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YidC-mediated Membrane Insertion of Assembly Mutants of Subunit c of the F$_1$F$_0$ ATPase*

YidC is a member of the Oxal family of membrane proteins that has been implicated in the membrane insertion of inner membrane proteins in *Escherichia coli*. We have recently demonstrated that proteoliposomes containing only YidC support both the stable membrane insertion and the oligomerization of the c subunit of the F$_1$F$_0$ ATP synthase (F$_0$c). Here we have shown that two mutants of F$_0$c unable to form a functional F$_1$F$_0$ ATP synthase interact with YidC, require YidC for membrane insertion, but fail to oligomerize. These data show that oligomerization is not essential for the stable YidC-dependent membrane insertion of F$_0$c consistent with a function of YidC as a membrane protein insertase.

Membrane protein targeting and insertion in *Escherichia coli* generally occurs via the highly conserved SRP/SecYEG pathway (1, 2). In addition, another membrane protein, YidC, that associates with SecYEG has been implicated in membrane protein insertion (3). YidC is a member of the Alb3/Oxa1/YidC family of membrane proteins that function in promoting membrane insertion in chloroplasts, mitochondria, and bacteria (4, 5). YidC has been implicated in various steps of membrane protein insertion and assembly. YidC was shown to cross-link to the cytosolic side of the membrane. The F$_1$,F$_0$ ATP synthase interacts with YidC, require YidC for membrane insertion, but fail to oligomerize. These data show that oligomerization is not essential for the stable YidC-dependent membrane insertion of F$_0$c consistent with a function of YidC as a membrane protein insertase.

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Membrane protein targeting and insertion in *Escherichia coli* generally occurs via the highly conserved SRP/SecYEG pathway (1, 2). In addition, another membrane protein, YidC, that associates with SecYEG has been implicated in membrane protein insertion (3). YidC is a member of the Alb3/Oxa1/YidC family of membrane proteins that function in promoting membrane insertion in chloroplasts, mitochondria, and bacteria (4, 5). YidC has been implicated in various steps of membrane protein insertion and assembly. YidC was shown to cross-link to the transmembrane regions of nascent membrane proteins (3, 6, 7) and was therefore proposed to function in concert with the Sec translocase to transfer transmembrane regions of Sec-dependent proteins into the lipid bilayer. Indeed, recent evidence shows that YidC is essential for the Sec translocase-mediated membrane insertion of subunit a of the cytochrome bo$_3$ oxidase subunit (8–10). YidC has also been implicated in the folding of LacY but appears not to be essential for its membrane insertion (11). Importantly, YidC also functions independently of the Sec translocase in membrane protein insertion. This was first demonstrated for small phage proteins such as M13 procoat (12) and Pf3 coat (13) that were previously suggested to insert “spontaneously” into the lipid bilayer (14). The c subunit of the F$_1$F$_0$ ATP synthase (F$_0$c) was shown to be an authentic substrate for this YidC-only pathway (15, 16). F$_0$c is a small inner membrane protein with two transmembrane segments that assembles into a decameric ring structure in *E. coli* (17). The c-ring is associated with two b subunits and one a subunit (F$_0$b and F$_0$a, respectively) and forms the membrane-embedded F$_0$ sector. The catalytic F$_i$ domain is peripherally bound to the F$_0$ sector at the cytosolic side of the membrane. The F$_1$,F$_0$ ATP synthase converts the energy stored in a transmembrane electrochemical proton gradient into ATP and plays a central role in the energy metabolism of the cell. ATP synthesis or hydrolysis occurs on three catalytic sites in the F$_i$ sector, whereas H$^+$ transport occurs through the F$_0$ subcomplex at the interface between F$_0$a and the F$_0$c ring (18–20).

Using an in vitro system it was demonstrated that YidC is required and sufficient for the stable membrane insertion of F$_0$c in its correct topology (15). Membrane-inserted F$_0$c was found to assemble into a large oligomeric complex with a size reminiscent of that of the F$_0$c ring observed in vivo. Interestingly, all known proteins that strictly require YidC for membrane insertion, the major coat proteins of the bacteriophages Pf3 and M13 (21), F$_0$c of the F$_1$,F$_0$ ATP synthase, and subunit a of the cytochrome bo$_3$ oxidase subunit, assemble into large oligomeric structures, which may point to a role of YidC in the assembly of macromolecular protein complexes. Furthermore, this raises the question of whether insertion and oligomerization are concerted processes and whether these both require the function of YidC. YidC may act solely as a membrane protein insertase mediating the membrane integration of F$_0$c monomers. Alternatively, YidC may function as a membrane chaperone that stabilizes the “spontaneously” membrane-inserted monomeric form of F$_0$c in its correct conformation to facilitate its oligomerization. Another possibility is that YidC catalyzes both the insertion and assembly reactions.

To distinguish between the putative insertase and chaperone functions of YidC we have analyzed the YidC dependence of the membrane insertion of two F$_0$c mutants, *i.e.* G23D and L31F, that do not support the formation of an active F$_1$,F$_0$ ATP synthase (22, 23). These mutants have been suggested to be defective in assembly of the F$_0$ sector, but the exact nature of the assembly defect has not been elucidated (24). Here we have shown that the two F$_0$c mutants still insert *in vitro* into the membrane in a YidC-dependent manner but that they are unable to assemble into an oligomer. This shows that the oligomerization into the c-ring *per se* is not essential for the stable
YidC-dependent membrane insertion of \( F_0c \) consistent with a function of YidC as a membrane protein insertase.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids** — The *uncE114* mutant strain MM944Iq (25) was used in the *in vivo* complementation experiments and ATPase activity and \( \Delta \text{pH} \) measurements. pET20AtpE was used for the *in vitro* transcription of wild-type \( F_0c \) (15). pET20AtpE-G23D and pET20AtpE-L31F were constructed by replacing the GGT codon at position 23 with GAT or the CTC codon at position 31 with TTC in the *atpE* gene, respectively. For *in vivo* expression, the Ndel/EcoRI sites surrounding *atpE* were replaced by *NcoI/BamHI* sites, and the resulting fragment was cloned into the plasmid pTrc99a, yielding pTrc99AtpE and its derivatives pTrc99AtpE-G23D and pTrc99AtpE-L31F.

**In Vivo Complementation** — The pTrc99a-based plasmids were used to transform strain MM944Iq. Cells were streaked on M63 minimal medium plates containing 0.6% succinate or 0.2% glucose with the appropriate supplements and 25 \( \mu \text{g/ml} \) kanamycin and 100 \( \mu \text{g/ml} \) ampicillin (25). Cells were grown for 20 h at 37°C.

**ATPase and \( \Delta \text{pH} \) Measurements** — Strain MM944Iq transformed with the appropriate plasmids was grown on M63 medium supplemented with 0.6% glucose, the appropriate supplements, 25 \( \mu \text{g/ml} \) kanamycin, 100 \( \mu \text{g/ml} \) ampicillin, and 400 \( \mu \text{M} \) isopropyl-1-thio-\( \beta \)-D-galactopyranoside for induction until an \( \text{OD}_{600} \) of 1.0. Cells were collected by centrifugation, and inner membrane vesicles (IMVs) were isolated by French pressure treatment (26). The ATPase activity of isolated IMVs at 37°C was measured by the malachite green assay as described (27). To inhibit the activity of coupled \( F_0/F_1 \) complexes 250 \( \mu \text{M} \) dicyclohexylcarbodiimide was used. The \( \Delta \text{pH} \) in IMVs was measured by means of the fluorescent dye 9-amino-6-chloro-2-methoxyacridine (ACMA). Reaction mixtures contained 1.25 \( \mu \text{M} \) IMVs and 1 \( \mu \text{M} \) ACMA in 25 \( \mu \text{M} \) HEPES-KOH, pH 8.0, 25 \( \mu \text{M} \) KCl, 2.5 \( \mu \text{M} \) MgCl\(_2\), 1 \( \mu \text{M} \) dithiothreitol, and 0.1 \( \mu \text{g/ml} \) bovine serum albumin. Reactions were started by the addition of 1 \( \mu \text{M} \) ATP or 1.25 \( \mu \text{M} \) NADH, and the ACMA fluorescence was monitored in a PerkinElmer LS50B luminescence spectrophotometer at excitation and emission wavelengths of 409 and 474 nm, respectively.

**In Vitro Insertion Reaction Assays** — The RiboMax transcription kit (Promega) was used for the synthesis of mRNA in a coupled transcription/translation system. Reactions were carried out for 20 min at 37°C in the presence of proteoliposomes as described previously (28). A small sample of the reaction mixture was used as a synthesis control, and the remainder was treated with 0.4 \( \mu \text{g/ml} \) proteinase K for 30 min on ice in the presence or absence of 1% Triton X-100. Samples were trichloroacetic acid precipitated and analyzed by 18% SDS-PAGE and phosphorimaging.

**Blue Native PAGE Analysis** — \( F_0c \) was synthesized radioactively in the presence of YidC proteoliposomes, empty liposomes, or no membranes present. After synthesis, 0.1% DDM-solubilized YidC proteoliposomes were added to the reactions without membranes present. All reactions were subsequently treated with 0.1% DDM, and after 15 min on ice nonsolubilized proteins were removed by centrifugation through a glycerol cushion consisting of 10 \( \mu \text{M} \) HEPES-KOH, pH 7.5, 0.1% DDM, 20% glycerol. The supernatant was mixed with Ni\(^{2+}\)-NTA-agarose beads (Qiagen, Hilden, Germany) equilibrated with buffer A (10 \( \mu \text{M} \) HEPES-KOH, pH 7.5, 100 \( \mu \text{M} \) KCl, 20% glycerol, 0.1% DDM) containing 10 \( \mu \text{M} \) imidazole. The suspension was gently shaken for 2 h at 4°C. Beads were washed with 10 volumes of buffer A containing 60 \( \mu \text{M} \) imidazole, and bound proteins were eluted with buffer A containing 250 \( \mu \text{M} \) imidazole, transferred to gel-loading buffer, and analyzed by 18% SDS-PAGE and phosphorimaging.

**Other Methods** — YidC was purified and reconstituted into *E. coli* phospholipids (Avanti Polar Lipids, Inc., Alabaster, AL).
as described (30). YidC polyclonal antiserum was raised in rabbit against purified hisYidC by Agrisera (Umeå, Sweden). F0c monoclonal antiserum was a generous gift from G. Deckers-Hebestreit (University of Osnabrück, Germany).

RESULTS

F0c G23D and L31F Mutants Are Defective in the Formation of an Active F1F0 ATP Synthase—Previously, two F0c mutants (G23D and L31F) (22, 23) have been isolated that do not support the assembly of a functional F1F0 ATP synthase, possibly as a result of a defect in the formation of a functional F0 sector. As both mutants have not been analyzed biochemically, we first validated their phenotype in vivo. For this purpose, the atpE (G23D) and atpE (L31F) mutant genes were cloned behind the inducible trc promoter and the resulting plasmids were transformed into strain MM944Iq. This strain contains a chromosomal mutation in the atpE gene encoding subunit c (Q42E) that disturbs the binding of F1 to the F0 subcomplex (25). Strain MM944Iq is unable to grow on minimal medium with succinate as an energy and carbon source, whereas growth on glucose is undisturbed. Introduction of a plasmid containing wild-type atpE into MM944Iq fully restored growth on succinate minimal medium plates (Fig. 1A, right panel), whereas an empty plasmid or a plasmid containing atpE (G23D) had no effect. A plasmid containing atpE (L31F) also restored growth of strain MM944Iq on succinate minimal medium plates, but after overnight growth at 37 °C observed colonies were approximately half the size of the colonies with the wild-type atpE plasmid (Fig. 1A, left panel). Immunoblotting showed that the levels of F0c in IMVs derived from cells expressing wild-type F0c and F0c L31F are elevated as compared with background levels of the chromosomal atpE gene (Fig. 1B, insert), whereas with F0c G23D IMVs the level of F0c is only marginally increased.

To obtain quantitative information on the activity of the F0c mutants, IMVs from strain MM944Iq were analyzed for their ATPase activity and ability to generate a proton motive force. In contrast to the wild-type, both F0c mutants supported only low levels of ATPase activity. When dicyclohexylcarbodiimide, an inhibitor of proton translocation by F1F0 ATP synthase, was added, the ATPase activity of the wild-type was strongly reduced whereas the activity of the mutant complexes was unaffected (Fig. 1B). To examine the effect of the F0c mutants on the ability of the F1F0 ATP synthase to generate a proton motive force, IMVs were incubated with either ATP or NADH and the generation of a transmembrane proton gradient (ΔpH) was monitored with the fluorescent dye ACMA. The ionophore valinomycin that converts the transmembrane electrical potential (Δψ) into a ΔpH, while nigericin (1 μM) was used to collapse the ΔpH.

FIGURE 2. F0c G23D and F0c L31F are defective in proton motive force generation. MM944Iq IMVs containing wild-type F0c (A, E), F0c G23D (B, F), F0c L31F (C, G), or no F0c (D, H) were analyzed for the generation of a ΔpH as monitored by the fluorescence quenching of ACMA. Where indicated 1 mM ATP (A–D) or 1.25 mM NADH (E–H) was added to generate a proton motive force. Valinomycin (1 μM) was used to convert the Δψ into a ΔpH, while nigericin (1 μM) was used to collapse the ΔpH.
what more pronounced with the F_{0c} L31F mutant, suggesting the presence of a very small ΔpH (Fig. 2C). When the oxidizable substrate NADH was added as an energy source all IMVs were capable of generating a ΔpH, showing that the expression of the F_{0c} mutants does not interfere with the integrity of the membrane (Fig. 2, D–G). These data demonstrate that the F_{0c} mutants G23D and L31F are defective in the formation of an active F_{1}F_{0} ATP synthase complex.

**F_{0c} G23D and L31F Insert in a YidC-dependent Manner into the Membrane but Are Unable to Form Oligomeric Complexes—**YidC is required for the efficient insertion of wild-type F_{0c} into liposomes and IMVs (15) and in intact cells (15, 16). To test the membrane insertion requirement of the F_{0c} mutants, the proteins were synthesized in a cell-free transcription/translation system and assayed for membrane insertion into liposomes and YidC proteoliposomes using a protease protection assay (15). In the presence of YidC proteoliposomes, wild-type F_{0c} was protease resistant as shown before (15) (Fig. 3A, lane 2). F_{0c} G23D and F_{0c} L31F were also found to be protease protected in the presence of YidC proteoliposomes (Fig. 3A, lanes 4 and 6), although insertion was less efficient as compared with wild-type F_{0c}. The formation of a second proteolytic fragment of F_{0c} L31F was observed at ~4 kDa (Fig. 3A, lane 8), which may point to an effect of the L31F mutation on the conformation of F_{0c} resulting in an altered susceptibility to the added protease. Similar results were obtained when IMVs were used instead of YidC proteoliposomes (data not shown). No or very little protease-protected F_{0c} could be detected in protein-free liposomes (Fig. 3B).

These data demonstrate that the F_{0c} G23D and L31F mutants insert into the membrane in a YidC-dependent manner.

**In vivo, the F_{0c} subunits assemble into a ring-like structure that forms part of the rotor domain of F_{1}F_{0} ATP synthase (31–33).** We have previously demonstrated that in vitro, YidC membrane-inserted wild-type F_{0c} assembles into an oligomer (15). To determine whether the membrane-inserted F_{0c} mutants are capable of oligomerization, YidC proteoliposomes containing membrane-inserted F_{0c} were re-isolated, solubilized with the detergent dodecyl maltoside, and subjected to Blue Native PAGE (Fig. 3C). Radioactively labeled wild-type F_{0c} was detected as a high molecular mass complex of ~80 kDa (Fig. 3C, lane 1) (15). The F_{0c} G23D and L31F mutants both failed to form such a complex and are only visible as a broad and a weak band, most likely the monomeric species (Fig. 3C, lanes 2 and 3). These data demonstrate that, at least in vitro, the YidC membrane-inserted F_{0c} G23D and L31F mutants are defective in the formation of a stable oligomer.

**F_{0c} Associates with YidC in Vitro—**Because the wild-type F_{0c} and assembly mutants require YidC for membrane insertion, the physical interaction between YidC and F_{0c} during membrane integration was addressed. Co-affinity purification was used to assay for the association of membrane-integrated YidC with in vitro synthesized and inserted radiolabeled F_{0c}. After an insertion reaction, membranes were solubilized with DDM. Unsolubilized membrane proteins were removed by centrifugation, and the supernatant was incubated with Ni^{2+}-NTA
beads to purify His-tagged YidC. Co-purification of F₀c was subsequently analyzed by autoradiography. Immunoblotting showed that the recovery of YidC in all samples was identical (Fig. 4B). Only a low level of background binding of F₀c to Ni²⁺-NTA beads is observed with protein-free liposomes (Fig. 4A, lanes 4–6), but in the presence of YidC-containing proteoliposomes significant levels of in vitro synthesized F₀c co-purified (Fig. 4A, lanes 1–3). Remarkably, the F₀c G23D mutant reproducibly co-purifies to an up to 2-fold higher extent than wild-type and F₀c L31F. Strikingly, a much lower level of association was observed when solubilized YidC proteoliposomes were added after the in vitro synthesis reaction had been completed (Fig. 4A, lanes 7–9). These data demonstrate that YidC directly interacts with F₀c within the membrane and that the G23D mutation in F₀c stabilizes this interaction.

**DISCUSSION**

The functional assembly of energy-transducing membrane protein complexes such as the cytochrome bo₃ oxidase and the F₁F₀ ATP synthase in *E. coli* is strongly affected by the cellular depletion of YidC (34). Recent in vivo and in vitro studies demonstrate that key subunits of these energy transducing complexes strictly require YidC for membrane insertion (8–10, 15, 16, 35, 36). Reconstitution studies demonstrate that YidC alone mediates the stable membrane insertion of subunit c (F₀c) of the F₉ sector of the F₁F₀ ATP synthase. Upon membrane insertion, a large oligomeric complex is formed with a molecular mass reminiscent of that of the F₀c ring (15). Here we have characterized two mutants of F₀c that are unable to form a functional F₁F₀ ATP synthase. Our data suggest that the assembly defect of the mutants is due to a deficiency in the F₀c ring formation, whereas the membrane insertion of these mutant proteins still strictly depends on YidC. This study therefore separates the proposed chaperone and insertase functions of YidC in the biogenesis of the F₀ sector. *In vivo*, the defect of the two F₀c mutants may be even further strengthened by a rapid degradation of the unassembled F₀c subunits by FtsH (37). Although we cannot exclude the possibility that YidC is also actively involved in the oligomerization of F₀c, this process appears not to be mechanistically linked to the initial insertion. YidC may facilitate the hydrophobic partitioning of F₀c into the membrane by providing an amphiphilic surface (38), and subsequent oligomerization of correctly inserted F₀c units upon their release from YidC may be spontaneous events as suggested previously (39).

By co-affinity purification we have demonstrated that YidC directly interacts with its substrate F₀c. This interaction was found to be more pronounced with one of the assembly mutants, F₀c G23D, which suggests a slower release of F₀c G23D by YidC. Because this effect is not as dramatic as the oligomerization defect, assembly is most likely interrupted because of the inability of the mutant c subunits to interact (see below). Chen *et al.* (13) have previously shown by photo-cross-linking that YidC interacts with an arrested nascent chain of the Sec-independent phage protein, Pf3 coat. However, photo-cross-linking is not a suitable approach to study the interaction between YidC and F₀c, because the formation of a helical hairpin, which has been suggested to drive the membrane integration of F₀c (38, 40), can only occur when both termini of the polypeptide chain have been released from the ribosome. Our co-affinity-purified YidC-F₀c complex most likely represents an insertion and assembly intermediate of the catalytic cycle.
The in vivo and biochemical characterizations of the mutant F$_0$F$_1$ ATP synthase complexes demonstrate that the F$_0$c G23D and L31F mutants are unable to support the formation of an active F$_0$ sector. The crystal structure of the c-ring of an F-type Na$^+$ ATP synthase of *Ilyobacter tartaricus* (33) now provides clues as to why these mutants are disturbed in oligomerization. An aspartate residue introduced at position 23 (*I. tartaricus* Gly-27) protrudes laterally from one F$_0$c subunit, resulting in both intra- and intermolecular steric hindrances (Fig. 5A), whereas the bulkier phenylalanine residue at position 31 (*I. tartaricus* Ala-35) causes a more extensive hindrance, but only within a single F$_0$c molecule (Fig. 5B). This difference in interactions might explain why the F$_0$c L31F mutant, in contrast to the F$_0$c G23D mutant, shows some ability to transport protons and, when overexpressed, rescues the growth defect on succinate. The intramolecular hindrances of the L31F mutant seem to destabilize the oligomer so that it does not survive native electrophoresis. However, oligomer formation is not completely abolished, because in vivo, partial activity of the F$_1$F$_0$ ATPase is retained. Moreover, the other F$_0$ subunits and the F$_1$ subcomplex may have a further stabilizing effect on the defective c-ring in vivo. The intermolecular hindrance of F$_0$c G23D disturbs the interactions between different subunits and consequently severely affects the assembly of the c-ring. It should be stressed that the level of F$_0$c in IMVs derived from cells expressing F$_0$c G23D is only marginally increased, which may point to stressed that the level of F$_0$c in IMVs derived from cells expressing F$_0$c G23D is only marginally increased, which may point to

The biogenesis of the active F$_1$F$_0$ ATPase is likely a coordinated process that requires the membrane insertion of the separate F$_0$ subunits, the stabilization of these subunits prior to their assembly into the F$_0$ subcomplex with the correct stoichiometry of a, b, and c subunits, and finally the coupling of F$_0$ to the F$_1$ sector. It is not clear if the F$_1$-sector and the F$_0$-sector are fully assembled before coupling occurs, but in vitro studies demonstrate that mixing of the separately purified complexes results in the restoration of coupled ATPase activity (41). Previous studies have shown that F$_0$a is not present in the membrane unless F$_0$c and F$_0$b are co-expressed (42). In vitro, F$_0$a and F$_0$b can be isolated as a subcomplex that when co-reconstituted with F$_0$c yields a functional F$_0$ sector (43). F$_0$a is unstable when uncomplexed, and it is degraded by the FtsH protease (37). On the other hand, stable F$_1$F$_0$ complexes occur without F$_0$a present (44), and the activity of the subunit a-deprived complexes of the thermophilic *Bacillus* PS3 can be restored by the simple addition of purified F$_0$a (45). Fluorescence studies have shown that F$_0$b and a fully assembled c-ring suffice for the binding of the F$_1$ subcomplex (46). These data imply that a correct and timed assembly of the c-ring is crucial for the formation of a functional ATP synthase and that F$_0$a might be the last component that in vivo is added to a complex of F$_0$b and the c-ring.

The Alb3/Oxa1/YidC family of proteins are not only structurally but also functionally related (47, 48). In mitochondria, Oxa1p is involved in the membrane insertion of a subset of proteins synthesized in the mitochondrial matrix and interacts with translating ribosomes (49). Oxa1p is needed for the assembly of large energy-transducing complexes such as the F$_1$F$_0$ ATP synthase and the cytochrome c oxidase (50, 51). The thykoidal Alb3 protein is required for the membrane insertion and assembly of light harvesting proteins (52). The results presented in this study do not exclude the possibility that YidC also actively participates in the assembly of large membrane complexes. In the case of F$_0$c, YidC might accumulate single or several membrane-inserted subunits prior to the formation of the ring structure. Our observation that stable YidC-F$_0$c subcomplexes can be isolated is consistent with this hypothesis. Future studies should address the mechanism by which the various subunits assemble into a functional F$_0$-sector and how protein substrates are released by YidC.

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