Subunit a of cytochrome o oxidase requires both YidC and SecYEG for membrane insertion
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The Escherichia coli YidC protein belongs to the Oxa1 family of membrane proteins that facilitate the insertion of membrane proteins. Depletion of YidC in E. coli leads to a specific defect in the functional assembly of major energy transducing complexes such as the F$_0$F$_1$ ATPase and cytochrome bo$_3$ oxidase. Here we report on the in vitro reconstitution of the membrane insertion of the CyoA subunit of cytochrome bo$_3$ oxidase. Efficient insertion of in vitro synthesized pre-CyoA into proteoliposomes requires YidC, SecYEG, and SecA and occurs independently of the proton motive force. These data demonstrate that pre-CyoA is a substrate of a novel pathway that involves both SecYEG and YidC.

Approximately 20% of the Escherichia coli proteome concerns inner membrane proteins (1). Most of these proteins insert into the membrane via the Sec translocase (for review, see Ref. 2). Recently, YidC has been identified as a novel membrane protein that facilitates insertion of a subset of membrane proteins on its own (3–5). YidC also associates with SecYEG (5), where it contacts transmembrane (TM) segments of newly synthesized membrane proteins (6–8). YidC is homologous to Oxa1 in mitochondria and Alb3 in chloroplasts (5). The latter two proteins act as membrane protein insertases and play an important role in the membrane insertion of subunits from major energy transducing complexes (for review, see Refs. 9 and 10). In analogy, in E. coli the functional assembly of the F$_0$F$_1$ ATPase and cytochrome bo$_3$ oxidase is shown to be dependent on YidC (11), and YidC is also implicated in lipoprotein translocation (12). We have recently demonstrated that membrane insertion and assembly of the F$_0$c subunit of the F$_0$F$_1$ ATPase solely depend on YidC (4). CyoA is the quinol binding subunit of the cytochrome bo$_3$ oxidase complex (13). Unlike F$_0$c, CyoA is a polytopic membrane protein with a lipoprotein signal sequence and a large periplasmic domain (Fig. 1A). Here we report on the minimal requirements for insertion of pre-CyoA into the E. coli membrane using an in vitro approach. The data demonstrate that pre-CyoA is a substrate of a novel pathway that requires both the Sec translocase and YidC.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**E. coli strain SF100 was used for the isolation of inner membrane vesicles (IMVs)$^2$ and for overexpression of SecYEG and YidC (14). The S135 lysate was prepared from E. coli MC4100. Plasmids pBSKftsQ (15) and pET27bCyoA (generous gift from Dr. M. Lübken, Department of Biophysics, Ruhr-Universität-Bochum) were used for in vitro transcription of FtsQ and CyoA, respectively.

**In Vitro Transcription, Translation, and Insertion Reaction—**In vitro transcription was performed using the Ribomax® kit (Promega) with plasmids pBSKftsQ and pET27bCyoA as templates. In vitro translation-insertion reactions were performed as described (15) except that the reaction was coupled to the transcription and performed for 40 min at 37°C.

**RESULTS**

Co-translational Insertion of Pre-CyoA into Inverted E. coli Inner Membrane Vesicles—Subunit II (CyoA) of cytochrome bo$_3$ ubiquinol oxidase (315 residues) from E. coli is synthesized as a precursor with an N-terminal signal sequence (pre-CyoA) that upon lipid modification of the mature N terminus is cleaved by signal peptidase II (18). Mature CyoA with a mass of 32 kDa is composed of two domains, an N-terminal membrane region with two TM domains and a large periplasmic C-terminal domain (13) (Fig. 1A). To study its membrane insertion, pre-CyoA was synthesized in vitro using an E. coli S135 lysate and [35S]methionine. In vitro synthesis of CyoA results in the formation of a 35-kDa protein visualized on SDS-PAGE (Fig. 1B, lane 1). When the in vitro transcription/translation reaction was performed in the presence of SecYEG-overexpressed IMVs, trypsin treatment of pre-CyoA resulted in the formation of a 25-kDa protease-protected fragment (Fig. 1B, lane 2). Solubilization of IMVs with Triton X-100 resulted in complete degradation of pre-CyoA (Fig. 1B, lane 3). In its correct topology, the large periplasmic domain of CyoA is translocated into the vesicle lumen and thus becomes protected from externally added trypsin. The cytoplasmic loop connecting TM1 and TM2, however, will be accessible to trypsin. Based on the available crystal structure of CyoA (19), this cytoplasmic loop contains four possible trypsin cleavage sites (at amino acid positions 70, 74, 77, and 87). Trypsin cleavage at one or all of these sites will result in the removal of the signal sequence and part of N-terminal region of the mature CyoA yielding an ~25-kDa fragment ($\Delta$N-CyoA). Correspondingly, trypsin treatment of endogenous CyoA in inside-out IMVs yielded a 25-kDa protease-protected fragment that degraded upon solubilization of the membrane vesicles with Triton X-100 (Fig. 1C). We therefore conclude that the in vitro observed 25-kDa trypsin-
phenicol, and SecYEG absence of IMVs. Next, protein synthesis was blocked by chloramphenicol. Pre-CyoA translocation into IMVs upon SecYEG overexpression (20) in the presence of 25 μg of SecYEG (SecYEG WT) showed only a low level of inserted CyoA (Fig. 2, lane 2), overproduction of SecYEG (SecYEG Δ) enhanced membrane insertion more than 5-fold (lane 5). This correlates well with the observed 5–6-fold stimulation of preOMPα translocation into IMVs upon SecYEG overexpression (20) and shows that insertion of pre-CyoA is a SecYEG-mediated process. The low level of membrane insertion with wild-type IMVs has been observed more often with in vitro systems (15) and likely results from a general inefficiency of in vitro translation/translocation reactions for inner membrane proteins and competing reactions such as aggregation. Other missing factors may contribute to the efficiency of membrane insertion, such as an intact lipid modification pathway needed to modify the mature N terminus of pre-CyoA prior to its processing by the lipoprotein peptidase. Finally, co-factor assembly and CyoB maturation may contribute to the overall efficiency of stably inserted CyoA.

In the in vitro assays, pre-CyoA was synthesized in the presence of IMVs (co-translational insertion). To investigate whether CyoA also inserts post-translationally, pre-CyoA was first synthesized in the absence of IMVs. Next, protein synthesis was blocked by chloramphenicol, and SecYEG WT IMVs were added to allow insertion (Fig. 3, lanes 4–6). Although efficient insertion of pre-CyoA was observed under co-translational conditions (Fig. 3, lane 5), no pre-CyoA insertion could be detected under post-translationally conditions (lane 2). These data demonstrate that membrane insertion of pre-CyoA occurs co-translationally.

The Proton Motive Force Is Not Required for Membrane Insertion of CyoA—The proton motive force (PMF) has been shown to play a pivotal role in the insertion of some membrane proteins such as M13 procoat (21) and FtsQ (15). Previously, we have shown that YidC depletion from E. coli SF100 and 6). As a reference, trypsin was loaded in lane 7. These results demonstrate that membrane insertion of pre-CyoA occurs independently of the PMF. protected fragment in the presence of IMVs represents correctly membrane-inserted CyoA.

To examine the insertion mechanism of pre-CyoA, IMVs with high levels of SecYEG were used as described previously (16). Levels of overexpression for SecYEG were calculated to be at least 10-fold that of wild-type levels of SecYEG (see also Fig. 7). Although wild-type IMVs showed only a low level of inserted CyoA (Fig. 2, lane 2), overproduction of SecYEG (SecYEG WT) enhanced membrane insertion more than 5-fold (lane 5). This correlates well with the observed 5–6-fold stimulation of preOMPα translocation into IMVs upon SecYEG overexpression (20) and shows that insertion of pre-CyoA is a SecYEG-mediated process. The low level of membrane insertion with wild-type IMVs has been observed more often with in vitro systems (15) and likely results from a general inefficiency of in vitro translation/translocation reactions for inner membrane proteins and competing reactions such as aggregation. Other missing factors may contribute to the efficiency of membrane insertion, such as an intact lipid modification pathway needed to modify the mature N terminus of pre-CyoA prior to its processing by the lipoprotein peptidase. Finally, co-factor assembly and CyoB maturation may contribute to the overall efficiency of stably inserted CyoA.

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CyoA Membrane Insertion

Membrane Insertion of CyoA Requires Both SecYEG and YidC—To investigate the minimal requirements for insertion of pre-CyoA, proteoliposomes were used that contained purified YidC, SecYEG, or both YidC and SecYEG. Herein, a molecular YidC/SecY ratio of 3 was used as described previously (14). No insertion was observed when pre-CyoA was synthesized in the presence of empty liposomes (Fig. 5A, lane 2) or proteoliposomes reconstituted with YidC only (lane 11). A low level of insertion was observed with proteoliposomes containing purified SecYEG (Fig. 5, lane 5), but co-reconstitution of YidC with SecYEG resulted in a drastic increase in the membrane insertion efficiency of pre-CyoA (lane 8). The increased level of pre-CyoA insertion was not because of differences in SecYEG reconstitution as the liposomes equally effectively translocated the precursor protein proOmpA (Fig. 5B, lanes 3 and 4). A further increase in the amount of YidC in the proteoliposomes only marginally improved the insertion (data not shown). Taken together, the above results indicate that both SecYEG and YidC are required for efficient membrane insertion of pre-CyoA.

Membrane Insertion of Pre-CyoA Is Dependent on SecA—Membrane proteins with large periplasmic domains such as FtsQ (23), and YidC (24) have been shown to require SecA for membrane insertion. As CyoA contains a large periplasmic domain (Fig. 1A), we next determined the SecA dependence of the insertion reaction. Pre-CyoA was synthesized in the presence of SecYEG/YidC proteoliposomes in a SecA-immunodepleted E. coli lysate. Although the lysate supported synthesis of pre-CyoA, no insertion could be observed (Fig. 6, lanes 4–6). When the lysate was supplemented with purified SecA, pre-CyoA insertion was restored (Fig. 6, lanes 7–9). This demonstrates a catalytic requirement for SecA.

Mutations in SecY have been described that differently affect protein translocation and membrane protein insertion (25). SecY39 (R357E mutation in the C5 cytoplasmic loop of SecY) is blocked in protein translocation (25, 26) and exhibits a functional defect in the SecA/SecY interaction (27). This mutant is also defective in the insertion of some signal recognition particle-dependent membrane proteins (27, 28). SecY40 (A363S) is defective in signal recognition particle-dependent membrane protein insertion but supports normal protein translocation (26, 29). As pre-CyoA is a protein that contains both TM domains and a large periplasmic domain, we determined the effect of the SecY mutations on the membrane integration of pre-CyoA. IMVs were isolated from cells overproducing wild-type (WT) IMVs and pre-CyoA was synthesized in an E. coli wild-type (WT) lysate and SecA-immunodepleted lysate without (lanes 4–6) or with 0.5 μg of purified SecA (lanes 7–9). Insertion assays were performed with SecYEG/YidC proteoliposomes. 

FIGURE 4. Insertion of pre-CyoA does not require a PMF. A, insertion assays with wild-type (WT) and SecYEG−/H9262 IMVs were performed as described in the legend to Fig. 2 in the absence (lanes 1–6) and presence (lanes 7–12) of 3 μM nigericin/valinomycin (nig/val) to dissipate the PMF. B, a coupled transcription/translation of FtsQ was performed with 25 μg of wild-type IMVs in the absence (lanes 1–3) and presence (lanes 4–6) of 3 μM nigericin/valinomycin to dissipate the PMF.

FIGURE 5. Efficient insertion of CyoA into proteoliposomes requires both SecYEG and YidC. Liposomes were reconstituted with purified SecYEG (20 μg) and/or YidC (60 μg) as described under “Experimental Procedures.” A, pre-CyoA was synthesized in the presence of proteoliposomes containing SecYEG (lanes 4–6), SecYEG and YidC (lanes 7–9), YidC (lanes 10–12), or liposomes (lanes 1–3). Samples were treated with trypsin in the absence (lanes 2, 5, 8, and 11) or presence (lanes 3, 6, 9, and 12) of 1% Triton X-100. B, fluorescein-labeled proOmpA was translocated into liposomes (lane 2) or proteoliposomes containing SecYEG (lane 3), SecYEG and YidC (lane 4), or YidC alone (lane 5).

FIGURE 6. SecA is required for membrane insertion of pre-CyoA. Pre-CyoA was synthesized in an E. coli wild-type (WT) lysate (lanes 1–3) and SecA-immunodepleted lysate without (lanes 4–6) or with 0.5 μg of purified SecA (lanes 7–9). Insertion assays were performed with SecYEG/YidC proteoliposomes.
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FIGURE 7. SecY mutations interfere with insertion of pre-CyoA into IMVs. A, pre-CyoA was synthesized in the presence of wild-type (WT) (lanes 1–3), SecYEG (lanes 4–6), SecY(R357E)EG (lanes 7–9), or SecYA3635E (E) (lanes 10–12) IMVs. Samples were treated with trypsin in the absence (lanes 2, 5, 8, and 11) or presence (lanes 3, 6, 9, and 12) of 1% Triton X-100. B, Coomassie Brilliant Blue-stained SDS-PAGE of equal amount of wild-type, SecYEG, SecY(R357E)EG (SecY39), and SecYA3635E (SecY40) IMVs. With the SecY level of SecYEG IMVs set at 100%, SecY39 and SecY40 IMVs contained a SecY level of 107 and 103%, respectively.

DISCUSSION

Recently, we have shown that in E. coli the functional assembly of major energy-transducing complexes such as the H+−translocating F0F1 ATPase and cytochrome bo3 oxidase is strongly affected by the depletion of YidC (11). In vitro experiments demonstrate that the membrane insertion of F1 and SecYEG is solely mediated by YidC (4), thus establishing a novel function for YidC (11).

In vitro translation and cross-linking approaches have shown that it contacts the TMs of newly inserted membrane proteins (5–8). The role of YidC in the membrane insertion of these Sec-dependent membrane proteins is less understood as no strict requirement for YidC is demonstrated for their functional assembly (5, 15). We now show that pre-CyoA, the precursor of subunit a of the cytochrome bo3 oxidase complex, utilizes both the Sec translocase and YidC for its insertion. We used an in vitro assay, which employed proteoliposomes with a defined protein composition, to reveal the minimal requirements for membrane insertion of pre-CyoA. For the first time, our data demonstrate a catalytic requirement for YidC by a membrane protein that inserts into the membrane in a Sec-dependent manner. This study explains why depletion of YidC in cells results in a loss of functional cytochrome bo3 oxidase complex. Pre-CyoA insertion also requires SecA for its assembly, which most likely relates to the translocation of the large periplasmic domain of CyoA as expected for membrane proteins with periplasmic domains larger than 60 amino acids (30).

Pre-CyoA membrane insertion presumably occurs in the following manner. First, the signal sequence and the first transmembrane segment insert into the SecYEG channel as a helical hairpin. This step may resemble the recent cryo-electron microscopy reconstruction of a ribosome-SecYEG complex in which the N-terminal TM domain of PstS is inserted as a hairpin structure (31). This process is likely followed by the lipid modification of the cysteine position of the mature N terminus of CyoA and then by the removal of the signal sequence by signal peptidase II. There are processes that are not monitored in the in vitro system as described in this study. During the lipid modification, TM2 of CyoA (Fig. 1A) must insert into the membrane, whereverupon the large periplasmic domain of CyoA needs to be translocated across the membrane. TM2 likely loops into the SecYEG pore together with the N-terminal region of the periplasmic domain of CyoA. The translocation of the periplasmic domain likely involves SecA as this reflects a true translocation reaction. YidC may be involved in various stages of the insertion reaction. It may facilitate clearance of the SecYEG pore and promote transfer of the hairpin of the signal sequence and TM1 into the lipid phase. Alternatively, YidC may be involved in the insertion of TM2 that needs to loop into the translocation pore. The latter process resembles the insertion mechanisms of F1c and M13 in which YidC may facilitate looping in of a single or of both TM domains of these small membrane proteins. Future experiments should reveal how YidC facilitates membrane insertion of the various regions of CyoA.

CyoA is the quinol binding subunit of the cytochrome o oxidase complex. CyoB is a very large heme-binding membrane protein of 74 kDa with 15 predicted TM domains, whereas CyoC is a smaller membrane protein of 20 kDa with 5 TM domains and an unknown function. Our current study deals with pre-CyoA, but in vivo, insertion of the subunits and their assembly into the cytochrome o oxidase complex is likely a coordinated process that also involves timely incorporation of the various co-factors. It will be a major challenge to elucidate the exact mechanism by which this energy-transducing complex assembles.

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