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,FATPase 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,FATPase 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,FATPase subunit of the F,FATPase solely depend on YidC (13). CyoA is the quinol binding subunit of the cytochrome bo_{3} oxidase complex (13). Unlike F,F, 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) 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. Drijessen) 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 the N-terminal region of the mature CyoA yielding an ~25-kDa fragment (ΔN-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-

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2 The abbreviations used are: IMVs, inner membrane vesicles; PMF, proton motive force; TM, transmembrane.
phenicol, and SecYEG. Next, protein synthesis was blocked by chloramphenicol, and SecYEG inserts post-translationally, pre-CyoA was first synthesized in the presence of 25 μg of SecYEG complex (lane 1, 10% of the total translation). Samples were treated with trypsin in the absence (lane 2) or presence (lane 3) of 1% Triton X-100. Full-length pre-CyoA and the trypsin-protected fragment (ΔN-CyoA) are indicated. Coomassie Blue-stained SDS-polyacrylamide gels of IMVs from E. coli SF100 with (lanes 1-3) and without (lanes 4-6) overexpressed CyoA. Samples were treated with trypsin in the absence (lanes 2 and 3) or presence of 1% Triton X-100 (lanes 3 and 6). As a reference, trypsin was loaded in lane 7.

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+) enhanced membrane insertion more than 5-fold (lane 5). This correlates well with the observed 5–6-fold stimulation of proOmpA translocation into IMVs upon SecYEG overexpression (3) 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+ 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 cells results in a reduced capacity of cells to generate a PMF (11). The observed assembly defect of CyoA in YidC-depleted cells could therefore relate to a PMF requirement of the insertion reaction. Therefore, the role of the PMF in pre-CyoA insertion was examined in vitro. Insertion of pre-CyoA into wild-type and SecYEG+ IMVs was only marginally affected by the ionophores nigericin and valinomycin that collapse the entire PMF (Fig. 4A). In contrast, ionophore addition completely blocked membrane insertion of the control membrane protein FtsQ (Fig. 4B) (15). These results demonstrate that membrane insertion of pre-CyoA occurs independently of the PMF.

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**FIGURE 1. In vitro insertion of pre-CyoA into E. coli inner membrane vesicles.** A, schematic representation of the membrane topology of pre-CyoA before removal of the signal sequence by signal peptidase II (Spase II). Trypsin cleavage of the cytoplasmic loop is indicated. N, N terminus of the protein. B, coupled in vitro transcription/translation of pre-CyoA in the presence of 25 μg of SF100 IMVs containing high levels of SecYEG complex (lane 1, 10% of the total translation). Samples were treated with trypsin in the absence (lane 2) or presence (lane 3) of 1% Triton X-100. Full-length pre-CyoA and the trypsin-protected fragment (ΔN-CyoA) are indicated. Coomassie Blue-stained SDS-polyacrylamide gels of IMVs from E. coli SF100 with (lanes 1-3) and without (lanes 4-6) overexpressed CyoA. Samples were treated with trypsin in the absence (lanes 2 and 3) or presence of 1% Triton X-100 (lanes 3 and 6). As a reference, trypsin was loaded in lane 7.

**FIGURE 2. Membrane insertion of pre-CyoA is facilitated by SecYEG.** Pre-CyoA was synthesized in the presence of 25 μg of wild-type (WT) or SecYEG+ IMVs. After 40 min at 37 °C, samples were treated with trypsin without (lanes 2 and 5) or with 1% Triton X-100 (lanes 3 and 6) for 30 min on ice and analyzed by SDS-PAGE and autoradiography. Lanes 1 and 4 represent 10% of the total translation.

**FIGURE 3. Pre-CyoA inserts co-translationally into IMVs.** Co-translational in vitro insertion of pre-CyoA (lanes 4-6) was performed using a coupled transcription/translation reaction in the presence of 25 μg of SecYEG+ IMVs. Lane 4 represents 10% of the total translation. After 40 min of incubation at 37 °C, samples were treated with trypsin in the absence (lane 5) or presence of 1% Triton X-100 (lane 6). Post-translational insertion of pre-CyoA (lanes 1-3) was done as for co-translational insertion but in the absence of IMVs (lane 1 represents 10% of the total translation). Translation was terminated by the addition of 25 μg/ml chloramphenicol; and subsequently 25 μg of SecYEG+ IMVs was added, and the incubation was continued for 40 min at 37 °C.

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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 effective 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 (22), AcrB (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 SecYEG/YidC proteoliposomes containing 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).
CyoA Membrane Insertion

Recently, we have shown that in E. coli the functional assembly of major energy-transducing complexes such as the H⁺-translocating F₁F₀ ATPase and cytochrome bo₃ oxidase is strongly affected by the depletion of YidC (11). In vitro experiments demonstrate that the membrane insertion of F₁c is solely mediated by YidC (4), thus establishing a novel route for membrane insertion of authentic E. coli membrane proteins, which involves only YidC. YidC is also required for membrane insertion of foreign small phage proteins such as M13 and Pf3 (3–5) that apparently usurp the YidC pathway for their insertion. YidC also interacts with the SecYEG complex, 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 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 assemblies.

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REFERENCES
CyoA Membrane Insertion