THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 15, pp. 10002–10009, April 14, 2006

Distinct Requirements for Translocation of the N-tail and C-tail of the *Escherichia coli* Inner Membrane Protein CyoA*

Received for publication, October 19, 2005, and in revised form, January 26, 2006 Published, JBC Papers in Press, February 15, 2006, DOI 10.1074/jbc.M511357200

Edwin van Bloois[‡1], Gert-Jan Haan[‡2], Jan-Willem de Gier[§], Bauke Oudega[‡], and Joen Luirink[‡2,3]

From the [‡]Department of Molecular Microbiology, Institute of Molecular Cell Biology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands and [§]Department of Biochemistry and Biophysics, Arrhenius Laboratories, Stockholm University, SE-106 91 Stockholm, Sweden

Inner membrane proteins (IMPs) of *Escherichia coli* use different pathways for membrane targeting and integration. YidC plays an essential but poorly defined role in the integration and folding of IMPs both in conjunction with the Sec translocon and as a Sec-independent insertase. Depletion of YidC only marginally affects the insertion of Sec-dependent IMPs, whereas it blocks the insertion of a subset of Sec-independent IMPs. Substrates of this latter "YidC-only" pathway include the relatively small IMPs M13 procoat, Pf3 coat protein, and subunit c of the F_1F_0 ATPase. Recently, it has been shown that the steady state level of the larger and more complex CyoA subunit of the cytochrome *o* oxidase is also severely affected upon depletion of YidC. In the present study we have analyzed the biogenesis of the integral lipoprotein CyoA. Collectively, our data suggest that the first transmembrane segment of CyoA rather than the signal sequence recruits the signal recognition particle for membrane targeting. Membrane integration and assembly appear to occur in two distinct sequential steps. YidC is sufficient to catalyze insertion of the N-terminal domain consisting of the signal sequence, transmembrane segment 1, and the small periplasmic domain in between. Translocation of the large C-terminal periplasmic domain requires the Sec translocon and SecA, suggesting that for this particular IMP the Sec translocon might operate downstream of YidC.

In *Escherichia coli*, IMPs[4] are targeted and inserted into the inner membrane via different pathways (1). Targeting of most IMPs analyzed until now depends on a conserved system consisting of the signal recognition particle (SRP) and its receptor FtsY (2, 3). The *E. coli* SRP, which consists of a signal sequence binding protein (Ffh for Fifty-four homologue) and a 4.5 S RNA, samples nascent chains at the ribosomal exit site for the presence of particularly hydrophobic targeting signals that are primarily present in nascent IMPs. The ribosome nascent chain-SRP complex is transferred to the Sec translocon located in the inner membrane by the SRP receptor FtsY that is located both in the cytoplasm and inner membrane. The mechanism of transfer by FtsY is not well understood but most likely involves interaction of FtsY with membrane lipids and with the Sec translocon component SecY (4). GTP binding and hydrolysis by Ffh and FtsY regulates the vectorial transfer process and recycling of the components involved (5). The heterotrimeric core of the Sec translocon comprises the integral membrane components SecY, SecE, and SecG. It functions in membrane insertion of most IMPs and in translocation of secretory proteins (6). The peripheral ATPase SecA is dynamically associated with the cytoplasmic side of the SecYEG core and drives the translocation of secretory proteins and large periplasmic domains of IMPs (7).

YidC has been identified as a novel Sec-associated component by cross-link and pull-down experiments and plays a role in the biogenesis of IMPs (8). *In vivo* depletion of YidC (which is an essential protein) generally does not affect protein translocation and only slightly affects the insertion of Sec-dependent IMPs into the membrane (9, 10). The function of Sec-associated YidC has been investigated for only a limited number of IMPs and is currently not well understood. Available evidence suggests that it acts downstream of the Sec translocon to facilitate the lateral transfer and assembly of TMs and to assist the folding of polytopic IMPs (9, 11–13).

On the other hand, TMs in Sec-dependent nascent IMPs contact YidC very early in biogenesis, suggesting an additional role in the reception and recognition of TMs in the context of the Sec translocon (14, 15). The versatility of YidC is emphasized by the fact that it also functions independent of the Sec translocon (16, 17). Depletion of YidC almost completely inhibits membrane insertion of the Sec-independent small Pf3 and M13 phage coat proteins that were initially considered to insert spontaneously into the membrane (18). *In vitro* reconstitution studies have shown that YidC is not only required but also sufficient for membrane insertion of Pf3 coat protein including the translocation of its small N-terminal domain (16). It is as yet not clear if this Sec-independent insertase function of YidC is also reflected in a physical separation of a fraction of YidC from the Sec translocon.

A recent study has shown that the loss of YidC rapidly leads to severe defects in the functional assembly of cytochrome *o* oxidase and the F_0F_1-ATPase complex (19). Specifically, the amount of F_0c (subunit c of the F_0F_1-ATPase) and CyoA (subunit of the cytochrome *o* oxidase) decreased rapidly upon the depletion of YidC reminiscent of the effect on the insertion of small phage coat proteins. YidC is homologous to the mitochondrial OxaI that mediates membrane insertion of the mitochondrial IMPs Atp9 and Cox2p that are, in turn, homologous to F_0c and CyoA. Hence, it has been suggested that YidC plays a pivotal, perhaps exclusive role in the membrane insertion of (domains of) F_0c and CyoA (19). Three independent studies indeed demonstrate that YidC, but not the SecYEG complex, is required for membrane insertion of F_0c, a double spanning IMP with very short periplasmic tails at the N and C termini (20–22). In contrast to F_0c, CyoA is a lipoprotein that is initially synthesized with a signal sequence containing a conserved "lipobox" sequence that includes the signal peptidase II (SPaseII) cleavage site (23). Like F_0c, mature CyoA has two closely spaced TMs, but unlike F_0c, the second TM is followed by a large translocated domain.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the Council for Chemical Sciences of the Netherlands Society for Scientific Research.

[2] Supported by European Union Network Grant QLK-C-T2000-00082.

[3] To whom correspondence should be addressed. Tel.: 31-20-5987175; Fax: 31-20-5986979; E-mail: joen.luirink@falw.vu.nl.

[4] The abbreviations used are: IMP, inner membrane protein; Ffh, Fifty-four homologue; SRP, signal recognition particle; TF, trigger factor; TM, transmembrane segment; SPaseII, signal peptidase II; HA, hemagglutinin.

In the present study we have analyzed the targeting and insertion of CyoA using complementary *in vivo* protease mapping experiments and an *in vitro* photocross-linking technique. Collectively, our data demonstrate that the SRP interacts with the first TM of CyoA (rather than with its signal sequence) to mediate targeting of CyoA to the Sec/YidC insertion site in the inner membrane. Membrane insertion appears to take place in two distinct stages. YidC is required and sufficient for membrane assembly of the N-terminal part of the protein, whereas translocation of the large C-terminal periplasmic domain requires the Sec translocon, including SecA. This suggests that for membrane assembly of CyoA, the Sec translocon operates downstream of YidC.

EXPERIMENTAL PROCEDURES

Reagents, Enzymes, and Sera—Restriction enzymes, Expand long template PCR system, and Lumi-Light^{PLUS} Western blotting substrate were from Roche Applied Science. Megashort script T7 transcription kit was from Ambion Inc. [^{35}S]Methionine and protein A-Sepharose were from Amersham Biosciences. T4 ligase and alkaline phosphatase were from Invitrogen. Pansorbin was from Merck. All other chemicals were supplied by Sigma. The SPaseII inhibitor globomycin was from our own stock. Antisera against YidC, Ffh, Trigger factor, OmpA, CyoA, and SecY have been described previously or were from our own collection (20, 24). Antiserum against influenza hemagglutinin (HA) was from Sigma.

Strains, Plasmids, and Growth Conditions—*E. coli* strain MRE600 was used to prepare a lysate for translation of *in vitro* synthesized mRNA and suppression of UAG stop codons in the presence of (Tmd)Phe-tRNA^{sup}. Strain MC4100 was used for the preparation of inverted membrane vesicles. Strain Top10F' (Invitrogen) was used as routine host for all plasmid constructs. The 4.5 S RNA depletion strain FF283, the SecE depletion strain CM124, and the YidC depletion strain JS7131 were grown as described (10). The temperature-sensitive amber suppressor YidC depletion strain KO1672 and its isogenic control strain were grown as described (25). The Ffh depletion strain HDB51, strain HTP-406, which expresses a mutant *ffh* allele, and its control strain JP313 were grown as described previously (26, 27). The temperature-sensitive SecY24 mutant IQ85 and its isogenic parent strain IQ86 (28) were cultured overnight in M9 minimal medium at 30 °C. To inactivate SecY, overnight cultures were back-diluted 1:50 and grown to early log phase at 42 °C in the same medium. All strains were routinely grown in Luria Bertani medium unless indicated otherwise. Where appropriate, antibiotics were added to the culture medium.

For *in vivo* protease accessibility assays, the *cyoA* gene was PCR-amplified from *E. coli* K-12 genomic DNA including an HA tag (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) preceded by a flexible linker peptide (Pro-Gly-Gly) at the C terminus of CyoA. The PCR fragment was cloned into pC4Meth (24), yielding pC4Meth-CyoA-HA. Subsequently, CyoA-HA coding sequences were introduced into pEH1, pEH3 (29), and into pASKIBA3 (IBA GmbH) to allow expression in strains of different genetic background. The plasmid pEH1-CyoA-wt, encoding the wild-type protein, was constructed by PCR using pC4Meth-CyoA-HA as the template. pEH1-CyoA-HAΔ77–315, encoding a truncated CyoA derivative, was constructed in a PCR-based approach using pC4Meth-CyoA-HA as template. CyoA-HAΔ77–315 comprises residues 1–76 fused to a C-terminal HA tag preceded by the flexible linker peptide (Pro-Gly-Gly) and a Lys-Lys-Lys-Lys stretch.

For *in vitro* cross-link experiments, the plasmids pC4Meth-118CyoA-TAG56 and pC4Meth-54CyoA-TAG10 were constructed by nested PCR as described (24) using pC4Meth-CyoA-HA as template. The plasmids encode a truncated CyoA of 118 and 54 amino acids, respectively, including a C-terminal 4× methionine tag to improve the labeling efficiency. Moreover, the CyoA derivatives contain an amber mutation at position 56 or 10 to allow sup-tRNA-mediated incorporation of a photocross-linking probe, as described (24). The nucleotide sequence of all constructs was confirmed by DNA sequencing.

Assay for in Vivo Membrane Assembly—Strains MC4100, HDB51, FF283, HTP406, JP313, IQ85, IQ86, CM124, JS713, KO1670, and KO1672 were grown to early log phase. Cells harboring derivatives of pEH1 or pEH3 (MC4100, HDB51, IQ85, IQ86, CM124, JS7131, KO1670, and KO1672) were induced for 3 min by adding isopropyl 1-thio-β-D-galactopyranoside (1 mM), and cells harboring derivatives of pASKIBA3 (FF283, HTP406 and JP313) were induced for 1 min by adding anhydrotetracycline (500 ng/ml). Cells were labeled with [^{35}S]methionine (30 μCi/ml) and converted to spheroplasts as described previously (10). Aliquots of the spheroplast suspension were incubated with or without proteinase K (0.3 mg/ml) for 1 h on ice. Subsequently, phenylmethylsulfonyl fluoride (0.33 mg/ml) was added to the spheroplast suspension to inhibit proteinase K. Finally, proteins were acid-precipitated and immunoprecipitated with antisera against OmpA, Trigger factor, or the HA tag (24). Samples were analyzed by SDS-PAGE, and proteins were visualized by phosphorimaging. When indicated, the SPaseII inhibitor globomycin (100 μg/ml) was added 5 min before induction, or sodium azide (2 mM) was added 4 min before induction to block SecA function.

Sodium Carbonate Extraction of Cells—Strain JS7131 harboring derivatives of pEH1 was grown to mid-log phase. Aliquots containing 1 A_{660} unit of cells were harvested and resuspended in 1 ml of M9 minimal medium. Isopropyl 1-thio-β-D-galactopyranoside (1 mM) was added for 1 min to induce CyoA expression. Subsequently, cells were pulse-labeled with [^{35}S]methionine (30 μCi/ml), converted to spheroplasts as described (10), and supplied with sodium carbonate (0.2 M final concentration). Samples were vigorously vortexed, incubated on ice for 15 min, and centrifuged (30 min at 110,000 × *g*). The carbonate-insoluble fraction was dissolved in 1% SDS and immunoprecipitated with SecB, Lep, or HA antiserum (24). The carbonate soluble fraction was acid-precipitated, dissolved in 1% SDS, and immunoprecipitated. Samples were analyzed by SDS-PAGE, and proteins were visualized by phosphorimaging.

In Vitro Cross-linking—Truncated mRNA was prepared as described previously (24) from HindIII-linearized CyoA-derivative plasmids. For photocross-linking, (Tmd)Phe was site-specifically incorporated into CyoA nascent chains by suppression of an UAG stop codon using (Tmd)Phe-tRNA^{sup} in an *E. coli in vitro* translation system containing [^{35}S]methionine to label the nascent chains. Targeting to inverted membrane vesicles, photocross-linking, and carbonate extraction were performed as described (24). Carbonate-soluble and -insoluble fractions were acid-precipitated or immunoprecipitated. Samples were analyzed by SDS-PAGE and visualized by phosphorimaging as described (24).

RESULTS

Expression and Detection of CyoA—In the present study we have investigated the targeting and membrane integration of CyoA. To permit immunodetection in protease accessibility experiments, the nine-amino acid-long influenza virus HA tag preceded by a flexible linker was attached to the C terminus of the protein (Fig. 1*A*). We first verified that addition of the tag does not interfere with the complex lipid modification and processing of CyoA. Lipoproteins share a common modification pathway by virtue of a conserved lipobox pentapeptide that includes the N-terminal cysteine of the mature protein (30). The lipoproteins are modified with diglyceride at the SH group of the cysteine, cleaved between the lipid-modified cysteine and the preceding

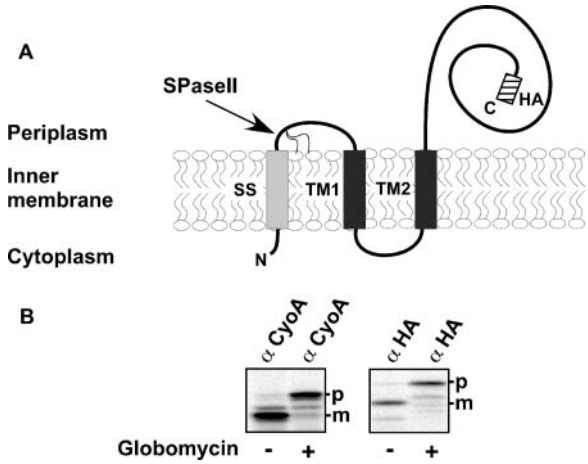

FIGURE 1. **Membrane assembly of CyoA-HA.** *A*, topology model of CyoA-HA. CyoA is synthesized with a lipoprotein-type signal sequence that is processed by SPaseII (*arrow*). Mature CyoA comprises two hydrophobic TMs connected by a small cytoplasmic loop and two translocated termini; that is, a lipid-modified N terminus and a large C terminus. An HA epitope tag is attached to the C terminus of CyoA to facilitate detection and verification of membrane assembly. The HA tag is accessible to proteinase K added to spheroplasts derived from wild-type cells. *B*, translocation and processing of pulse-labeled CyoA and CyoA-HA in wild-type cells in the absence or presence of the SPaseII inhibitor globomycin. CyoA was immunoprecipitated with antisera against the wild-type protein (α-*CyoA*) or HA tag (α-*HA*). The precursor and mature forms of CyoA-HA are denoted p and m, respectively.

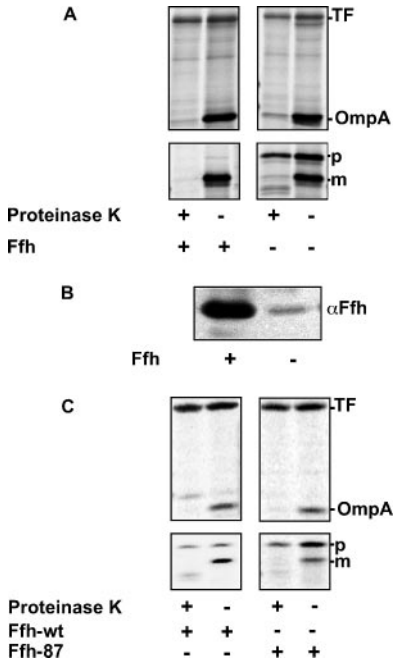

FIGURE 2. **The SRP is required for membrane targeting of CyoA.** *A*, protease mapping of CyoA-HA in Ffh depletion strain HDB51. HDB51 cells harboring a CyoA-HA expression plasmid were grown under non-depleting or depleting conditions, pulse-labeled, converted to spheroplasts, and treated with proteinase K as described under "Experimental Procedures." The samples were immunoprecipitated using antibodies directed against the HA-tag (*bottom panel*) or antibodies directed against OmpA and trigger factor (*top panel*). OmpA (outer membrane protein A) and trigger factor (cytoplasmic protein) were analyzed to monitor the efficacy of proteinase K treatment and spheroplast formation, respectively. Immunoprecipitated material was analyzed by SDS-PAGE and visualized by phosphorimaging. *B*, immunoblot analysis of Ffh levels in 0.1 A_{660} units of cells used in *panel A*. *C*, protease mapping of CyoA-HA in Ffh-87 mutant strain HTP406 and its wild-type control strain (Ffh-wt). Both strains harboring a CyoA-HA expression plasmid were grown in parallel, pulse-labeled, and converted to spheroplasts. Samples were prepared and processed as described under A. The precursor and mature forms of CyoA-HA are denoted *p* and *m*, respectively.

amino acid by SPaseII, and acylated with fatty acid at the NH_2 group of the cysteine residue. SPaseII only cleaves lipid-modified precursor and can be specifically inhibited by the antibiotic globomycin leading to the accumulation of lipid-modified prolipoprotein.

Cells expressing wild-type or HA-tagged CyoA were pulse-labeled in the absence or presence of globomycin, and CyoA was immunoprecipitated from the samples using antibodies against CyoA or the HA tag, respectively. As shown in Fig. 1*B*, processing of both wild-type and HA-tagged CyoA is almost completely inhibited by globomycin, indicating that pre-CyoA-HA is lipid-modified and processed by SPaseII, like wild-type CyoA. As expected, the HA-tagged CyoA species migrate slightly slower than wild-type CyoA in SDS-PAGE. These data indicate that attachment of an HA tag to the C terminus of CyoA does not interfere with its processing and maturation, consistent with a recent study using two other HA-tagged lipoproteins (25).

CyoA Is Targeted by the SRP to the Inner Membrane—Many IMPs that have uncleaved signal anchor sequences require the SRP for efficient targeting. To investigate the role of the SRP in targeting of CyoA, which is synthesized with a cleavable signal sequence, we used a protease mapping assay to analyze membrane assembly of CyoA-HA in strains in which the expression or activity of both components of the SRP, Ffh and the 4.5 S RNA, can be modulated. Cells of strain HDB51 in which *ffh* expression is under control of an arabinose-inducible promoter were grown in the presence or absence of arabinose, pulse-labeled, converted to spheroplasts, and treated with proteinase K to degrade external (periplasmic) protein domains. As shown in Fig. 2*A*, in the presence of Ffh mature CyoA-HA was only detected in mock-treated samples, demonstrating that the HA tag is translocated and sensitive to proteinase K. Apparently, the HA tag does not interfere with membrane assembly of CyoA, validating our assay conditions. Trigger factor (TF) and OmpA are cytoplasmic and outer membrane control proteins used to monitor spheroplast formation and the efficacy of proteinase K treatment, respectively. Depletion of Ffh resulted in accumulation of protease-protected precursor Cyo-HA, suggesting that the SRP is required for targeting of CyoA to the inner membrane.

Translocation and processing of the Sec-dependent protein OmpA were not affected under these conditions, indicating that inactivation of the Sec translocon had not occurred. Protease insensitivity of a small fraction of mature OmpA upon depletion of Ffh has been observed before (20, 31) and remains to be investigated. Depletion of Ffh was verified by analyzing the Ffh content in a cell sample taken before pulse-labeling by immunoblotting (Fig. 2*B*). CyoA targeting was also analyzed in the relatively mild and viable Ffh-87 mutant strain (27) (Fig. 2*C*). Quantitation of the data in Fig. 2*C* (correcting for the methionine content of the precursor and mature forms) showed that the precursor accumulated ~6-fold in cells of the Ffh-87 mutant when compared with cells of the wild-type control strain. OmpA processing was not affected under these conditions, as expected for this SRP-independent protein. A possible role for the SRP in CyoA targeting was further investigated in the 4.5 S RNA depletion strain FF283. In accordance with the effects of depletion of Ffh, depletion of 4.5 S RNA also resulted in precursor accumulation (data not shown). Together, the results strongly suggest that CyoA requires the SRP for efficient membrane targeting.

The SecYEG Translocon and SecA Are Required for Translocation of the C-terminal Polar Domain of CyoA—The requirements for membrane assembly of CyoA were studied using strains that are conditional for the expression or activity of subunits of the Sec translocon and YidC employing the same protease accessibility procedure. To evaluate the role of the Sec translocon, we used the temperature-sensitive SecY mutant IQ85 (and its isogenic parent strain IQ86) and the SecE depletion strain CM124 in which the essential *secE* gene is under control of an

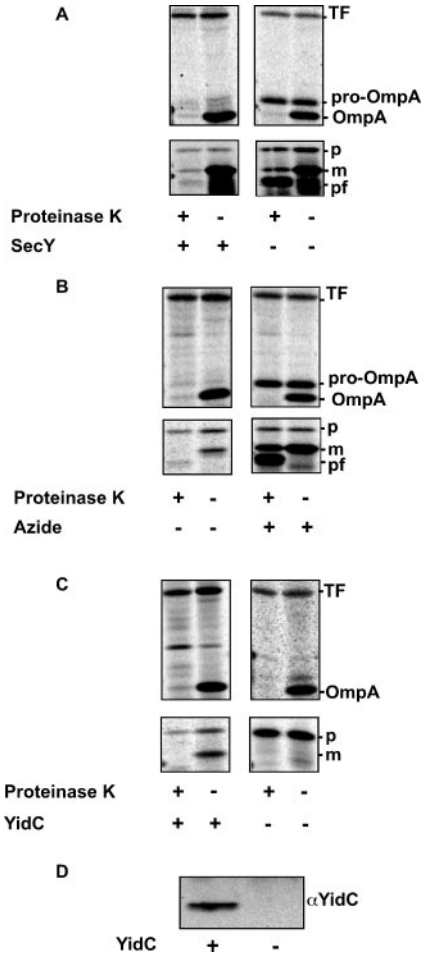

FIGURE 3. **Distinct requirements for translocation of the N terminus and C terminus of CyoA.** *A*, protease mapping of CyoA-HA in the temperature-sensitive SecY mutant strain IQ85 and its isogenic parent IQ86. Both strains harboring a CyoA-HA expression plasmid were grown in parallel at 42 °C, pulse-labeled, and converted to spheroplasts. Samples were prepared and processed as under Fig. 2*A*. *B*, protease mapping of CyoA-HA in cells treated with azide to inhibit SecA function. Strain MC4100 harboring a CyoA-HA expression plasmid was grown in M9 medium, pulse-labeled in the presence or absence of azide, and converted to spheroplasts. Samples were prepared and processed as under Fig. 2*A*. *C*, protease mapping of CyoA-HA in YidC depletion strain JS7131. Cells of strain JS7131 harboring a CyoA-HA expression plasmid were grown under non-depleting or depleting conditions, pulse-labeled, and converted to spheroplasts. Samples were prepared and processed as under Fig. 2*A*. *D*, immunoblot analysis of YidC levels in 0.1 A_{660} units of cells used in *panel C*. The precursor and mature forms of CyoA-HA are denoted *p* and *m*, respectively. *pf* is the proteinase K proteolytic fragment of CyoA-HA.

arabinose-inducible promoter. Inactivation of SecY or depletion of SecE results in the loss of the complete SecY/E core of the translocon. As shown in Fig. 3*A*, inactivation of SecY had a strong effect on the accessibility of CyoA toward proteinase K. Under control conditions, no immunoprecipitable material of CyoA was left after proteinase K treatment, confirming that the C-terminal tail is translocated and the HA tag is degraded by proteinase K. However, upon inactivation of SecY, mature CyoA was to a large extent converted into a protected proteolytic fragment (*pf*) that migrated slightly faster than mature CyoA. This form was absent when proteinase K was added to detergent-solubilized spheroplasts (not shown). Most likely, this fragment represents membrane-inserted mature CyoA with an untranslocated C terminus and a translocated, hence degraded, N-terminal loop. Strikingly, processing of CyoA was hardly affected under these conditions, indicating that signal sequence insertion, lipid modification, and accessibility to SPaseII were not perturbed. As a control, protease-protected proOmpA accumulated under these conditions (Fig. 3*A*), consistent with the well documented role of the Sec machinery in the transfer of proOmpA across the inner membrane (6). These results suggest that the small N-terminal domain of CyoA upstream of TM1 does not require the Sec translocon to reach the periplasmic side of the membrane, in contrast to the large C-terminal domain. To confirm that the observed effects were due to inactivation of the SecY/E core of the translocon, the assembly of CyoA was also analyzed in the SecE depletion strain CM124. Depletion of SecE had a similar effect on the translocation of the CyoA domains as inactivation of SecY (data not shown).

The ATPase SecA is required to energize the translocation of large periplasmic domains of IMPs through the SecYEG translocon (1). To investigate whether SecA is involved in transfer of the large C-terminal polar domain of CyoA, a protease accessibility experiment was carried out in the absence or presence of azide to block SecA function (Fig. 3*B*). In the absence of azide, no immunoprecipitable material was left after proteinase K treatment, indicative of correct localization of the HA tag. In the presence of azide, the same effect was observed as upon inactivation of SecY or depletion of SecE, strongly indicating that SecA is required to catalyze membrane transfer of the C-terminal polar domain. However, translocation of the N-terminal region and signal sequence cleavage processing were not affected by azide. Inhibition of SecA function by azide treatment was confirmed by a reduced processing and translocation of proOmpA (Fig. 3*B*). Together, the results suggest that the Sec translocon and SecA act in concert to translocate the CyoA C terminus, whereas translocation of the N terminus neither requires the Sec translocon or SecA.

YidC Is Required for Translocation and Membrane Assembly of the CyoA N Terminus—To analyze the role of YidC in the membrane assembly of CyoA, we used the YidC depletion strain JS7131 in which the *yidC* gene is under control of an arabinose-inducible promoter (Fig. 3*C*). In the presence of YidC, the HA tag was correctly translocated across the inner membrane and degraded by proteinase K. Depletion of YidC resulted in inhibition of processing and accumulation of protease-resistant precursor CyoA. As a control, the translocation and processing of OmpA was monitored and appeared unaffected, suggesting that inactivation of the Sec translocon had not occurred under these conditions, consistent with unaltered levels of Sec translocon components (data not shown). YidC depletion was confirmed by analyzing the YidC content in a cell sample taken before pulse labeling by immunoblotting using YidC antiserum (Fig. 3*D*).

To study the localization of the accumulated CyoA precursor upon depletion of YidC, spheroplasts derived from JS7131 cells depleted and not depleted for YidC were subjected to sodium carbonate extraction, which separates integral membrane proteins from peripheral membrane proteins and soluble proteins. As expected, mature CyoA was recovered in the carbonate-insoluble fraction in the presence of YidC, indicative of an integral membrane localization (data not shown). Upon depletion of YidC, the accumulated precursor CyoA was recovered in the carbonate-insoluble fraction (data not shown), suggesting that YidC is not required for the initial membrane insertion of CyoA.

The data indicate that YidC is required for translocation of the N-terminal region and subsequent signal sequence cleavage. In the absence of YidC, the C-terminal domain is also incapable of translocation, which is probably due to an obligatory vectorial assembly from N to C terminus.

Translocation of the N Terminus of CyoA Does Not Require the Sec Translocon—The data so far indicate that membrane insertion and translocation of the N and C terminus of CyoA follow different pathways. To further delineate the requirements for translocation of the CyoA N terminus, we constructed a CyoA mutant that lacks TM2 and the large polar C-terminal tail (Fig. 4*A*). The construct contains an HA

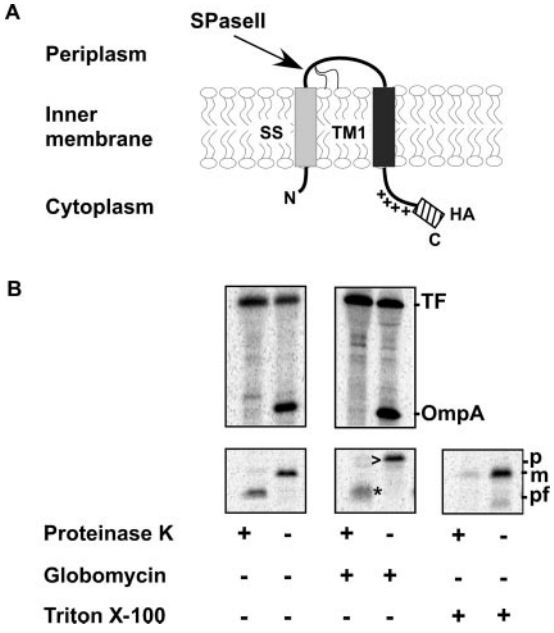

FIGURE 4. **Membrane assembly of CyoA-HAΔ77–315.** *A*, topology model of CyoA-HAΔ77–315. This truncated CyoA derivative comprises the N-terminal signal sequence, TM1, and the periplasmic domain in between. A stretch of four lysines was added to the C terminus to anchor it at the cytoplasmic side of the inner membrane followed by an HA tag to facilitate detection and verification of membrane assembly. In spheroplasts derived from wild-type cells, proteinase K degrades the translocated N terminus, resulting in a distinct proteolytic fragment. *B*, protease mapping of CyoA-HAΔ77–315 in cells treated with globomycin. MC4100 cells harboring a CyoA-HAΔ77–315 expression plasmid were grown in M9 medium and pulse-labeled in the presence or absence of globomycin. Samples were prepared and processed as under Fig. 2*A*. The precursor form of CyoA-HAΔ77–315 is denoted *p*, *m* is the mature form, and *pf* is the proteinase K proteolytic fragment. For clarity, the lipid-modified precursor is indicated by an *arrowhead*, and the proteolytic fragment of the precursor is indicated by an *asterisk*.

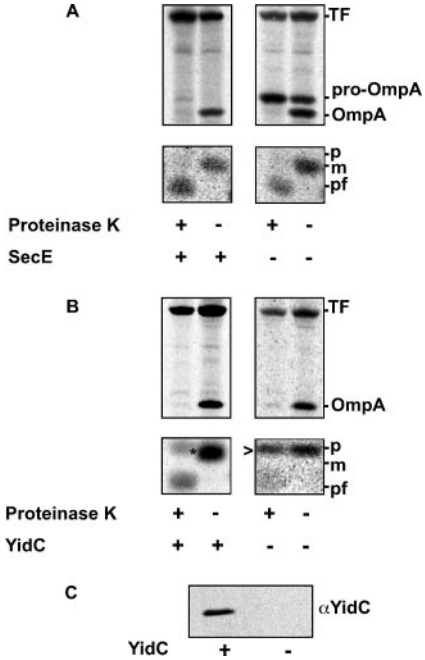

FIGURE 5. **Assembly of CyoA-HAΔ77–315 requires YidC but not the Sec translocon.** *A*, protease mapping of CyoA-HAΔ77–315 in SecE depletion strain CM124. Cells of CM124 containing a CyoA-HAΔ77–315 expression plasmid were grown under non-depleting or depleting conditions, pulse-labeled, and converted to spheroplasts. Samples were prepared and processed as under Fig. 2*A*. *B*, protease mapping of CyoA-HAΔ77–315 in the temperature-sensitive YidC strain KO1672 and its isogenic parent KO1670. Both strains containing a CyoA-HAΔ77–315 expression plasmid were grown in parallel at 42 °C, pulse-labeled, and converted to spheroplasts. Samples were prepared and processed as under Fig. 2*A*. *C*, immunoblot analysis of YidC levels in 0.1 A_{660} units of cells used in *panel B*. The precursor form of CyoA-HAΔ77–315 is denoted *p*, *m* is the mature form, and *pf* is the proteinase K proteolytic fragment. For clarity, the precursor is indicated by an *arrowhead*, and the mature form is indicated by an *asterisk*.

tag at the C terminus to enable immunodetection preceded by a positively charged stretch of four lysine residues to ensure correct topology of the truncate. According to the positive inside rule, positively charged residues of IMPs are predominantly localized at the cytoplasmic face of the inner membrane and contribute to the topology of IMPs by preventing translocation of adjacent domains (1). Processing and topogenesis of this CyoA-HAΔ77–315 construct were verified using a protease accessibility assay (Fig. 4*B*). In spheroplasts derived from a wild-type MC4100 strain, the construct is expressed and clipped at the N terminus by externally added proteinase K, indicating that the construct is membrane-integrated and has acquired its expected topology. The clipped form is degraded in detergent-solubilized cells, confirming that this form is not intrinsically protease-resistant but is inaccessible in intact spheroplasts (Fig. 4*B*). Globomycin treatment caused an upward shift in migration of the truncate, indicative of compromised processing, implying that the construct is normally lipid-modified and processed by SPaseII. As expected and similar to the mature form, the N terminus of the unprocessed precursor is clipped by proteinase K, resulting in a slightly faster migrating protected species.

In subsequent experiments we investigated the requirements for membrane assembly of CyoAHAΔ77–315 using the conditional strains described above. First, involvement of the Sec translocon was studied in the SecE depletion strain CM124. Clearly, depletion of SecE had no discernible effect on the processing or accessibility of CyoA-HAΔ77–315 toward proteinase K (Fig. 5*A*). Efficient depletion of SecE was confirmed by the reduced processing and translocation of OmpA (Fig. 5*A*). Similar results were obtained using the temperature-sensitive SecY mutant IQ85 and its isogenic control strain IQ86 (data not shown). Next, YidC involvement was studied using the temperature-sensitive YidC strain KO1672 and its isogenic control strain KO1670 (Fig. 5*B*). Depletion of YidC strongly inhibited processing of CyoA-HAΔ77–315, resulting in accumulation of the precursor form that appeared mostly inaccessible to proteolysis by proteinase K. Apparently, the N terminus of the truncate is unable to reach the periplasm under these conditions. Processing and translocation of OmpA appeared unaffected, demonstrating that inactivation of the Sec translocon had not occurred. Depletion of YidC was verified by analyzing the YidC content in a cell sample taken before pulse labeling by immunoblotting (Fig. 5*C*). Together, the *in vivo* results indicate that the N terminus of CyoA can insert independent from the C terminus in a YidC-dependent, Sec-independent mechanism.

Nascent CyoA Interacts with Ffh, SecY, SecE, and YidC—The *in vivo* experiments described above indicate that efficient targeting and membrane assembly of the complete CyoA protein requires the SRP, YidC, the SecYEG translocon, and SecA. To independently confirm the contribution of these factors, we used a combined *in vitro* translation/photocross-linking approach to evaluate the interactions of nascent CyoA variants that are trapped during targeting and membrane assembly. First, we analyzed the contacts of TM1 during biogenesis of CyoA. TM1 is more hydrophobic than the signal sequence (not shown) and, thus, more likely to play a role in SRP-mediated membrane targeting (32). Nascent chains of CyoA were synthesized in an *E. coli* cell-free extract from truncated mRNA to a length of 118 residues and labeled by the incorporation of [^{35}S]methionine. Assuming that the ribosome covers ~35 amino acids, the signal sequence and first TM are well exposed out of the ribosome at this nascent chain length (Fig. 6*A*). The truncated CyoA construct contained a single amber stop codon (TAG) at position 56 in the TM1 that was suppressed during *in vitro* translation by the

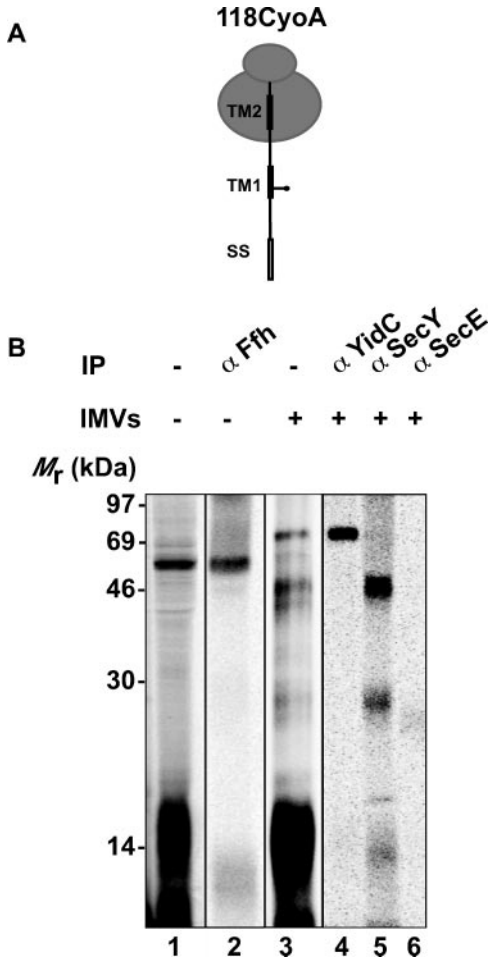

FIGURE 6. **The molecular environment of nascent CyoA.** *A*, schematic representation of the nascent CyoA 118-mer with a photocross-linking probe at position 56. The TMs and signal sequence (*SS*) are indicated. *B*, *in vitro* translation of nascent CyoA was carried out in the presence of (Tmd)Phe-tRNA^{sup} in the absence (*lanes 1* and *2*) or presence of inverted membrane vesicles (*IMVs*) (*lanes 3–6*). After translation, samples were either UV-irradiated and acid-precipitated (*lanes 1* and *2*) or extracted with sodium carbonate (*lane 3*-6). Acid precipitates and carbonate pellet fractions were immunoprecipitated (*IP*) using antisera against the indicated proteins (*lanes 2* and *4–6*) or directly analyzed (*lanes 1* and *3*).

addition of a suppressor tRNA that carried a phenylalanine with a photo-activable Tmd group ((Tmd)-Phe-tRNA^{sup}). This allowed us to specifically probe interactions of TM1 in nascent CyoA. We confirmed that the TAG mutation was efficiently suppressed by the (Tmd)Phe-tRNA^{sup}, resulting in nascent CyoA of the expected molecular weight (not shown). Next, 118CyoA-TAG56 was synthesized in the absence of inverted membrane vesicles and UV-irradiated to activate the cross-linking probe and secure contacts of the CyoA TM1 in the *E. coli* cytosol. A prominent cross-linking product of ~60 kDa was generated that could be immunoprecipitated using anti-Ffh serum (Fig. 6*B*, *lane 2*). This suggests association of TM1 with the SRP, consistent with the *in vivo* SRP data described above.

To investigate the contacts of 118CyoA-TAG56 during membrane assembly, inverted membrane vesicles were added during translation, allowing co-translational membrane insertion of nascent CyoA. Subsequent cross-linking induced by UV irradiation and recovery of the membrane-integrated nascent chains by sodium carbonate extraction generated cross-linking products of ~70 and ~45 kDa in which YidC and SecY were identified as partners by immunoprecipitation (Fig. 6*B*, *lanes 4* and *5*). Moreover, a weak band at ~29 kDa represented cross-linking to SecE (Fig. 6*B*, *lane 6*). This indicates that TM1 of nascent CyoA is primarily in contact with YidC and SecY at this stage during membrane insertion.

To probe initial interactions from the signal sequence, 54CyoA-TAG10 was constructed in which the signal sequence (with a photocross-link probe in the core region) is expected to be exposed outside the ribosome. Notably, cross-linking was restricted to TF and the ribosomal protein L23; no significant cross-linking was observed to Ffh or membrane components (data not shown). These results are consistent with the relatively modest hydrophobicity of the signal sequence and previously obtained cross-link data using other signal sequences of similar hydrophobicity (15, 32). Together, the cross-link data argue that TM1 recruits the SRP for targeting of nascent CyoA and inserts into the membrane at the Sec-YidC insertase.

DISCUSSION

CyoA is one of the four integral membrane subunits of the cytochrome *o* oxidase complex in the *E. coli* inner membrane. CyoA is an unusual polytopic IMP in the sense that it is synthesized with an N-terminal, lipoprotein type signal sequence that is cleaved from the precursor protein (23). This generates mature CyoA with two hydrophobic TMs and an N-terminal cysteine residue that is probably anchored to the inner membrane via three covalently attached acyl chains. It has been recently shown that, unlike most other polytopic IMPs, the level of CyoA in the inner membrane drops dramatically upon depletion of YidC, suggesting an important role of YidC in the biogenesis of CyoA (19). Evidence presented in this work suggests that YidC is required and sufficient for assembly of the N-terminal region, explaining its pivotal role in the assembly of the complete CyoA that probably proceeds in a vectorial co-translational mechanism.

In Fig. 7 a model is depicted that describes the targeting and membrane assembly of CyoA based on the *in vivo* protease mapping and *in vitro* cross-linking experiments described in this study. The SRP targets nascent CyoA via TM1 to the insertion site in the membrane judged by the observation that precursor CyoA accumulates in the cells in three SRP mutant strains under conditions where proOmpA, an outer membrane protein that is targeted via the SecB pathway, is normally processed. The *in vitro* cross-link experiments suggest that co-translational targeting is not initiated until TM1 is exposed at the ribosomal nascent chain exit site and associates with Ffh. The signal sequence emerging from the ribosome in shorter nascent chains does not contact the SRP nor inserts stably into the membrane. Rather, at this stage the signal sequence is proximal to the chaperone trigger factor and the ribosomal protein L23. The recently presented crystal structure of a ribosome-trigger factor complex demonstrates that trigger factor binds to L23 near the nascent chain exit site, arching over the exit region and, thus, providing a secluded cavity (33). This architecture may prevent premature contact of nascent CyoA with the translocon. Selection of TM1 rather than the signal sequence by the SRP is probably dictated by the more hydrophobic core of TM1 that is clearly above the threshold level for SRP binding in contrast to the much less hydrophobic core of the signal sequence (not shown).

At this stage, TF and SRP may sequester the signal sequence and TM1 near L23 to facilitate the formation of a hairpin-like conformation that is required for the subsequent concerted membrane integration at YidC of the signal sequence, TM1, and intercalated periplasmic domain that contains the SPaseII cleavage site. The SRP is proposed to guide the ribosome-nascent CyoA complex to YidC, but mechanistic details of this step and a possible involvement of the SRP receptor FtsY remain unclear. Interestingly, in thylakoid membranes a functional interaction between chloroplast SRP, FtsY, and the YidC homologue Alb3 has been

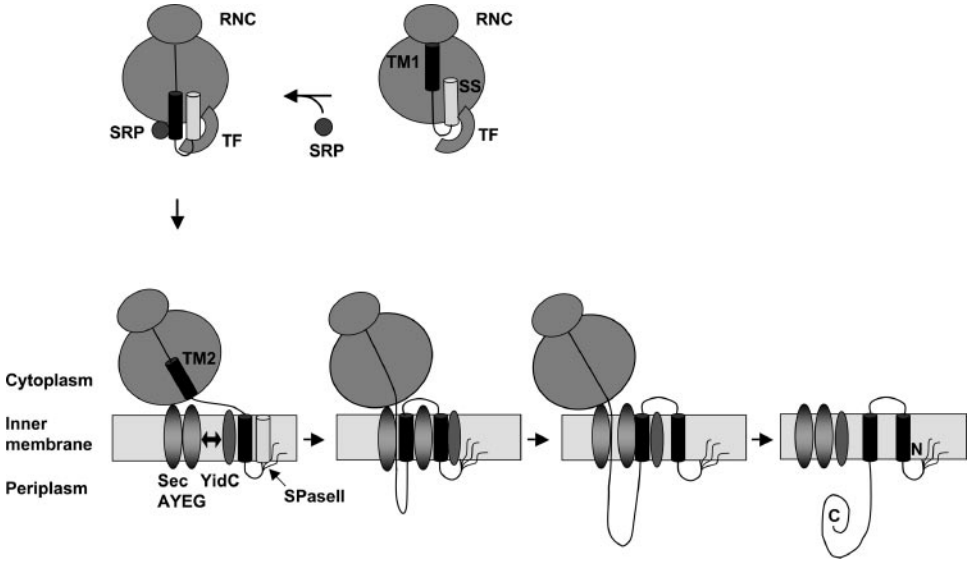

FIGURE 7. **Model for the membrane assembly of CyoA.** The model is described under "Discussion." The (transient) association between YidC and the SecYEG complex is indicated by a *double arrow*. *RNC*, ribosome nascent chain complex.

demonstrated (34). It should be noted, however, that involvement of the SRP in targeting of F_oc, another IMP that requires YidC for membrane insertion, is controversial (20–22). Targeting of membrane proteins via the second hydrophobic sequence is not unprecedented. Studies on the insertion of M13 procoat, the cystic fibrosis transmembrane regulator (CFTR), and the *Shaker*-like K^+ channel revealed a prominent role for the second, more hydrophobic sequence in targeting and initiation of membrane insertion (M13 procoat) or TM1 (CFTR, *Shaker*-like K^+ channel) (35–37).

If not necessary for targeting *per se*, what is the role for the CyoA signal sequence? It may be required to transfer the N-terminal hydrophilic domain in between the signal sequence and TM1 to the periplasmic side of the membrane in a concerted hairpin-type insertion. Notably, deletion of Arg-2 to Ser-9 of the CyoA signal sequence eliminates incorporation of the oxidase in the membrane, although the stage of the defect was not determined (23). Furthermore, lipid modification of the conserved N-terminal cysteine, located at the SPaseII cleavage site, might be required to anchor the N terminus in the membrane. Interestingly, mutation of this conserved cysteine into an alanine abolishes lipid modification and signal sequence cleavage but does not affect membrane assembly and functioning of the oxidase (23). In this mutant the uncleaved signal sequence probably functions as an additional TM, anchoring the downstream sequence to the membrane in the absence of lipid modification.

Translocation of the periplasmic loop that precedes TM1 in CyoA requires YidC, whereas the Sec machinery is dispensable. Interestingly, the structure of the ubiquinol oxidase shows that this loop is only 11 residues long including one negatively and one positively charged residue (38). It has been demonstrated recently that YidC is necessary and sufficient for translocation of the periplasmic domain in between the signal sequence and TM1 of M13 procoat (39). It was shown that translocation of this loop only becomes Sec-dependent when the net number of negatively charges (−3 in wild type) or the length of the loop (20 residues in wild type) is increased (39). Apparently, YidC is able to translocate small, relatively uncharged domains in between a signal sequence and a TM. It remains to be investigated whether this translocation involves a channel-like property of YidC or whether it is a "side effect" of YidC-assisted formation of transmembrane helices.

Cross-linking of membrane-inserted nascent CyoA in which both the signal sequence and TM1 have emerged from the ribosome revealed that at this stage TM1 is not only close to YidC, as expected, but also to SecY and SecE, which are only needed at a later stage in translation when TM2 is inserted and the large C-terminal domain is translocated into the periplasm. Possibly, the Sec translocon is already recruited near the YidC insertase in response to sequences of CyoA that are still buried in the ribosomal exit tunnel. Alternatively, the YidC insertase is permanently or dynamically connected with the Sec translocon. In this respect, it should be noted that there is no direct evidence for strictly separate pools of Sec-associated and "free" YidC. Interestingly, a cryo electron microscopy reconstruction of the dimeric *E. coli* SecYEG channel tethered to a ribosome-nascent FtsQ complex has been reported very recently, giving an impression of the translocon structure caught in the act of inserting a membrane protein (40). The structure suggests a dynamic and asymmetric distribution of features with greater lipid accessibility at the front of the channel. As proposed by the authors, YidC could be situated near this front side to regulate the transfer of nascent peptides between the lipid bilayer and the interior of the Sec translocon.

Clearly, the Sec translocon is required for translocation of the large (~200 residues) periplasmic domain downstream of TM2. Most likely, TM2 acts as a signal anchor sequence and inserts in the SecYEG translocon to initiate translocation of the C terminus in a SecA-dependent manner (Fig. 7). This step is probably mechanistically similar to the assembly and translocation of other large periplasmic domains in IMPs such as those present in FtsQ and Lep (1). It remains uncertain whether TM1 is kept near YidC at this stage before its transfer into the lipid bilayer. Finally, TM2 should move laterally from the Sec translocon into the lipid bilayer, possibly via YidC, and assemble with TM1 into the tightly packed hetero-oligomeric oxidase complex. Notably, these latter steps were not explored by cross-linking and remain speculative.

In conclusion, we have presented evidence that membrane assembly of CyoA occurs in two distinct sequential steps. Although the precise molecular mechanism awaits further analysis, the data suggest that in this case the Sec translocase operates downstream of the YidC insertase, explaining the strongly reduced levels of CyoA in the inner membrane upon depletion of YidC. It remains to be investigated whether other membrane proteins follow this pathway for membrane integration. It is of interest that the data are to some extent reminiscent of Lep, a model IMP that has been extensively used in biogenesis studies. Lep has the same overall topology as CyoA (2 TMs, small N-terminal and large C-terminal periplasmic domain) but is synthesized without an N-terminal signal sequence. Lep is targeted by the SRP that interacts with TM1 (32, 41). Similar to CyoA, translocation of the small N-terminal periplasmic domain does not require the Sec machinery, in contrast to

the large periplasmic domain downstream of TM2 (42). On the other hand, depletion of YidC has little effect on the overall assembly of Lep (10), indicating that Lep is rather flexible in the use of different insertase components, in contrast to the proposed hairpin-type insertion of the signal sequence/TM1 pair in CyoA and M13 procoat that appear strictly YidC-dependent.

Interestingly, Cox2p, the CyoA homologue in the inner membrane of mitochondria in *Saccharomyces cerevisiae*, displays similar distinct requirements for membrane assembly; the YidC homologue Oxa1p is required for translocation of both the N and C terminus across the inner membrane into the intermembrane space (43, 44), whereas a distant YidC homologue, Cox18 (Oxa2p), is specifically involved in topogenesis of the C-terminal domain (45, 46). Therefore, despite the fact that mitochondria do not contain a Sec translocon, the principle of a segmented insertion mechanism appears conserved.

Acknowledgments—We are grateful to Corinne ten Hagen-Jongman for technical support and to all laboratory members for discussions and critical reading of the manuscript. Harris Bernstein, Jon Beckwith, Ross Dalbey, and Bob Gennis are thanked for strains and sera.

REFERENCES

1. Gier, J. W. (2005) *Annu. Rev. Microbiol.* **59,** 329–355
2. Luirink, J., and Sinning, I. (2004) *Biochim. Biophys. Acta* **1694,** 17–35
3. Koch, H. G., Moser, M., and Muller, M. (2003) *Rev. Physiol. Biochem. Pharmacol.* **146,** 55–94
4. Angelini, S., Deitermann, S., and Koch, H. G. (2005) *EMBO Rep.* **6,** 476–481
5. Shan, S. O., and Walter, P. (2005) *FEBS Lett.* **579,** 921–926
6. Veenendaal, A. K., van der Does, C., and Driessen, A. J. (2004) *Biochim. Biophys. Acta* **1694,** 81–95
7. Vrontou, E., and Economou, A. (2004) *Biochim. Biophys. Acta* **1694,** 67–80
8. Yi, L., and Dalbey, R. E. (2005) *Mol. Memb. Biol.* **22,** 101–111
9. Urbanus, M. L., Scotti, P. A., Froderberg, L., Saaf, A., deGier, J. W. L., Brunner, J., Samuelson, J. C., Dalbey, R. E., Oudega, B., and Luirink, J. (2001) *EMBO Rep.* **2,** 524–529
10. Froderberg, L., Houben, E., Samuelson, J. C., Chen, M., Park, S. K., Phillips, G. J., Dalbey, R., Luirink, J., and de Gier, J. W. (2003) *Mol. Microbiol.* **47,** 1015–1027
11. Houben, E. N., ten Hagen-Jongman, C. M., Brunner, J., Oudega, B., and Luirink, J. (2004) *EMBO Rep.* **5,** 970–975
12. Beck, K., Eisner, G., Trescher, D., Dalbey, R. E., Brunner, J., and Muller, M. (2001) *EMBO Rep.* **2,** 709–714
13. Nagamori, S., Smirnova, I. N., and Kaback, H. R. (2004) *J. Cell Biol.* **165,** 53–62
14. Houben, E. N. G., Urbanus, M. L., van der Laan, M., ten Hagen-Jongman, C. M., Driessen, A. J. M., Brunner, J., Oudega, B., and Luirink, J. (2002) *J. Biol. Chem.* **277,** 35880–35886
15. Houben, E. N., Zarivach, R., Oudega, B., and Luirink, J. (2005) *J. Cell Biol.* **170,** 27–35
16. Serek, J., Bauer-Manz, G., Struhalla, G., Van Den Berg, L., Kiefer, D., Dalbey, R., and Kuhn, A. (2004) *EMBO J.* **23,** 294–301
17. Preuss, M., Ott, M., Funes, S., Luirink, J., and Herrmann, J. M. (2005) *J. Biol. Chem.* **280,** 13004–13011
18. Samuelson, J. C., Chen, M. Y., Jiang, F. L., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G. J., and Dalbey, R. E. (2000) *Nature* **406,** 637–641
19. 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.* **10,** 5801–5806
20. van Bloois, E., Jan Haan, G., de Gier, J. W., Oudega, B., and Luirink, J. (2004) *FEBS Lett.* **576,** 97–100
21. van der Laan, M., Bechtluft, P., Kol, S., Nouwen, N., and Driessen, A. J. (2004) *J. Cell Biol.* **165,** 213–222
22. Yi, L., Celebi, N., Chen, M., and Dalbey, R. E. (2004) *J. Biol. Chem.* **279,** 39260–39267
23. Ma, J., Katsonouri, A., and Gennis, R. B. (1997) *Biochemistry* **36,** 11298–11303
24. Scotti, P. A., Urbanus, M. L., Brunner, J., de Gier, J. W., von Heijne, G., van der Does, C., Driessen, A. J., Oudega, B., and Luirink, J. (2000) *EMBO J.* **19,** 542–549
25. Froderberg, L., Houben, E. N., Baars, L., Luirink, J., and de Gier, J. W. (2004) *J. Biol. Chem.* **279,** 31026–31032
26. Lee, H. C., and Bernstein, H. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3471–3476
27. Tian, H. P., and Beckwith, J. (2002) *J. Bacteriol.* **184,** 111–118
28. Shiba, K., Ito, K., Yura, T., and Ceretti, D. P. (1984) *EMBO J.* **3,** 631–635
29. Hashemzadeh-Bonehi, L., Mehraein-Ghomi, F., Mitsopoulos, C., Jacob, J. P., Hennessey, E. S., and Broome-Smith, J. K. (1998) *Mol. Microbiol.* **30,** 676–678
30. Hayashi, S., and Wu, H. C. (1990) *J. Bioenerg. Biomembr.* **22,** 451–471
31. van Bloois, E., Nagamori, S., Koningstein, G., Ullers, R. S., Preuss, M., Oudega, B., Harms, N., Kaback, H. R., Herrmann, J. M., and Luirink, J. (2005) *J. Biol. Chem.* **280,** 12996–13003
32. Valent, Q. A., de Gier, J.-W. L., von Heijne, G., Kendall, D. A., ten Hagen-Jongman, C. M., Oudega, B., and Luirink, J. (1997) *Mol. Microbiol.* **25,** 53–64
33. Ferbitz, L., Maier, T., Patzelt, H., Bukau, B., Deuerling, E., and Ban, N. (2004) *Nature* **431,** 590–596
34. Moore, M., Goforth, R. L., Mori, H., and Henry, R. (2003) *J. Cell Biol.* **162,** 1245–1254
35. Rohrer, J., and Kuhn, A. (1990) *Science* **250,** 1418–1421
36. Lu, Y., Xiong, X., Helm, A., Kimani, K., Bragin, A., and Skach, W. R. (1998) *J. Biol. Chem.* **273,** 568–576
37. Tu, L., Wang, J., Helm, A., Skach, W. R., and Deutsch, C. (2000) *Biochemistry* **39,** 824–836
38. 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
39. Chen, M., Xie, K., Yuan, J., Yi, L., Facey, S. J., Pradel, N., Wu, L. F., Kuhn, A., and Dalbey, R. E. (2005) *Biochemistry* **44,** 10741–10749
40. Mitra, K., Schaffitzel, C., Shaikh, T., Tama, F., Jenni, S., Brooks, C. L., III, Ban, N., and Frank, J. (2005) *Nature* **438,** 318–324
41. de Gier, J. W. L., Mansournia, P., Valent, Q. A., Phillips, G. J., Luirink, J., and von Heijne, G. (1996) *FEBS Lett.* **399,** 307–309
42. Lee, J.-I., Kuhn, A., and Dalbey, R. E. (1992) *J. Biol. Chem.* **267,** 938–943
43. He, S., and Fox, T. D. (1997) *Mol. Biol. Cell* **8,** 1449–1460
44. Hell, K., Herrmann, J., Pratje, E., Neupert, W., and Stuart, R. A. (1997) *FEBS Lett.* **418,** 367–370
45. Funes, S., Nargang, F. E., Neupert, W., and Herrmann, J. M. (2004) *Mol. Biol. Cell* **15,** 1853–1861
46. Saracco, S. A., and Fox, T. D. (2002) *Mol. Biol. Cell* **13,** 1122–1131