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 F0,F1 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 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 F0,F1-ATPase complex (19). Specifically, the amount of F0c (subunit c of the F0,F1-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 Oxal that mediates membrane insertion of the mitochondrial IMPS Atp9 and Cox2p that are, in turn, homologous to F0c and CyoA. Hence, it has been suggested that YidC plays a pivotal, perhaps exclusive role in the membrane insertion of (domains of) F0c and CyoA (19). Three independent studies indeed demonstrate that YidC, but not the SecYEG complex, is required for membrane insertion of F0c, a double spanning IMP with very short periplasmic tails at the N and C termini (20–22). In contrast to F0c, CyoA is a lipoprotein that is initially synthesized with a signal sequence containing a conserved “lipobox” sequence that includes the signal peptidease II (SPaseII) cleavage site (23). Like F0c, mature CyoA has two closely spaced TMs, but unlike F0c, the second TM is followed by a large translocated domain.
In the present study we have analyzed the targeting and insertion of CyoA using complementary \textit{in vivo} protease mapping experiments and an \textit{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 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 Lumif-LightPLUS 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 Sfasell inhibitor globomycin was from our own stock. Antiserum against YidC, Ffh, Trigger factor, OmpA, CyoA, and SecY have been described previously or were from our own collection (20, 24). Antibodies 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 \textit{in vitro} synthesized mRNA and suppression of UAG stop codons in the presence of (Tmd)Phe-\textit{tRNA}\textsuperscript{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 (20). 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. Isopropyl-\(\beta\)-D-galactopyranoside (1 mM) was added for 1 min to induce CyoA expression. Subsequently, cells were pulse-labeled with [\(^{35}\)S]methionine (30 \(\mu\)Ci/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 \textit{Vivo} Cross-linking—Truncated \textit{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-t\textit{tRNA}\textsuperscript{sup} in an \textit{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. 1A). 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 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, JS7131, 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-\(\beta\)-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 \(\mu\)Ci/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 Sfasell inhibitor globomycin (100 \(\mu\)g/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\textsubscript{660} unit of cells were harvested and resuspended in 1 ml of M9 minimal medium. Isopropyl-1-thio-\(\beta\)-D-galactopyranoside (1 mM) was added for 1 min to induce CyoA expression. Subsequently, cells were pulse-labeled with [\(^{35}\)S]methionine (30 \(\mu\)Ci/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.
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Amino acid by SPases II, and acylated with fatty acid at the NH2 group of the cysteine residue. SPases II 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. 1B, 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 SPases II, 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 IMs 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. 2A, 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. 2B). CyoA targeting was also analyzed in the relatively mild and viable 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.

The SecYEG Translocon and SecE 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 deletion strain CM124 in which the essential secE gene is under control of an...
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 SecY/E 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. 3ug). 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. 38). 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 nor 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. 3C). 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. 3D).

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.

**Translation 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. 4A). The construct contains an HA
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![Diagram A: Membrane assembly of CyoA-HAΔ77–315. A, topology model of CyoA-HAΔ77–315. B, panel A: proteinase K proteolytic fragment indicated by an asterisk; and the mature form is indicated by an arrowhead. For clarity, the lipid-modified precursor is indicated by an arrowhead, and the proteolytic fragment of the precursor is indicated by an asterisk.](Image 72x501 to 276x733)

![Diagram B: Assembly of CyoA-HAΔ77–315 requires YidC but not the Sec translocon. A, proteinase K proteolytic fragment of CyoA-HAΔ77–315 is indicated by an asterisk. B, SecE, proteinase K, OmpA, and YidC are indicated by the arrows. C, the YidC content in the cell sample is determined by immunoblotting.](Image 361x489 to 517x733)

Tag at the C terminus to enable immunodetection preceded by a positively charged stretch of four lysines 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. 4B). 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. 4B). 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 CyoA-HAΔ77–315 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. 5A). Efficient depletion of SecE was confirmed by the reduced processing and translocation of OmpA (Fig. 5A). 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. 5B). 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. 5C). Together, the in vivo experiments indicate that the N terminus of CyoA can insert independently 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/photoscross-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 [35S]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. 6A). 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
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-linking 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 c 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 (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 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 observed.

**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(writer)</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 (lanes 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).
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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.

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 captured 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 procot 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 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.

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