F$_1$F$_0$ ATP synthase subunit c is a substrate of the novel YidC pathway for membrane protein biogenesis

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Summary

The Escherichia coli YidC protein belongs to the Oxa family of membrane proteins that have been suggested to facilitate the insertion and assembly of membrane proteins either in cooperation with the Sec translocase or as a separate entity. We have recently shown that depletion of YidC causes a specific defect in the functional assembly of F$_1$F$_0$ ATP synthase and cytochrome o oxidase. We now demonstrate that the insertion of in vitro synthesized F$_1$F$_0$ ATP synthase subunit c (F$_0$c) into inner membrane vesicles requires YidC. Insertion is independent of the proton motive force, and proteoliposomes containing only YidC catalyze the membrane insertion of F$_0$c in its native transmembrane topology whereupon it assembles into large oligomers. Co-reconstituted SecYEG has no significant effect on the insertion efficiency. Remarkably, signal recognition particle (SRP) and its membrane-bound receptor FtsY are not required for the membrane insertion of F$_0$c. In conclusion, a novel membrane protein insertion pathway in Escherichia coli is described in which YidC plays an exclusive role.
**Chapter 5**

**Introduction**

All major energy-transducing cellular membranes contain one or more members of the Cytochrome Oxidase Biogenesis (Oxa) family of membrane proteins (140). In the inner membrane of yeast mitochondria, Oxa1 is required for the insertion of several membrane proteins among which are subunits of the F_{1}F_{0} ATP synthase and cytochrome c oxidase (220). These include mitochondrion-encoded proteins, like Cox2 (15, 99), as well as nucleus-encoded proteins, like Oxa1 itself (107). The latter are first imported into the mitochondrial matrix and subsequently inserted into the inner membrane. The chloroplast homologue Alb3 is required for the insertion of the light-harvesting complex protein (LHCP) into the thylakoid membrane (172). In the Gram-negative bacterium *Escherichia coli*, an Oxa1 homologue termed YidC was found that could be crosslinked to the transmembrane segments of nascent membrane proteins, like FtsQ (213), leader peptidase (115) and MtlA (17). This process involves targeting via the signal recognition particle (SRP) pathway (109) and the initial membrane insertion by the SecYEG translocase (48). However, YidC is not essential for the insertion of the Sec-dependent model protein FtsQ as the Δψ-dependent membrane integration of this protein can be reconstituted with proteoliposomes containing only SecYEG (243). Initially, only the Sec-independent phage proteins M13 procoat (205) and Pf3 coat (35) were found to depend strictly on YidC for membrane insertion. Therefore, it was not clear why YidC is essential for cell viability. Recently, we have shown that depletion of YidC causes a severe defect in the functional assembly of both cytochrome o oxidase and F_{1}F_{0} ATP synthase (242), which is reminiscent of the effects of mutations in OXA1 (7). In particular, the amounts of cytochrome o oxidase subunit a (CyoA) and the small, ring-forming F_{0} subunit c (F_{0}c) were exceptionally sensitive to YidC depletion. Consequently, YidC-depleted inner membrane vesicles (IMVs) are severely impaired in their ability to generate a proton motive force (PMF) with oxidizable substrates or ATP (242). Yi et al. have shown that in addition to F_{0}c, membrane insertion of F_{0} subunit a (F_{0}a) is also inhibited by depletion of YidC in vivo (268).

*E. coli* F_{1}F_{0} ATP synthase consists of a membrane-integral F_{0} part (subunit composition a_{3}b_{2}c_{10}) and a peripherally bound, catalytic F_{1} subcomplex (α_{3}β_{3}γ_{3}ε) (33). During the catalytic cycle, the reversible protonation of F_{0}c at residue Asp61
induces a rotation of $\gamma$, $\epsilon$ and the c ring relative to the $\alpha_3\beta_3$ hexagon. Subunits $F_{0b}$ and $F_{1\delta}$ form a so-called "stator" that ensures that $F_{0a}$ and the $\alpha_3\beta_3$ hexagon do not rotate together with $\gamma e_c$ring. This causes a rotor torque that is believed to be translated into conformational changes of the catalytic residues by elastic power transmission finally leading to the synthesis of ATP from ADP and phosphate (257). Although the mechanisms of energy-transduction have been studied in great detail, remarkably little is known about the assembly of large energy-transducing membrane protein complexes like $F_{1F_0}$ ATP synthases or cytochrome oxidases. In yeast, proteins have been identified that are required for their biogenesis, but their precise function is not understood (1, 2, 34). The $E. coli$ $F_{1F_0}$ ATP synthase represents the simplest form of this enzyme containing only the core subunits described above. The observed YidC requirement for the assembly of functional $F_{1F_0}$ ATP synthase in vivo and particularly of the membrane insertion of the $F_{0c}$ rotor ring subunit indicates that the biogenesis of this key enzyme is more complex than anticipated so far (10). In order to understand the role of YidC in the membrane insertion and oligomeric assembly of $F_{0c}$, we have employed an in vitro translation/insertion assay that has been successfully used to determine the minimal requirements for the Sec-dependent membrane insertion of FtsQ (243). Here we demonstrate that in vitro membrane insertion of $F_{0c}$ is blocked by YidC depletion and can be reconstituted with proteoliposomes containing only YidC, while SecYEG, the PMF and the SRP pathway are not required. Recently, Serek et al. reported that YidC alone reconstituted into proteoliposomes stimulates the in vitro membrane insertion of Pf3 indicating that YidC can function as a separate membrane protein insertase (214). Our data demonstrate an essential role of the YidC insertase in the assembly of a Sec-independent, authentic $E. coli$ membrane protein. $F_{0c}$ is the first described natural substrate of this novel membrane protein biogenesis pathway, which appears to be employed by bacteriophages to assemble their coat proteins in the host cell membrane.
Results

Insertion of $F_0c$ into inner membrane vesicles requires YidC but is independent of the proton motive force

$E. coli$ $F_0c$ is a very hydrophobic polypeptide of 79 amino acids length. It consists of two transmembrane helices connected by a small polar loop that is exposed to the cytoplasm (Fig. 1A, according to ref. 87). $[^{35}S]$methionine-labeled $F_0c$ was synthesized \textit{in vitro} in an $E. coli$ S135 lysate (Fig. 1B, lane 1). As the predicted molecular mass of $F_0c$ is 8.3 kDa, the translation product showed an aberrant running behavior on SDS-PAGE typically observed for hydrophobic membrane proteins. Western Blot analysis of wild-type $E. coli$ IMVs revealed that endogenous $F_0c$ migrates at an identical position (data not shown) indicating that the \textit{in vitro} translation product is full-length $F_0c$. When $E. coli$ IMVs were present during translation, a significant fraction of the $F_0c$ was found to be resistant to added proteinase K (Fig. 1B, lane 2), trypsin and pronase (data not shown). Furthermore, endogenous $F_0c$ in IMVs appeared protease-resistant as well. Solubilization of IMVs with detergent prior to protease treatment led to partial cleavage of $F_0c$ (Fig. 1B, lane 3), probably due to protection of the hydrophobic domains by the detergent micelles. In the absence of membranes and detergent $F_0c$ was completely degraded (Fig. 1B, lanes 4, 5). Taken together, this indicates that the protease-protected conformation of $F_0c$ represents its membrane-inserted state. When IMVs were pretreated with proteinase K before being added to the translation reaction, membrane insertion of $F_0c$ was almost completely blocked (Fig. 1C) indicating that a membrane protein is needed to facilitate this process. The PMF, however, is not required, as uncoupling of IMVs by the addition of the ionophores valinomycin and nigericin had no effect on the membrane insertion of $F_0c$ (Fig. 1D), while it severely inhibited the PMF-dependent membrane insertion of the Sec-dependent model protein FtsQ (Fig. 2C; Van der Laan et al., 2004).

We have previously shown that the amount of $F_0c$ in IMVs depleted from YidC is strongly reduced (242). To determine if YidC plays a direct role in the membrane insertion of $F_0c$, \textit{in vitro} insertion assays were performed with YidC-depleted IMVs prepared from strain $E. coli$ JS7131 (205) grown in the presence of 0.2% glucose. To achieve optimal depletion (Fig. 2A) and to avoid pleiotropic effects related to the stationary phase, cells were diluted 1:1 after every generation. This procedure led to a
**Fig. 1:** Membrane topology model of F$_0$c (A) based on the NMR solution structure (1A91.pdb). The arrows indicate the positions of the introduced cysteine mutations for topology analysis. *In vitro* synthesized F$_0$c inserts into *E. coli* wild type IMVs (B). Translation reactions were carried out in the presence (B, lanes 1-3) or absence (B, lanes 4 and 5) of 5 µg IMVs. After the translation reaction samples were treated with 0.4 mg/ml proteinase K (B, lanes 2, 3 and 5) in the presence (B, lane 3) or absence (B, lane 1, 2, 4 and 5) of 1 % (v/v) Triton X100. In the presence of Triton X100, a proteolytic fragment of F$_0$c is formed (*). Where indicated, 20% standards of the translation reactions are shown.

A membrane protein is required for the membrane insertion of F$_0$c (C). IMVs were pretreated in the presence (C, lanes 1 and 3) or absence (C, lanes 2 and 4) of proteinase K as described in the Methods section. The PMF is not required for the membrane insertion of F$_0$c (D). Insertion reactions were carried out in the absence (D, lanes 1 and 3) or in the presence (D, lane 2 and 4) of 1 µM valinomycin (Val.) and 1 µM nigericin (Nig.).

Very efficient YidC depletion (Fig. 2A) and an almost complete inability to generate a PMF with ATP (data not shown). Consequently, PMF-dependent processes such as the translocation of proOmpA (Fig. 2B, lanes 2, 3) and the membrane insertion of FtsQ (Fig. 2C, lanes 1-4) were strongly affected in these YidC-depleted IMVs. The extents of inhibition caused by the depletion of YidC were comparable to that...
Fig. 2: YidC is required for the membrane insertion of F₀c. YidC can be efficiently depleted from *E. coli* JS7131 IMVs (A). Cells were grown as described in the Methods sections in the presence of 0.2% arabinose (A, lane 1) or 0.2% glucose (A, lane 2) and the amounts of YidC in IMVs were monitored by Western Blotting. YidC-depleted IMVs exhibit reduced proOmpA translocation (B, lanes 1-3) and FtsQ insertion activities (C, lanes 1-4). The same extents of reduction are observed, when the reactions are carried out with wild type IMVs in the presence of 1 µM valinomycin (Val.) and 1 µM nigericin (Nig.) to uncouple the PMF (B, lane 4; C, lanes 5 and 6). Post-translational proOmpA translocation as well as co-translational FtsQ insertion reactions contained 25 µg IMVs. YidC depletion blocks membrane insertion of F₀c (D). F₀c insertion reactions were performed as described in the legend to Fig. 1.
observed with wild-type IMVs in the presence of uncouplers (Fig. 2B, lane 4; Fig. 2C, lanes 5, 6). Remarkably, the membrane insertion of F₀c into IMVs was almost completely blocked upon depletion of YidC (Fig 2D). As we have shown that F₀c inserts independently of the PMF (Fig. 1D), it can be concluded that this effect is directly caused by the loss of YidC.

**YidC proteoliposomes catalyze the SecYEG-independent membrane insertion of F₀c**

The observation that YidC is required for the insertion of F₀c into IMVs did not exclude that other membrane proteins, like the Sec translocase or other unknown components, are involved as well. Only the reconstitution of F₀c insertion into proteoliposomes could clearly define the minimal requirements for this process. Therefore, we prepared (proteo-)liposomes containing either YidC together with SecYEG, YidC or SecYEG alone, or no protein and titrated them into the translation mixture. Upon addition of protein-free liposomes, small amounts of protease-protected F₀c were detected (Fig. 3A), indicating a low level of spontaneous membrane insertion. Proteoliposomes containing YidC, however, supported highly efficient membrane insertion of F₀c (Fig. 3A, B). Insertion efficiency achieved in the presence of saturating amounts of proteoliposomes was ~ 40 %. Co-reconstitution of SecYEG did not significantly increase the amount of membrane-inserted F₀c (Fig. 3A, B). Moreover, the presence of SecYEG alone did not stimulate F₀c insertion compared to protein-free liposomes (Fig. 3A, B). In contrast, proteoliposomes containing SecYEG mediated the translocation of proOmpA independently of YidC showing that the reconstituted Sec translocase is functional (Fig. 3A). These data indicate that YidC alone is able to catalyze membrane integration of F₀c independently of the Sec translocase. To further examine a possible role of SecYEG in YidC-mediated insertion of F₀c, experiments were performed using limiting YidC concentrations. YidC was reconstituted at different protein to lipid ratios in the presence or absence of a fixed amount of SecYEG. However, in no case a significant stimulation of F₀c insertion by SecYEG was observed (Fig. 4A, B).

Recently, we have shown that the insertion of the Sec-dependent membrane protein FtsQ is a strictly co-translational process. **In vitro**, no insertion is observed when the membranes are added after translation has been terminated by the addition of chloramphenicol (115, 243). Under identical conditions, post-translational insertion of F₀c into YidC proteoliposomes could also not be detected (Fig. 4C).
Fig. 3: YidC alone is sufficient to catalyze the membrane insertion of F₀c into proteoliposomes (A). Co-translational F₀c insertion as well as post-translational proOmpA translocation reactions were performed in the presence of the indicated amounts of (proteo-)liposomes reconstituted with YidC and SecYEG (YidC/SecYEG), YidC alone, SecYEG alone or without any proteins. F₀c insertion efficiency at different (proteo-)liposome concentrations was quantified from the amount of protease-protected material in the presence of SecYEG/YidC (■), YidC (○), SecYEG (□) or protein-free (●) liposomes (B). The amount of F₀c synthesized was comparable in all reactions (not shown) and was set as 100%. All data points shown in (B) are averages from five independent experiments, in which proteoliposomes from three independent reconstitutions were used. Purified SecYEG and YidC were both used at a protein / lipid ratio of 1:100 [w/w].
**Fig. 4:** SecYEG is not required for F₀c membrane insertion. (A). Reactions were carried out as described in the legend of figure 3 in the presence of 100 µg lipid/ml proteoliposomes. For the reconstitutions, YidC was used at the indicated protein to lipid ratios together with (+SecYEG) or without (-SecYEG) a fixed amount of SecYEG (protein / lipid ratio 1:100 [w/w]). F₀c insertion efficiencies were quantified from the amount of protease-protected material in the presence (○) or absence (●) of SecYEG (B). Synthesis of F₀c was comparable in all reactions (not shown). All data points shown in (B) are averages from three independent experiments. Proteoliposomes from two independent reconstitutions were used. When added after translation has been stopped with chloramphenicol, YidC proteoliposomes do not stimulate membrane insertion of F₀c (C). Translation reactions were carried out in the absence of membranes. Reactions were stopped by the addition of 25 µg/ml chloramphenicol. After 5 min indicated amounts of YidC proteoliposomes or protein-free liposomes were added and reaction mixtures were incubated for 20 min at 37°C.
**YidC-mediated membrane insertion of F\(_{\theta}c\) does not require the SRP pathway**

Co-translational targeting to the Sec translocase occurs via the SRP pathway (109). SRP binds to particularly hydrophobic signal sequences or transmembrane segments as they emerge from the ribosome. Upon interaction of SRP with its membrane-bound receptor FtsY, GTP hydrolysis drives the release of SRP from the nascent chain and the transfer of the translating ribosome to the Sec translocase. It is not clear, whether the SRP pathway also delivers proteins directly to YidC. The YidC-dependent phage proteins M13 procoat (47) and Pf3 (35) do not require SRP. However, Fröderberg *et al.* have constructed a fusion protein that does not depend on SecYEG, but requires YidC as well as the SRP pathway *in vivo* (79). In addition, the chloroplast YidC homologue Alb3 forms a complex with cpSRP and cpFtsY that can be stabilized by the addition of the non-hydrolysable GTP analogue GMP-PNP (173). As F\(_{\theta}c\) represents the first "native" substrate of a novel membrane protein insertion pathway in which YidC seems to play a key role, we analyzed the involvement of the SRP pathway as well as the SecA motor protein. SecA is strictly required for the SecYEG-dependent translocation of the large periplasmic domain of FtsQ (243). As expected, immunodepletion of SecA from the translation lysate had no effect on the insertion of F\(_{\theta}c\) into YidC proteoliposomes (Fig. 5A, lane 2), whereas under identical conditions, it completely blocks FtsQ insertion (243).

Remarkably, also immunodepletion of the SRP receptor FtsY did not significantly affect YidC-mediated F\(_{\theta}c\) insertion (Fig 5A, lane 3). Efficient FtsY depletion was demonstrated by Western Blotting (Fig. 5 B) and by the inhibitory effect on membrane insertion of FtsQ (Fig. 5C) as described before (243). To confirm this observation we applied a second, independent experimental approach using a translation lysate that had been depleted from SRP *in vivo*. *E. coli* SRP consists of a 4.5 S RNA and a 48 kDa protein called Ffh. Strain HDB51 (145) carries the \(ffh\) gene under control of the arabinose promotor. Therefore, cells can be depleted from Ffh by growing them in the presence of glucose. Translation lysates were prepared from cells grown under Ffh depletion conditions as well as from Ffh-containing control cells grown in the presence of arabinose. Efficiency of depletion was monitored by Western Blotting (Fig. 5D). In agreement with the results obtained with FtsY-depleted lysate, we did not observe any major effect of Ffh depletion on the insertion of F\(_{\theta}c\) into IMVs, YidC proteoliposomes or YidC/SecYEG proteoliposomes (Fig. 5E).
**Fig. 5:** Membrane insertion of F₀c does not require the SRP pathway or SecA. Translation lysate was immunodepleted from either FtsY or SecA and F₀c insertion reactions were carried out in the presence of YidC proteoliposomes (100 µg lipid/ml; protein / lipid ratio 1:100) (A). A Western Blot with α-FtsY antiserum (B) using 20 µg of wild type (B, lane 1) or FtsY-depleted (B, lane 2). Depletion of FtsY from the lysate inhibits the FtsQ insertion into SecYEG proteoliposomes (C). Reactions were carried out with FtsY-depleted (C, lanes 1 and 2) or wild type lysate (C, lanes 3 and 4) in the presence of Na⁺-loaded SecYEG proteoliposomes (200 µg lipid/ml) and 1 µM valinomycin to generate a transmembrane electrical potential. Ffh can be efficiently depleted *in vivo* in strain *E. coli* HDB51 (D). A Western Blot performed with 25 µg of HDB51 wild type (D, lane 1) or Ffh-depleted (D, lane 2)
lysate is shown. Ffh depletion has no effect on F₀c insertion into IMVs or proteoliposomes containing YidC or YidC together with SecYEG (E). Reactions were carried out with wild type or Ffh-depleted HDB51 lysate in the presence of 5 µg IMVs or 100 µg lipid/ml proteoliposomes. Purified YidC and SecYEG were reconstituted at a protein to lipid ratio of 1/100. Ffh depletion inhibits membrane insertion of FtsQ (F). Reactions were carried out with Ffh-depleted (F, lanes 1 and 2) and wild type HDB51 lysate (F, lanes 1 and 2) in the presence of 25 µg IMVs or 200 µg lipid/ml Na⁺-loaded SecYEG proteoliposomes.

However, the insertion of FtsQ into either IMVs or SecYEG proteoliposomes was strongly inhibited upon depletion of Ffh (Fig. 5F). Taken together, these data demonstrate that membrane targeting and YidC-mediated membrane insertion of F₀c does not require the SRP pathway.

**F₀c inserted into YidC proteoliposomes has acquired the native transmembrane topology**

Next, we addressed the question if F₀c is inserted into YidC proteoliposomes in the correct transmembrane topology. F₀c exposes both the N- and the C-terminus to the periplasm while the small loop connecting the two hydrophobic helices is located in the cytoplasm (Fig. 1A). In our in vitro system, the lumen of the proteoliposomes corresponds to the periplasmic side of the membrane. If F₀c has acquired the correct transmembrane topology, the C-terminus should be inside the proteoliposomes and therefore not accessible for membrane-impermeable chemical reagents. In contrast, it should be possible to modify the cytoplasmic loop that remains on the outside of the proteoliposomes with such probes. We introduced unique cysteine residues into the protein at position 79 at the C-terminus (F₀c A79C) and at position 40 in the cytoplasmic loop (F₀c A40C) (Fig. 1A). Both modified proteins inserted into proteoliposomes in a YidC-dependent manner comparable to wild-type F₀c (Fig. 6A).

For the determination of the transmembrane topology of proteoliposome-inserted F₀c, the protein was synthesized without radioactive tracers in the presence of YidC proteoliposomes. After the translation/insertion reaction and subsequent proteinase K treatment, proteoliposomes were isolated from the reaction mixture and incubated with the membrane permeable, fluorescent cysteine-modifying reagent fluorescein maleimide. As expected both F₀c A79C and F₀c A40C were labeled, whereas the cysteine-less wild type F₀c was not (Fig. 6B, lanes 2, 3). Pretreatment of the
proteoliposomes with the membrane-impermeable cysteine-reactive reagent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMdiS) completely prevented subsequent fluorescein maleimide labeling of F0c A40C (Fig. 6B, lane 4), while F0c A79C could still be labeled after AmdsiS pretreatment (Fig. 6B, lane 4). Five independent experiments yielded an averaged labeling efficiency of ~60%. When the proteoliposomes were solubilized in detergent prior to incubation with AmdsiS, subsequent labeling of F0c A79C with fluorescein maleimide was not possible (data not shown). These results indicate that the cytoplasmic loop of F0c is indeed localized on the outside, whereas the C-terminus has been translocated into lumen of the proteoliposomes. These data show that the F0c has acquired the correct membrane topology after YidC-mediated insertion into proteoliposomes.

F0c inserted into YidC proteoliposomes oligomerizes into large complexes

*In vivo*, F0c subunits assemble into a ring-like structure that is part of the rotor domain of F1F0 ATP synthase (33). Therefore, we asked if *in vitro* synthesized F0c inserted into YidC proteoliposomes forms large oligomeric complexes as well. First, an insertion reaction as described above was carried out. Subsequently, the proteoliposomes were re-isolated from the reaction mixture, solubilized under mild conditions and subjected to Blue-Native PAGE (Fig. 6D). Radioactively labeled F0c was indeed detected almost exclusively in a distinct high molecular weight complex with a molecular mass of ~ 80-100 kDa after insertion into YidC proteoliposomes (Fig. 6D, lane 2). These data demonstrate that membrane inserted F0c is competent for oligomerization.

**Discussion**

About 30% of all structural genes in *E. coli* encode inner membrane proteins. These proteins have to be targeted to the membrane, inserted into the lipid bilayer, correctly folded and some of them have to be assembled into sophisticated supramolecular complexes. Examples of such complexes are the major energy-transducing membrane protein complexes such as the redox-driven proton pump cytochrome *o* oxidase or the PMF-driven mechanoenzyme F1F0 ATP synthase. Little is known about folding of
membrane proteins and complex assembly, but it has been well established that the majority of membrane proteins is targeted via the SRP pathway to the SecYEG translocase where membrane insertion takes place (48). In contrast, two special membrane proteins turned out to have completely different requirements for their

**Fig. 6:** $F_0$C inserted into YidC proteoliposomes has acquired its native transmembrane topology. $F_0$C A79C and $F_0$C A40C insert into YidC proteoliposomes comparable to the wild-type protein (A). Reactions were carried out as described in the legend to Fig. 3 in the presence of the indicated amounts of (proteo-)liposomes. For cysteine accessibility studies, wild type $F_0$C and both single cysteine derivatives were synthesized and inserted into YidC proteoliposomes in non-radioactive translation reactions (B). Subsequent labeling was performed with 1 mM fluorescein maleimide (B, lanes 2-4) as described in the Methods section. A pre-incubation with 0.5 mM AMdiS was performed where indicated (B, lane 4). To control specificity of labeling the same reactions where carried out in the absence of mRNA (B, lane 1) or with the cysteine-free wild type $F_0$C (B, lane 2). *In vitro* synthesized $F_0$C assembles into large oligomeric complexes after insertion into YidC proteoliposomes (C). Reactions were carried out in the presence of protein-free liposomes (C, lane 1) or YidC proteoliposomes (C, lane 2) as described in the Methods section and the high molecular mass oligomers were analyzed by Blue native PAGE. On this gel system, monomeric $F_0$C (in the presence of 1% SDS) migrates as a very diffuse polypeptide band (not shown).
insertion. The small bacteriophage proteins M13 procoat and Pf3 coat do not need the SRP pathway (35, 47) or SecYEG (83, 128) to become integrated into the membrane, but they depend on YidC \textit{in vivo} (35, 205). These two proteins do not stably integrate into the membrane, but in a second step assemble into the phage coat at the extracellular surface of the membrane (204). \textit{In vitro}, they insert into protein-free liposomes with low efficiency (83, 199, 214) and their topologically correct insertion is strongly stimulated by the PMF (45, 128, 214). Moreover, it has been shown that nascent chains of Pf3 coat can be crosslinked to YidC, but not SecY (35). These data indicate that in \textit{E. coli}, an alternatively membrane protein insertion pathway exists in which YidC plays a key role. Recently, Serek \textit{et al.} demonstrated that Pf3 coat can be incorporated into YidC proteoliposomes \textit{in vitro} indicating that YidC functions as an individual membrane protein insertase (214). However, the YidC requirement of the phage proteins M13 procoat and Pf3 coat does not explain why YidC is an essential protein for \textit{E. coli}. Other proteins that have been shown to strictly require YidC \textit{in vivo} are artificial fusion proteins (79, 268) or membrane proteins that have been modified by epitope tags that can alter their membrane insertion pathways (75).

The first step towards the discovery of the native substrates of YidC was the observation that YidC-depleted IMVs contain strongly reduced amounts of cytochrome \textit{o} oxidase and F\textsubscript{1}F\textsubscript{0} ATP synthase (242), two major energy-transducing membrane protein complexes. In the case of F\textsubscript{1}F\textsubscript{0} ATP synthase, especially the amount of the small ring-forming F\textsubscript{0c} protein was affected. Here, we show by means of \textit{in vitro} insertion experiments that YidC alone mediates the membrane integration of F\textsubscript{0c}. Proteoliposomes containing only YidC catalyze efficient F\textsubscript{0c} insertion. Although F\textsubscript{0c} shows some spontaneous insertion into liposomes, reconstituted YidC dramatically stimulates F\textsubscript{0c} integration into proteoliposomes. At all YidC concentrations tested, co-reconstitution of the SecYEG complex has no effect on the insertion efficiency, indicating that the Sec translocase is not required for membrane integration of F\textsubscript{0c}.

It has been shown that membrane partitioning of M13 procoat and Pf3 coat occurs in the absence of a PMF, but that the proteins do not acquire their native topology under these conditions (35, 45). Insertion of F\textsubscript{0c} does not depend on the PMF. We demonstrate by cysteine accessibility studies that the C-terminus of the protein, which is located in the periplasm \textit{in vivo}, is translocated into YidC proteoliposomes, as it is
protected from labeling with a membrane-impermeable probe. In contrast, the cytoplasmic loop connecting the two transmembrane helices remains accessible on the outer surface of the proteoliposomes. Thus, the final topology of F₀c differs from M13 procoat and Pf3 coat. Whereas with M13 procoat, the hydrophilic loop must be translocated across the membrane, the corresponding domain of F₀c remains cytosolic. The first step in the membrane insertion of both, M13 procoat and F₀c

**Fig. 7:** Two-step model of F₀c membrane integration. In the first step of the insertion reaction, F₀c interacts with the membrane mainly via electrostatic interactions with charged phospholipid headgroups. Subsequently, YidC facilitates the hydrophobic partitioning of the two TMSs into the lipid bilayer, thereby driving the translocation of both, the charged N-terminal and the C-terminal domain.

might be the binding to the membrane surface mediated by electrostatic interactions with the charged phospholipid headgroups (Fig. 7). Presumably, the three positive charges in the cytosolic loop of F₀c then prevent membrane translocation, while partitioning of the hydrophobic transmembrane domains into the lipid bilayer is assisted by YidC (Fig. 7). The short N-terminal, periplasmic tail of F₀c contains two negative charges. However, a possible electrophoretic contribution to their translocation is obviously not required. Interestingly, stepwise re-addition of charged amino acids into an uncharged Pf3 coat variant (Pf3-4N) demonstrated that negatively charged residues in the periplasmic N-terminal domain show a clear electrophoretic
response only when the hydrophobicity of the TMS is limiting (128). F_{0c}, however, is a very hydrophobic protein. This could explain why YidC-mediated hydrophobic interactions seems to be the sole driving force for F_{0c} membrane insertion. It has been established that the YidC-dependent proteins M13 procoat and Pf3 coat do not require the SRP targeting pathway to become inserted into the membrane (35, 47). Consistently, we now demonstrate by two independent approaches, i.e., the in vitro depletion of FtsY and in vivo depletion of Ffh, that inactivation of the SRP pathway has no effect on the insertion of the physiological YidC substrate F_{0c} into both, IMVs and YidC proteoliposomes. Although an SRP requirement was not observed, proteoliposomes have to be present co-translationally. Recently, a direct interaction between mitochondrial ribosomes and the YidC homologue Oxa1p has been reported (121, 222). YidC lacks the C-terminal cytosolic domain that is required for ribosome binding to Oxa1p (121). So far, there is no evidence for a direct coupling between translation and membrane insertion of F_{0c}. It, however, seems likely that a very hydrophobic protein like F_{0c} quickly aggregates in the absence of membranes and this may complicate the post-translational insertion reaction as the time window between translation and membrane insertion of such small membrane proteins is probably very short in vivo. In this respect, F_{0c} does not seem to differ much from M13 procoat, a protein of similar length that also does not require SRP for membrane targeting.

Our data represent the first direct demonstration of a physiologically important catalytic activity of YidC which casts a light on the essential role of YidC in *E. coli*. We have functionally reconstituted a novel membrane protein insertion pathway and we have shown that the F_{0c} protein is a substrate of it. Viruses and phages generally make use of key biogenesis pathways of their host cells in order to assure their own propagation. For the assembly of filamentous phages like M13 and Pf3, the accumulation of coat proteins in the cytoplasmic membrane of the host is an essential step. It now appears that M13 and Pf3 make use of a cellular machinery that plays a central role the biogenesis of major energy-transducing membrane protein complexes like F_{1}F_{0} ATP synthase. The mechanism of YidC-mediated membrane protein integration remains to be elucidated. Classical protein translocation and insertion machineries like Sec or Tat translocases use ATP and/or the PMF as energy source to actively transport proteins or domains of proteins across the membrane. In contrast, no PMF requirement was found for the YidC-mediated insertion of F_{0c} and YidC contains no obvious conserved nucleotide binding domain.
Chapter 5

It has been suggested that the function of YidC might be analogous to a chaperone which stabilizes folding and assembly intermediates of membrane proteins or membrane protein complexes. Remarkably, all three substrates of the YidC pathway are small and very hydrophobic proteins that assemble into large oligomeric structures like the phage coat or the rotor ring of the F1F0 ATP synthase. Also substrates of Oxa1, like Cox2p (99), and Alb3, like LHCP (172), are part of energy-transducing membrane protein complexes. YidC could play an important role as a chaperone in the assembly of these complexes. It might stabilize the transmembrane topology of a single F0c or act as a platform on which subunits can accumulate and organize into a ring structure. This ring is a very rigid complex (10). However, some intermediates in the process of complex formation might have to be stabilized by YidC. The role of YidC as well as the Sec translocase in the membrane insertion of other F0 subunits is unclear. Yi et al. have reported that the polytopic subunit F0a also requires of YidC to become integrated into the membrane (268). However, F0a is known to be unstable in the absence of F0c, so that the F0a insertion defect might be at least partly indirect (106).

The reconstituted system described here will be a valuable tool in the detailed analysis of YidC-mediated membrane protein integration. Important questions are, where the initial interaction of YidC and its substrates takes place and how binding to and release from YidC are regulated. To understand the role of YidC in the formation of membrane protein complexes, the reconstituted assay described in this paper should be expanded to all subunits of the F0 complex, e.g. by combination with the reconstitution of Sec-dependent membrane protein insertion on which we have recently reported (243). This will be a challenge for future studies.

Methods

Strains and Plasmids

Wild type IMVs were prepared from E. coli SF100 (12), which was used also for overexpression of SecYEG (238) and YidC (241). Strain JS7131 (205) was used to obtain YidC-depleted IMVs. Strain MC4100 was used to obtain S135 lysate. Strain HDB51 (145) was used to prepare Ffh-depleted and wild-type control lysate. Plasmid
pBSKftsQ (243) was used for in vitro transcription of ftsQ. For in vitro transcription of atpE, the gene encoding the F₀c protein, plasmid pET20atpE was constructed. atpE was PCR-amplified from pBWU13 (118) and cloned into pET20b (Novagen) yielding pET20atpE. pET20atpE-A40C and pET20atpE-A79C for the in vitro synthesis of F₀c single cysteine derivatives were constructed by replacing the GCG codon at position 40 or position 79 of atpE, respectively, by a TGC codon.

**Depletion of YidC and Ffh**

Depletion of YidC and Ffh was carried out essentially as described by Nouwen et al. for SecDFyajC (186) For YidC depletion, an overnight culture E. coli JS7131 grown in LB supplemented with 0.2% arabinose and 25 μg/ml spectinomycin was diluted 1:100 into the same medium without the antibiotic and grown until an OD₆₆₀ of 0.8. Wild type IMVs were prepared from these cells. A part of the same culture was washed once with warm LB and resuspended in LB containing 0.2% glucose at an OD₆₆₀ of 0.4. After every generation, the culture was diluted with one volume of the same medium, until the cells stopped growing. YidC-depleted IMVs were prepared from this culture. E. coli HDB51 was grown overnight in LB with 25 μg/ml kanamycin, 10 μg/ml tetracycline, 100 μg/ml ampicillin and 0.2% (w/v) arabinose. Cells were diluted 1:200 into the same medium without antibiotic and grown until an OD₆₆₀ of 0.2. HDB51 wild type lysate was prepared from these cells. Depletion of Ffh was induced in the same way as described for YidC, with the exception that cells were initially resuspended at an OD₆₆₀ of 0.1.

**Proteinase K pretreatment of IMVs**

To inactivate membrane proteins, IMVs were treated with 0.2 mg/ml proteinase K for 20 min on ice. Proteinase K activity was subsequently blocked by the addition of 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and IMVs were collected by centrifugation through a cushion consisting of 50 mM HEPES-KOH pH, 7.5, 0.5 mM PMSF and 20% [w/w] sucrose. IMVs were washed with 50 mM HEPES-KOH pH 7.5 and resuspended in 50 mM HEPES-KOH pH 7.5, 20% glycerol. Control IMVs were subjected to the same treatment with the exception that proteinase K was left out.
In vitro transcription, translation and insertion reaction

The RiboMax in vitro transcription kit (Promega) was used for the synthesis of mRNA. Plasmids pBSKftsQ (FtsQ), pET20atpE (wild type F0c), pET20atpE-A40C (F0c A40C, containing a unique cysteine residue at position 40) or pET20atpE-A79C (F0c A79C, containing a unique cysteine at position 79), respectively, served as DNA templates. In vitro translation/insertion reactions were carried out for 20 min at 37ºC in the presence of the indicated amounts of IMVs or (proteo-)liposomes as described (243).

Labeling of F0c A40C and F0c A79C

In order to determine the transmembrane topology of in vitro synthesized and membrane-inserted F0c, translation reactions using F0c A40C or F0c A79C mRNAs and YidC proteoliposomes were carried out in the absence of any radioactively labeled amino acid. Subsequently, 0.4 mg/ml protease K was added to degrade not incorporated F0c. After 30 min on ice, 0.5 mM PMSF was added to block proteinase K activity. Proteoliposomes were isolated from the reaction mixture by centrifugation through a cushion consisting of 50 mM HEPES-KOH, pH 7.0, 50 mM KCl and 20% [v/v] glycerol. Membranes were resuspended in 50 mM HEPES-KOH, pH 7.0, 50 mM KCl and incubated for 10 min on ice in the presence or absence of 0.5 mM AMdiS (Molecular Probes) as indicated. Subsequently, 1 mM fluorescein maleimide (Molecular Probes) was added and incubation was continued for another 10 min. Labeling reactions were quenched with 5 mM 1,4-dithiothreitol (DTT) and samples were subjected to precipitation with trichloroacetic acid (TCA). Samples were analyzed by 17.5% SDS-PAGE and fluorescently labeled proteins were visualized using a Lumimager F1 (Roche).

Oligomerization of in vitro synthesized F0c

F0c was synthesized as radioactively labeled protein in the presence proteoliposomes containing either YidC, SecYEG together with YidC or no proteins. 0.4 mg/ml proteinase K was added and samples were incubated for 30 min on ice to degrade not inserted F0c. 0.5 mM PMSF was used to inactivate proteinase K, and F0c-loaded proteoliposomes were isolated by centrifugation through a cushion consisting of 50 mM HEPES-KOH, pH 8.0, 50 mM KCl and 20% [v/v] sucrose. Pellets were resuspended in 50 mM solubilization buffer (50 mM HEPES pH 8.0, 50 mM KCl,
20% glycerol, 0.05% dodecyl maltoside) and incubated for 15 min on ice. Subsequently, samples were mixed with gel loading buffer. Blue Native PAGE analysis was performed on 8 - 18% gradient gels as described (209).

**Other methods**

SecYEG (156) and YidC (241) were purified as described. Reconstitutions were carried out as described (241). Where indicated, SecA or FtsY were removed from the lysate by immunodepletion (243), depletion was verified by immunoblotting. ProOmpA translocation experiments were carried out for 20 min at 37 ºC out using fluorescently labeled precursor protein (51).

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