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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_1F_0 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) insertion 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_1F_0 ATPase and cytochrome bo_3 quinol 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_0c subunit of the F_1F_0 ATPase solely depend on YidC (4). CyoA is the quinol binding subunit of the cytochrome bo_3 quinol oxidase complex (13). Unlike F_0c, CyoA is a polytopic membrane protein with a lipoprotein signal sequence and a large periplasmic domain (Fig. 1*A*). 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. Lübben, Department of Biophysics, Ruhr-Universitat-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.

Other Methods—IMVs containing overproduced SecYEG or YidC were isolated as described (16). SecYEG (16), YidC (14), and SecA (17) were purified and reconstituted into *E. coli* phospholipids (Avanti Polar Lipids, Alabaster, AL) at 1 μg of SecYEG and 3 μg of YidC per 40 μg of lipids using Bio-Beads SM-2 (Bio-Rad) (14). Proteoliposomes were analyzed by SDS-PAGE and silver staining to verify the reconstituted levels of SecYEG and YidC (14). Functional levels of reconstituted SecYEG and YidC were verified by proOmpA translocation (14) and F_0c membrane insertion (4) assays, respectively. SecA was removed from the S135 lysate by immunodepletion and verified by immunoblotting using monoclonal SecA antibodies (15).

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. 1*A*). To study its membrane insertion, pre-CyoA was synthesized *in vitro* using an *E. coli* S135 lysate and [^{35}S]methionine. *In vitro* synthesis of CyoA results in the formation of a 35-kDa protein visualized on SDS-PAGE (Fig. 1*B*, *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. 1*B*, *lane 2*). Solubilization of IMVs with Triton X-100 resulted in complete degradation of pre-CyoA (Fig. 1*B*, *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 (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. 1*C*). 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.

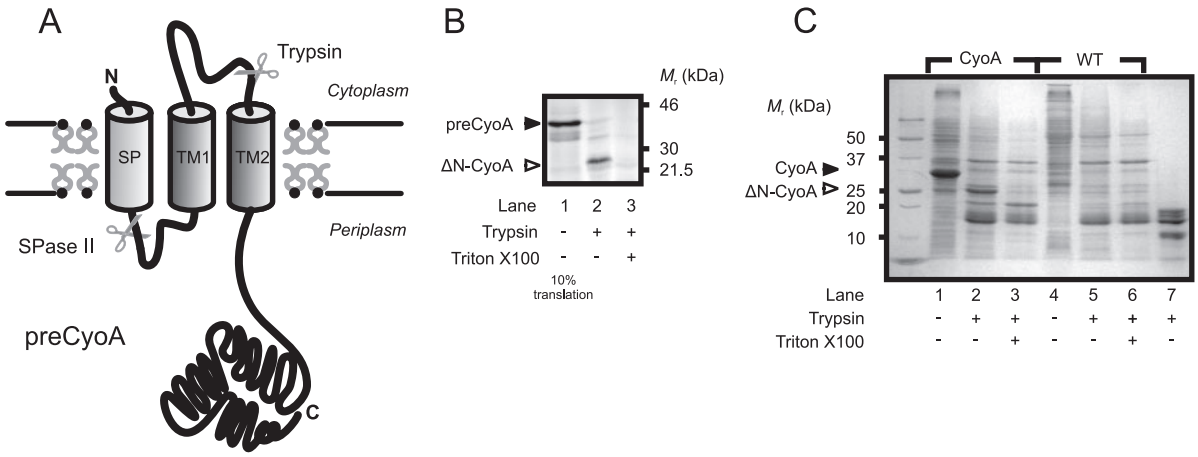

FIGURE 1. ***In vitro* insertion of pre-CyoA into *E. coli* inner membrane vesicles.** *A*, schematic representation of the membrane topology of pre-CyoA before removal of the signal sequence by signal peptidase II (*SPase II*). Trypsin cleavage of the cytoplasmic loop is indicated. *N*, N terminus of 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. *C*, 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 *5*) or presence of 1% Triton X-100 (*lanes 3* and 6). As a reference, trypsin was loaded in *lane 7*.

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$^+$) 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] (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 chloroamphenicol, 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. 4*A*). In contrast, ionophore addition completely blocked membrane insertion of the control membrane protein FtsQ (Fig. 4*B*) (15). These results demonstrate that membrane insertion of pre-CyoA occurs independently of the PMF.

[3] D. J. F. du Plessis, N. Nouwen, and A. J. M. Driessen, unpublished data.

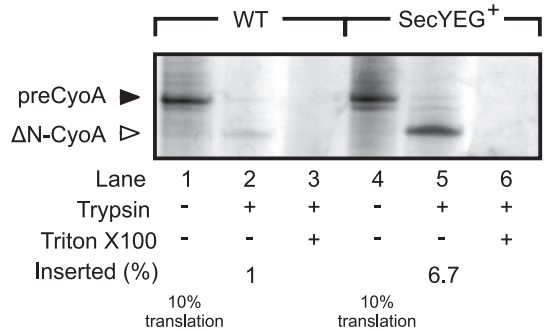

FIGURE 2. **Membrane insertion of pre-CyoA is facilitated by SecYEG.** Pre-CyoA was synthesized in the presence of 25 μg of wild-type (*WT*) or SecYEG$^+$ IMVs. After 40 min at 37 °C, samples were treated with trypsin without (*lanes 2* and *5*) or with 1% Triton X-100 (*lanes 3* and *6*) for 30 min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

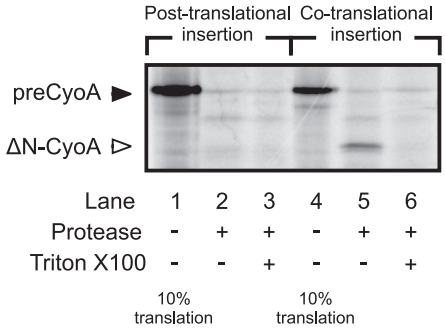

FIGURE 3. **Pre-CyoA inserts co-translationally into IMVs.** Co-translational *in vitro* insertion of pre-CyoA (*lanes 4-6*) was performed using a coupled transcription/translation reaction in the presence of 25 μg of SecYEG$^+$ IMVs. *Lane 4* represents 10% of the total translation. After 40 min of 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.

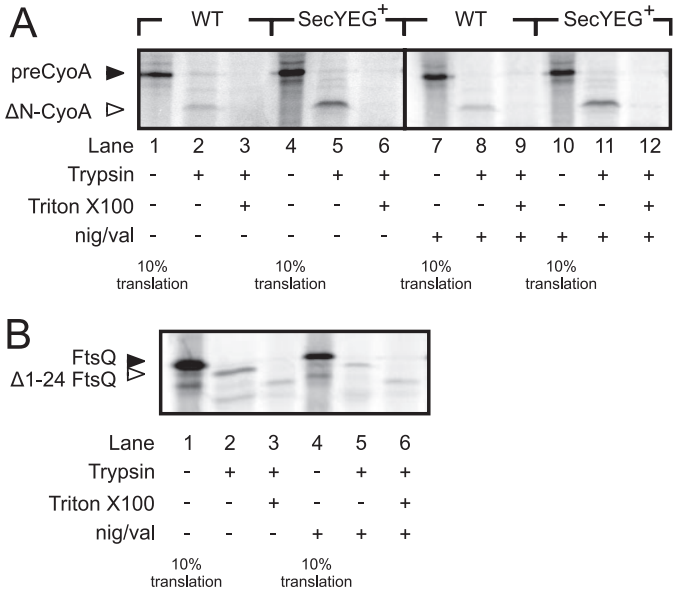

FIGURE 4. **Insertion of pre-CyoA does not require a PMF.** *A*, insertion assays with wild-type (*WT*) and SecYEG$^+$ IMVs were performed as described in the legend to Fig. 2 in the absence (*lanes 1–6*) and presence (*lanes 7–12*) of 3 μM nigericin/valinomycin (*nig/val*) to dissipate the PMF. *B*,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

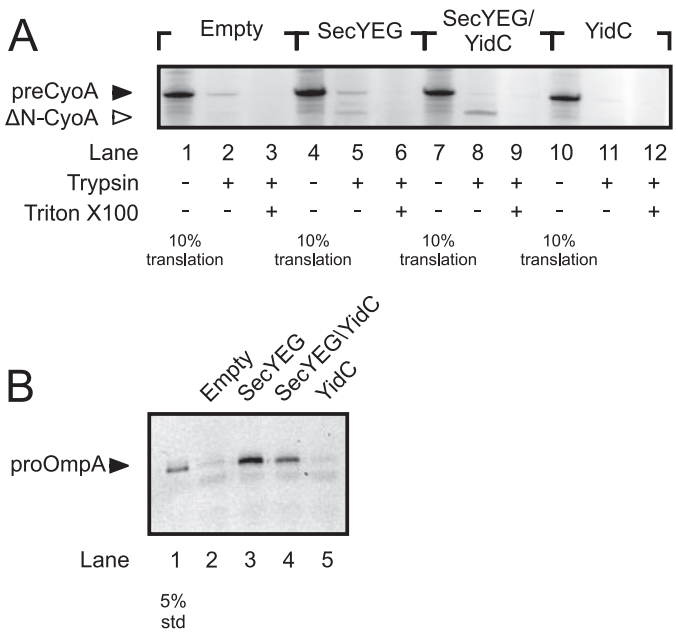

FIGURE 5. **Efficient insertion of CyoA into proteoliposomes requires both SecYEG and YidC.** Liposomes were reconstituted with purified SecYEG (20 μg) and/or YidC (60 μg) as described under "Experimental Procedures." *A*, pre-CyoA was synthesized in the presence of proteoliposomes containing SecYEG (*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*).

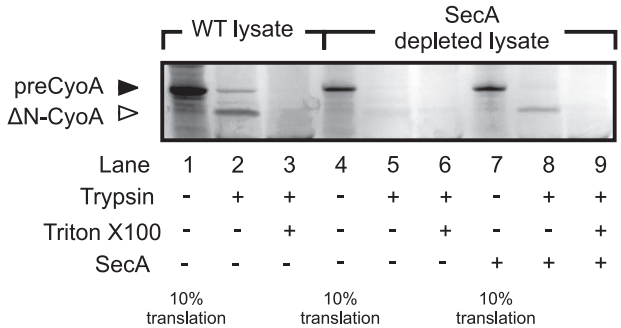

FIGURE 6. **SecA is required for membrane insertion of pre-CyoA.** Pre-CyoA was synthesized in an *E. coli* wild-type (*WT*) lysate (*lanes 1–3*) and SecA-immunodepleted lysate without (*lanes 4–6*) or with 0.5 μg of purified SecA (*lanes 7–9*). Insertion assays were performed with SecYEG/YidC proteoliposomes.

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. 5*A*, *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. 5*B*, *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 (15, 22), AcrB (23), and YidC (24) have been shown to require SecA for membrane insertion. As CyoA contains a large periplasmic domain (Fig. 1*A*), 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 SecY(R357E)EG and SecY(A363S)EG and analyzed for pre-CyoA insertion. Coomassie-stained SDS-PAGE analysis showed that the SecY mutant proteins were overproduced to the same level as wild-type SecY (Fig. 7*B*). Both SecY(R357E)EG (Fig. 7*A*, *lanes 7–9*) and SecY(A363S)EG (*lanes 10-12*) IMVs showed a severe defect in the membrane integration of pre-CyoA as compared with SecYEG$^+$ IMVs (*lanes 4-6*). The residual level of insertion above that of IMVs containing chromosomal levels of SecYEG (*lanes 1-3*) is in line with previous observations that these mutants are not completely defective (25). Taken together, these data indicate that pre-CyoA is a substrate of a novel route that involves both the Sec translocase and YidC.

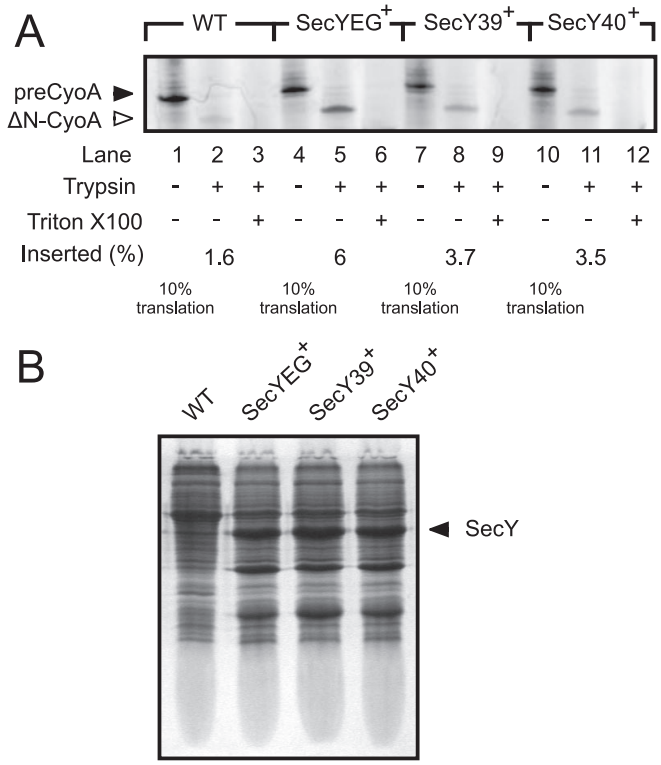

FIGURE 7. **SecY mutations interfere with insertion of pre-CyoA into IMVs.** *A*, pre-CyoA was synthesized in the presence of wild-type (*WT*) (*lanes 1–3*), SecYEG$^+$ (*lanes 4–6*), SecY(R357E)EG$^+$ (*lanes 7–9*), or SecY(A363S)EG$^+$ (*lanes 10–12*) IMVs. Samples were treated with trypsin in the 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 SecY(A363S)EG (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 F_1F_0 ATPase and cytochrome bo_3 oxidase is strongly affected by the depletion of YidC (11). *In vitro* experiments demonstrate that the membrane insertion of F_0c 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 is demonstrated for their functional assembly (5, 15). We now show that pre-CyoA, the precursor of subunit a of the cytochrome bo_3 quinol 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 *o* 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 state may resemble the recent cryo-electron microscopy reconstruction of a ribosome-SecYEG complex in which the N-terminal TM domain of FtsQ was 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. 1*A*) must insert into the membrane, whereupon 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 F_0c 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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REFERENCES

1. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) *J. Mol. Biol.* **305,** 567–580
2. Dalbey, R. E., and Chen, M. (2004) *Biochim. Biophys. Acta* **1694,** 37–53
3. Serek, J., Bauer-Manz, G., Struhalla, G., van den Berg, L., Kiefer, D., Dalbey, R. E., and Kuhn, A. (2004) *EMBO J.* **23,** 294–301
4. van der Laan, M., Bechtluft, P., Kol, S., Nouwen, N., and Driessen, A. J. M. (2004) *J. Cell Biol.* **165,** 213–222
5. Samuelson, J. C., Chen, M., Jiang, F., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G. J., and Dalbey, R. E. (2000) *Nature* **406,** 637–641
6. Scotti, P. A., Urbanus, M. L., Brunner, J., de Gier, J. W., von Heijne, G., van der Does, C., Driessen, A. J. M., Oudega, B., and Luirink, J. (2000) *EMBO J.* **19,** 542–549
7. Houben, E. N. G., Scotti, P. A., Valent, Q. A., Brunner, J., de Gier, J. W., Oudega, B., and Luirink, J. (2000) *FEBS Lett.* **476,** 229–233
8. Houben, E. N., ten Hagen-Jongman, C. M., Brunner, J., Oudega, B., and Luirink, J. (2004) *EMBO Rep.* **5,** 970–975
9. van der Laan, M., Nouwen, N. P., and Driessen, A. J. M. (2005) *Curr. Opin. Microbiol.* **8,** 182–187
10. Yi, L., and Dalbey, R. E. (2005) *Mol. Membr. Biol.* **22,** 101–111
11. van der Laan, M., Urbanus, M. L., ten Hagen-Jongman, C. M., Nouwen, N., Oudega, B., Harms, N., Driessen, A. J. M., and Luirink, J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5801–5806
12. Froderberg, L., Houben, E. N. G., Baars, L., Luirink, J., and de Gier, J. W. (2004) *J. Biol. Chem.* **279,** 31026–31032
13. Abramson, J., Riistama, S., Larsson, G., Jasaitis, A., Svensson-Ek, M., Laakkonen, L., Puustinen, A., Iwata, S., and Wikstrom, M. (2000) *Nat. Struct. Biol.* **7,** 910–917
14. van der Laan, M., Houben, E. N. G., Nouwen, N., Luirink, J., and Driessen, A. J. M. (2001) *EMBO Rep.* **2,** 519–523

15. van der Laan, M., Nouwen, N., and Driessen, A. J. M. (2004) *J. Biol. Chem.* **279,** 1659–1664
16. van der Does, C., de Keyzer, J., van der Laan, M., and Driessen, A. J. M. (2003) *Methods Enzymol.* **372,** 86–98
17. Cabelli, R. J., Chen, L., Tai, P. C., and Oliver, D. B. (1988) *Cell* **55,** 683–692
18. Ma, J., Katsonouri, A., and Gennis, R. B. (1997) *Biochemistry* **36,** 11298–11303
19. Abramson, J., Larsson, G., Byrne, B., Puustinen, A., Garcia-Horsman, A., and Iwata, S. (2000) *Acta Crystallogr. D Biol. Crystallogr.* **56,** 1076–1078
20. van der Does, C., den Blaauwen, T., de Wit, J. G., Manting, E. H., Groot, N. A., Fekkes, P., and Driessen, A. J. M. (1996) *Mol. Microbiol.* **22,** 619–629
21. Cao, G., Kuhn, A., and Dalbey, R. E. (1995) *EMBO J.* **14,** 866–875
22. Urbanus, M. L., Scotti, P. A., Froderberg, L., Saaf, A., de Gier, J. W., Brunner, J., Samuelson, J. C., Dalbey, R. E., Oudega, B., and Luirink, J. (2001) *EMBO Rep.* **2,** 524–529
23. Qi, H. Y., and Bernstein, H. D. (1999) *J. Biol. Chem.* **274,** 8993–8997
24. Koch, H. G., Moser, M., Schimz, K. L., and Muller, M. (2002) *J. Biol. Chem.* **277,** 5715–5718
25. Baba, T., Jacq, A., Brickman, E., Beckwith, J., Taura, T., Ueguchi, C., Akiyama, Y., and Ito, K. (1990) *J. Bacteriol.* **172,** 7005–7010
26. Shimohata, N., Akiyama, Y., and Ito, K. (2005) *J. Bacteriol.* **187,** 3997–4004
27. Mori, H., and Ito, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 5128–5133
28. Koch, H. G., and Muller, M. (2000) *J. Cell Biol.* **150,** 689–694
29. Angelini, S., Deitermann, S., and Koch, H. G. (2005) *EMBO Rep.* **6,** 476–481
30. Andersson, H., and von Heijne, G. (1993) *EMBO J.* **12,** 683–691
31. Mitra, K., Schaffitzel, C., Shaikh, T., Tama, F., Jenni, S., Brooks, C. L., III, Ban, N., and Frank, J. (2005) *Nature* **438,** 318–324