradicting with the assembly pathway described for E. coli F₀F₁ with respect to the formation of a b₂c₁₀ subcomplex as an intermediate state [37,38].

For single-subunit knockout mutants Δb, Δδ and Δα, in which one of the F₀F₁ subunits was knocked out by inserting an early stop codon into the corresponding gene, the following picture emerged (Figure 2): whereas in Δα and Δδ only the corresponding subunit is lacking in membrane vesicles, Δb also lacks, in addition to b₂, subunits a and δ [18]; both subunits are known to bind to b₂ with high affinity [39,40]. Accordingly, in Δb, a

Membrane insertion of F₀ subunits

The membrane insertion of subunit c is a YidC insertase-dependent process with an N-terminal type I and a C-terminal type II signal anchor as topogenic sequences [19-21]. Studies on the oligomerization of the c₁₀ subcomplex reveal that YidC [22] as well as the other F₀F₁ subunits are not involved [23,24], whereas Atp1 participates in a chaperone-like manner [25-28] (Figure 1). Atp1, an extremely hydrophobic protein of 14 kDa that can be purified by chloroform/methanol extraction [29], has been shown to be present in substoichiometric amounts in purified F₀ and F₀F₁ preparations [30]. Whereas Atp1 has been shown to be essential for the oligomerization of the c ring in Na⁺ translocating ATP synthases and therefore for the assembly of F₀F₁ [26,27], it is not absolutely required for the function of H⁺ translocating ATP synthases [25,28,31], although in its presence the stability as well as the activity of the membrane-bound ATPase activity is modestly increased [28,31].
Figure 2 | Subcomplexes characterized in studying the assembly of E. coli ATP synthase

WT, wild-type cells encoding F0F1 chromosomally. In addition to the functional F0F1 complex present in the cytoplasmic membrane (not shown), soluble F1 can be isolated from the cytoplasm. b + c, subunits b and c were synthesized in the absence of other F0F1 subunits. Δa, Δb and Δδ, early stop codons inserted into the corresponding genes of the atp operon. Time-delayed synthesis of the missing subunit allows the subsequent formation of functional F0F1 [17,18]. The individual subunits are presented as in Figure 1.

c10αβ3γε subcomplex is formed exhibiting membrane-bound ATPase activity. Furthermore, under wild-type conditions with a chromosomally expressed atp operon, in addition to the functional F0F1 complex present in the cytoplasmic membrane, soluble F1 can be isolated from the cytoplasm after insertion of codons encoding a His-tag into chromosomally expressed atpD. This F1 subcomplex exhibits ATPase activity and comprises subunits α, β, γ and ε (G. Deckers-Hebestreit, unpublished work). This implies that an independent c10 as well as αβ3γε subcomplex formation enables the generation of a membrane-bound F0F1 core complex c10αβ3γε (Figure 1) as has also been described for yeast mitochondrial ATP synthase [41,42].

In Δa, in addition to the F0F1 core complex exhibiting comparable ATPase activities as in Δb, a separate ab2 subcomplex is present in the membrane (Figure 1) as shown by disulfide cross-linking combining different cysteine pairs. Furthermore, no interaction between ab2 and the core complex could be observed when performing cross-linking experiments between subunit b and subunits α, β or c in their native environment in membrane vesicles [18] clearly demonstrating that a theoretically possible F0F1-δ is not formed as subcomplex. However, the question on the influence of subunit c on a stable membrane insertion of subunit a remains unsolved. Nevertheless, a comparable modular pathway for F0F1 assembly has been observed by Rak et al. [42] for yeast mitochondria performing pulse-chase experiments in organello. Furthermore, a time-delayed in vivo assembly system has been developed, which adjusted two expression systems (araBADp and T7p-laco) to provide a compatible, mutually independent and sufficiently stringent induction repression regime [17]. This in vivo assembly system revealed that the subcomplexes ab2 and c10αβ3γε remain in an assembly intermediate state that enables the formation of catalytically active F0F1 after time-delayed individual synthesis of subunit δ [18] (Figure 2). In detail, all structural atp genes except atpH (encoding subunit δ) were expressed under the control of the araBAD promoter by induction with arabinose. Following synthesis of F0F1 lacking subunit δ during growth, expression was repressed by glucose/D-fucose, and complete degradation of atp mRNA was controlled by real-time RT (reverse transcription)–PCR. A time-delayed expression of atpH under the control of promoter T7p-laco was subsequently induced in trans by addition of IPTG.

In mutant Δa, an F0F1 complex just lacking subunit a (b3c1αβ3γεδ2; F0F1-a) is present in the membrane and, again, the subsequent synthesis of subunit a yields functional F0F1 (Figure 2) as analysed by DCCD (dicyclohexylcarbodiimide)-sensitive ATPase activity, ATP-driven proton translocation as well as ATP synthesis [17]. An observation that has also been described for F0F1 of Bacillus PS3 expressed in E. coli using cell-free synthesized or separately purified subunit a to complete F0F1 to functional F0F1 [43,44]. In addition, from human ρ0 cells, a stable ATP synthase lacking only subunits a and A6L (a subunit not present in bacterial F0F1) was isolated [45].

At first sight it seems to be a contradiction that both subunits can each be assembled as the last subunit into the F0F1 complex. However, the use of mutants provokes an arrest in the assembly pathway at a certain point, whereas in wild-type cells all subunits are nearly isochronously at their location as well as ATP synthesis [17]. An observation that has also been described for FoF1 of Bacillus PS3 expressed in E. coli using cell-free synthesized or separately purified subunit a to complete FoF1 to functional FoF1 [43,44]. In addition, from human ρ0 cells, a stable ATP synthase lacking only subunits a and A6L (a subunit not present in bacterial F0F1) was isolated [45].
ATPase activity of αβγδε and c10α3β3γε

An αβγδε complex has been shown to form the minimal unit exhibiting ATPase activity and, furthermore, it exerted the strongest ATPase activity compared with αβγε or αβγδε [6,49,50]. In this context, it is worthwhile mentioning that FoF1 is known to undergo drastic conformational changes in the growth rate [50]. In addition, subunit α intracellular ATP/ADP ratio induced by the presence of LDAO (lauryldimethylamine oxide), which has been shown to perturb the inhibitory interaction of subunit ε on subunit β in a reversible manner [51]. The presence of αβγδε or c10α3β3γε as intermediates during assembly raises the question of how growing E. coli cells deal with such ATP-consuming products. The glycolytic flux in E. coli cells is controlled by the demand for ATP, indicating that glycolysis can compensate for the higher need. A lowered intracellular ATP/ADP ratio induced by the presence of overexpressed αβγδε resulted in a 1.7-fold stimulation of the glycolytic flux concomitant with only a smaller decrease in the growth rate [50]. In addition, subunit ε in bacterial FoF1 is known to undergo drastic conformational changes involving the transition of the two C-terminal helices between a hairpin-forming 'down'-state and an extended 'up'-state. Moreover, an enzyme complex with ε in the 'up'-state cannot catalyse ATP hydrolysis. Such a regulatory mechanism allows a prompt response to changes in the intracellular ATP/ADP ratio to avoid potentially wasteful ATP hydrolysis [52,53]. These observations imply that in vivo the assembly intermediates capable of performing ATP hydrolysis are probably mainly in a passive state due to a low intracellular ATP/ADP ratio, whereas in vitro ATPase activity assays, which are equipped with saturating concentrations of ATP, ATP hydrolysis could be observed with rates comparable with wild-type.

Generation of the proton-translocating F₀ complex

The F₀ complex is composed of subunits α, b and c present as ab2c10 in functional FoF1. Wild-type membranes stripped of F₁ revealed an F₀ complex functional in H⁺ translocation as well as rebinding of F₁ [54,55]. Furthermore, purification of Fo subunits from assembled FoF1 and their reconstitution into liposomes revealed the same characteristics [5,56]. In contrast, after synthesis of F₀ subunits in the absence of F₁, only low levels of proton permeability were observed in membranes as well as after reconstitution of purified subunits into liposomes, implying a dependence on F₁ for the proton translocation by F₀ [15,16], although the additional presence of subunit δ slightly enhances the proton conductance through F₀ [57,58].

The interdependence between F₀ and F₁ during assembly has been proposed to ensure that an open proton channel does not exist as an intermediate [59,16]. Studies with Δα and Δε mutants now revealed that subunit ε is the key player in generating the H⁺ -translocating unit composed of c10 and functions as a clamp to induce a first contact between a (via b2) and c10 (via αβγδε). In contrast, the exclusive presence of both subcomplexes is not sufficient to constitute an open H⁺ channel [18]. By this arrangement, two important

Table 1 | FoF1 subcomplexes formed in vivo during growth of E. coli cells that are possibly intermediate states during assembly of the ATP synthase

Subcomplex formation was verified by disulfide cross-linking in membranes or tag-based affinity purification and deduced by its catalytic activity respectively. Subcomplexes formed by in vitro reconstitution of subunits purified from dissociated FoF1 complexes were not taken into account. Once an FoF1 complex has been assembled, it cannot be excluded that the conformation of purified subunits/subcomplexes essential for protein-protein interaction within FoF1 was changed into an energetically favoured one during the preceding in vivo assembly process.

<table>
<thead>
<tr>
<th>Subcomplex formed</th>
<th>Expression of atp genes/genetic manipulation</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>Membrane-bound subcomplexes</td>
<td></td>
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<tr>
<td>b2</td>
<td>αtpF</td>
<td>[18]</td>
</tr>
<tr>
<td>c10</td>
<td>αtpE</td>
<td>[23]</td>
</tr>
<tr>
<td>ab2</td>
<td>αtpBEFHAGDC, 811 end (Δδ)</td>
<td>[18]</td>
</tr>
<tr>
<td>ab2c10δ</td>
<td>αtpBEFH</td>
<td>[57,58]</td>
</tr>
<tr>
<td>c10α3β1γε</td>
<td>αtpBEFHAGDC, 811 end (Δδ)</td>
<td>[18]</td>
</tr>
<tr>
<td>c10α3β1γε</td>
<td>αtpBEFHAGDC, b17 end (Δδ)</td>
<td>[18]</td>
</tr>
<tr>
<td>b2c10 αβγδε</td>
<td>αtpBEFHAGDC, aW231 end (Δδ)</td>
<td>[18]</td>
</tr>
<tr>
<td>b2c10 αβγδε</td>
<td>ΔαtpB</td>
<td>[17]</td>
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Subcomplexes present in the cytosol αβ3γε γε αβ3γε chromosomal expression of αtpBEFHAGDC (G. Deckers-Hebestreit, unpublished work) αβ3γε αtpBEFHAGDC (G. Deckers-Hebestreit, unpublished work) αtpBEFHAGDC (G. Deckers-Hebestreit, unpublished work) αtpBEFHAGDC (G. Deckers-Hebestreit, unpublished work) αtpBEFHAGDC (G. Deckers-Hebestreit, unpublished work) αtpBEFHAGDC (G. Deckers-Hebestreit, unpublished work) αtpBEFHAGDC (G. Deckers-Hebestreit, unpublished work) αtpBEFHAGDC (G. Deckers-Hebestreit, unpublished work)

*An atpB-C deletion strain was used for plasmid-encoded expression of different subsets of FoF1 subunits.
†A GST tag fused to y11-258 was used for purification of γε.
characteristics of F$_o$F$_1$ are respected. First, an intermediate exhibiting uncontrolled H$^+$ conduction across the membrane is avoided. Instead it is guaranteed that an open H$^+$ channel is concomitantly assembled within coupled F$_o$F$_1$, thereby maintaining a low membrane proton permeability essential for the viability of the cell. Secondly, the low affinity between stator and rotor subunits in F$_o$, which is essential for function, is elegantly bypassed by subunit $\delta$. Nonetheless, as described above, once the F$_o$ complex has been generated, a depletion factor from the isolated subunits of F$_1$ ATP synthase is avoided. Instead it is guaranteed that an open H$^+$ -conducting capabilities, indicating that the first contact between $ab_2$ and $c_{10}$ enabled by $\delta$ triggers a kind of an induced fit within subunits $a$ and $c$, that hereupon facilitates binding between F$_o$ subunits even in the absence of F$_1$.

It can be speculated that the (transient) salt bridge formed between the conserved amino acid residues aArg245 and cAsp61 during proton translocation [9] is involved in generating/maintaining an F$_o$ complex composed of $ab_2$ and $c_{10}$. From energetic considerations, one can assume that during insertion of subunit $c$ into the membrane as well as during formation of the oligomeric c ring, the carboxy groups of cAsp61 are in a protonated state. During assembly of subcomplexes $ab_2$ and $c_{10}$ by subunit $\delta$, the interface between subunit $a$ and the $c_{10}$ ring is established, protonated cAsp61 comes into close contact with aArg245 situated at the two half-channels of subunit $a$, which in turn elicits deprotonation of cAsp61 and simultaneously stabilizes the H$^+$-translocating unit $ac_{10}$; a hypothesis that demands further investigation.

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