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Review

Biogenesis of inner membrane proteins in *Escherichia coli* ☆

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ABSTRACT

The inner membrane proteome of the model organism *Escherichia coli* is composed of inner membrane proteins, lipoproteins and peripherally attached soluble proteins. Our knowledge of the biogenesis of inner membrane proteins is rapidly increasing. This is in particular true for the early steps of biogenesis — protein targeting to and insertion into the membrane. However, our knowledge of inner membrane protein folding and quality control is still fragmentary. Furthering our knowledge in these areas will bring us closer to understand the biogenesis of individual inner membrane proteins in the context of the biogenesis of the inner membrane proteome of *Escherichia coli* as a whole. This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

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1. Introduction

The proteins that make up the inner membrane proteome of *Escherichia coli* are integral inner membrane proteins (hereafter referred to as IMPs), lipoproteins and peripherally attached soluble proteins. Around a quarter of all genes in the *E. coli* genome encode IMPs and many other genes encode proteins that do not have any transmembrane segments (TMs), but are in some other capacity part of the inner membrane proteome (*e.g.*, [1–3]). Most IMPs appear to participate in (transient) complexes, and co-factors and lipids can also be part of these complexes [3–11]. Complexes in the inner membrane of *E. coli* are involved in key processes such as energy generation and conversion in the respiratory chain, cell division, signal transduction, and transport processes.

The assortment of IMPs is diverse in size and complexity. The number of TMs and the size and nature of periplasmic and cytoplasmic domains of IMPs are highly variable [2,7,9,12]. In this review, we will focus on IMP biogenesis (Fig. 1). We refer to other reviews for more detailed information about the biogenesis of the other constituents of the inner membrane proteome (*e.g.*, [3,13]).

2. Biogenesis of inner membrane proteins

2.1. A role of the ribosome in IMP biogenesis

Recent studies indicate that the ribosome is an unexpectedly dynamic and communicative machine. Cross-linking, Förster resonance energy transfer (FRET) and structural studies indicate that nascent peptides adopt some secondary structure and engage in specific interactions already in the ribosomal exit tunnel (*e.g.*, [14–18]). This information is transduced to the surface of the ribosome and influences the recruitment of chaperones and Sec-translocon components (see Section 2.4.1) near the exit site. *Vice versa*, these external factors may respond to the ribosome and influence translation and folding within the ribosome [19]. For instance, the presence of a signal anchor sequence in the ribosome has been shown to promote the binding of the signal recognition particle (SRP) (see Section 2.2) to the ribosome [20]. Signal anchor sequences form a compacted, presumably helical conformation in the lower part of the exit tunnel for optimal presentation to the SRP near the exit site [21–23]. It has been shown in the mammalian system that contacts of the ribosome with translocon subunits were affected by a signal anchor sequence that was still confined within the ribosome [24]. The timing, nature and extent of the conformational changes are important topics for future research. It should be noted, however, that this intra-ribosomal signaling may not be completely conserved in bacterial and mammalian systems given the heterogeneity of the key proteins at the nascent chain exit site [15].

2.2. SRP-targeting pathway

It is generally assumed that ribosomes synthesizing IMPs are targeted in a co-translational fashion to the inner membrane *via* the SRP-targeting pathway [25,26].

The SRP is a ubiquitous ribonucleoprotein particle, found in all three kingdoms of life [26]. The SRP was first identified in mammalian cells, where it targets both secretory and membrane proteins in a co-translational fashion to the membrane of the endoplasmic reticulum (ER). The *E. coli* SRP is more specialized in the targeting of IMPs whereas

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secretory proteins in *E. coli* are primarily routed *via* the post-translational pathway that involves chaperones such as SecB [26]. Both pathways converge at the Sec-translocon and the choice between the two pathways appears to be determined primarily by the preference of the SRP for particularly hydrophobic targeting signals [27–29].

In contrast to the mammalian SRP that consists of an extensive RNA scaffold and 6 proteins, the *E. coli* SRP is relatively simple. It consists only of one small RNA (4.5S RNA) and the Ffh (Fifty-four homologue) protein, which is homologous to the mammalian SRP54 [25]. Yet, this minimalistic SRP is able to coordinate the delivery of ribosome nascent chain complexes at the Sec-translocon. It does so in conjunction with its receptor, FtsY, that is conveniently located in the vicinity of the Sec-translocon [30].

Both Ffh and part of the 4.5S RNA participate in the formation of a largely hydrophobic groove that can accommodate a hydrophobic targeting sequence in an α-helical conformation [31,32]. In comparison with the mammalian SRP, the *E. coli* SRP lacks subunits that are involved in the translational pausing upon interaction of the ribosome associated SRP with the targeting signal in the nascent peptide. Although somewhat controversial, this would imply that no SRP-imposed arrest exists in *E. coli* and that this feature is not critical for the basics of SRP functioning [33,34].

E. coli SRP docks to the ribosome at the large subunit proteins L23 and L29 near the nascent exit site [14]. The binding site overlaps with that of the abundant general chaperone trigger factor and SecA that appears to have multiple functions in protein folding, targeting and translocation [35–37]. Although the precise orchestration of nascent chain interactions near the exit site remains elusive, it is clear that recruitment of the SRP to the ribosome is crucial to overcome the relatively low affinity of the SRP for the targeting sequence "in solution".

As mentioned above, recruitment of the SRP may be promoted by conformational changes near the exit site in response to the presence of a signal anchor sequence in the exit tunnel. Strikingly, one study suggests that any short nascent peptide (with or without a signal anchor sequence) has this effect [34]. When nascent peptides emerge from the ribosome, the targeting complex with ribosomes exposing a signal anchor sequence is maintained, whereas ribosomes exposing other sequences are released. This postponed sorting mechanism is proposed to take place at the membrane. This is in keeping with a recent study, which indicates that the SRP-targeting pathway rejects incorrect cargos through a series of checkpoints during subsequent steps of targeting [38]. The narrow window of SRP recruitment to short nascent peptides allows efficient use of the limited number of SRPs available (see Section 4). The targeting of translating ribosomes to the membrane even before the emergence of a signal anchor sequence could also explain the apparent absence of an SRP-mediated translational arrest in *E. coli*.

Ribosome nascent chain (RNC)–SRP complexes contact the SRP receptor FtsY at the membrane to mediate transfer of the nascent chain to the Sec-translocon. SRP–FtsY complex formation is accelerated by the 4.5S RNA that transiently binds to FtsY and by the RNCs on which SRP is docked [39–43]. Both SRP (*i.e.*, the Ffh component) and FtsY are GTPases [44]. GTP binding and hydrolysis in both the SRP and FtsY tightly regulate co-translational targeting [45]. The SRP binds, probably in a GTP-free state, to a targeting signal as it emerges from the ribosome [46]. Interaction of the SRP with the ribosome increases the affinity of SRP for GTP [46]. SRP bound to a targeting signal at the ribosome in an activated, GTP-bound form is primed for the interaction with FtsY [47]. Likewise, FtsY may be primed in a GTP-bound state suitable for complex formation with SRP by an interaction with membrane lipids and Sec-translocon components [48–50]. On the other hand, ribonucleotide competition experiments have shown that binding of RNC–SRP to FtsY precedes GTP binding by both Ffh and FtsY [51]. The GTPases of Ffh and FtsY act as GTPase activating proteins for each other [52]. GTP hydrolysis occurs only after the targeting signal

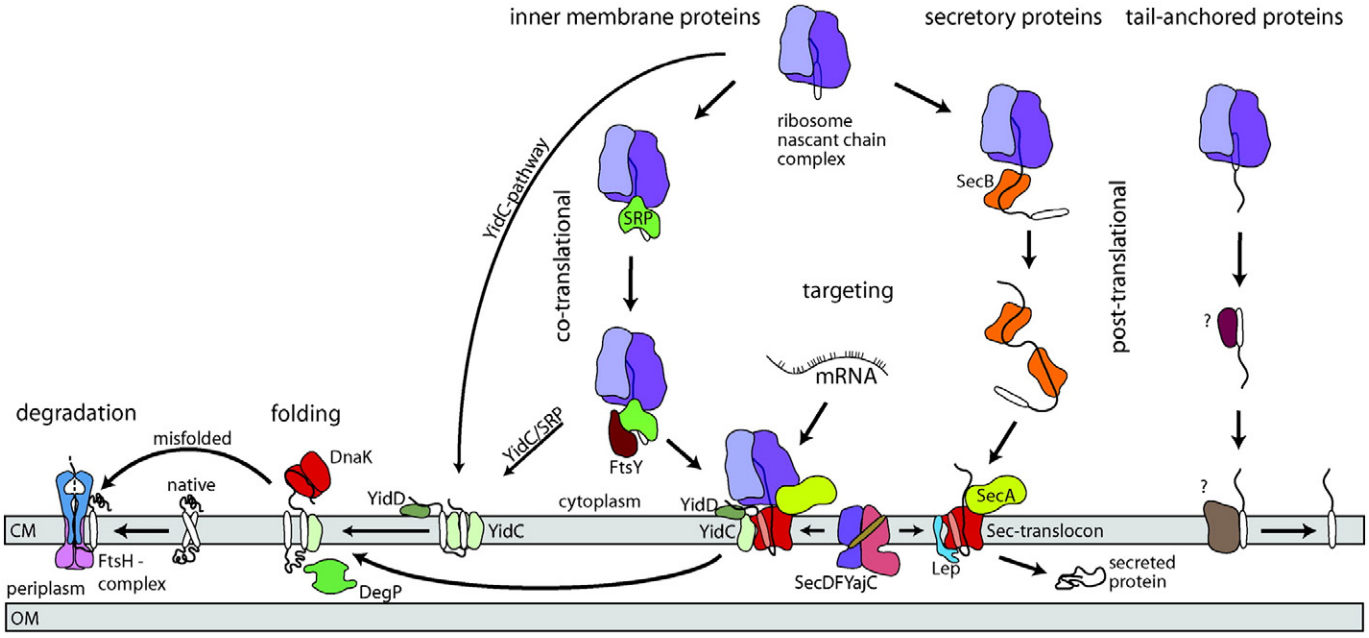

Fig. 1. The biogenesis of inner membrane proteins in *E. coli*. For a full description see the text. Ribosome–inner membrane protein nascent chain complexes (RNCs) are targeted in a cotranslational fashion to the inner membrane *via* the SRP pathway (comprising the signal recognition particle (SRP) and its receptor FtsY). At the inner membrane, the RNC docks at the Sec-translocon — a protein-conducting channel that facilitates both the translocation of hydrophilic polypeptide chains across the membrane and the insertion of transmembrane segments (TMs) into the lipid bilayer. The translocation of sizeable periplasmic loops requires the ATPase SecA. YidC has been proposed to mediate the transfer of TMs from the Sec-translocon into the lipid bilayer and can assist the folding of IMPs. The SecDFYajC complex can play a role in the biogenesis of IMPs as well as the translocation and folding of secreted proteins. Some IMPs are targeted *via* the SRP pathway or directly to YidC. YidD functions in the biogenesis of both YidC and Sec–YidC dependent IMPs. Folding of soluble cytoplasmic domains might be supported by cytoplasmic chaperones such as DnaK, whereas that of periplasmic domains might be supported by periplasmic chaperones such as DegP (which can also act as a protease). The FtsH complex is involved in quality control and degradation of IMPs. mRNAs encoding IMPs localize to the inner membrane in a translation-independent mechanism. Secretory proteins are targeted by the chaperone SecB in a mostly posttranslational manner to the Sec-translocon. The translocation of secretory proteins is SecA dependent. The signal sequence of secretory protein is removed by leaderpeptidase (Lep). Targeting and insertion of tail anchor (TA) proteins into the inner membrane may occur in a post-translational manner *via* the SRP/Sec-translocon system or an alternative pathway.

has been released, which then results in the dissociation of the SRP/FtsY complex [45,51,53].

Recently, it was shown that an SRP/FtsY fusion that is tethered to the inner membrane can support growth of cells devoid of SRP and FtsY arguing that SRP-mediated targeting of RNCs may also take place close to the membrane [54]. This might support the flexibility and efficiency of targeting by increasing the concentration of SRP near the Sec-translocon. In a more provocative model, ribosomes are targeted to the inner membrane *via* nascent FtsY that has affinity for membrane lipids [55]. This would imply that the SRP is recruited at a late stage after the FtsY-mediated targeting step but prior to engagement of the Sec-translocon.

The role of the SRP and FtsY in the targeting of IMPs has been deduced primarily from studies on a few model proteins. Recently, the effects of the depletion of the essential SRP have been determined for the whole inner membrane proteome [56]. SRP depletion in *E. coli* resulted in strongly impaired kinetics of the biogenesis of the inner membrane proteome. This observation is in keeping with an essential role of the SRP in the efficient biogenesis of the *E. coli* inner membrane proteome, but also suggests that it – at least for the biogenesis of most IMPs/IMP complexes – is not required *per se*.

2.3. Alternative IMP targeting pathways

Besides the SRP-targeting pathway other IMP targeting pathways appear to exist in *E. coli*.

Recently, it has been shown that mRNAs encoding IMPs localize to the inner membrane in a translation-independent mechanism [57]. mRNAs encoding IMPs have a significantly higher uracil content than mRNAs encoding cytoplasmic proteins [58], which suggests a correlation between the localization of mRNAs and their uracil content. mRNA targeting is well documented in eukaryotes (*e.g.*, [59]). The large subunit of the ribosome remains associated with the Sec-translocon at the ER but only when programmed with mRNA molecules that encode secretory and membrane proteins.

"Traditional" chaperones may also assist in the targeting of a subset of IMPs. In an *in vitro* study it has been shown that the cytoplasmic chaperone GroEL can mediate post-translational membrane insertion of the IMP lactose permease, LacY [60]. Similarly, bacteriorhodopsin is efficiently delivered to membranes by GroEL [61]. However, in a proteome wide screen no IMPs were identified as GroEL substrates [62].

The *E. coli* genome encodes around a dozen tail anchor (TA) proteins, *i.e.*, IMPs that are anchored to the membrane through a C-terminal TM [63,64]. Also, quite a number of very small (<50 amino acids) IMPs with only one TM are present in *E. coli*[65]. The location of the targeting signal in these IMPs conceptually precludes co-translational membrane insertion *via* the SRP/Sec-translocon pathway. How these proteins are targeted to and inserted into the inner membrane remains to be investigated in more detail.

In eukaryotes, a novel mechanism has been identified to prevent aggregation and promote targeting and insertion of TA proteins into the ER membrane, the GET (guided entry of TA proteins) pathway [66]. This nucleotide-dependent delivery system comprises dedicated chaperones, receptors and membrane proteins, but the molecular details are only recently being elucidated. In addition, evidence for a post-translational role of the SRP/Sec-translocon system in the biogenesis of a subset of TA proteins has been presented which may depend on the hydrophobicity of the targeting signal [67].

2.4. Membrane insertion and folding of IMPs

Nascent IMPs are primarily targeted to the Sec-translocon/YidC insertion site or to YidC that is not connected to the Sec-translocon. The Sec-translocon is a protein-conducting channel homologous to the Sec61–translocon complex in the ER [68]. The IMP YidC has been identified as an indispensable factor that assists in the integration, folding, and assembly of IMPs both in association with the Sec-translocon and as an independent entity (see also Sections 2.4.2–2.4.4).

2.4.1. The Sec-translocon

The core of the Sec-translocon is formed by an evolutionary conserved heterotrimeric complex of IMPs [68]. A major breakthrough in our thinking on Sec-mediated protein translocation and insertion came from the elucidation of the high-resolution crystal structure of the trimeric Sec-translocon complex obtained from the archaeon *Methanococcus jannaschii*[69]. It provides a glimpse on how the dual tasks of vectorial transport of secreted proteins and lateral movement of IMPs are combined in one complex. The ten TMs of SecY form the core of the channel that appears to be in the closed state and is plugged by a small α-helical domain. The TM of the clamp-like protein SecE and the two TMs of SecG flank the SecY channel. In contrast to SecE of *M. jannaschii E. coli* SecE consists of three TMs. However, only the C-terminal TM of *E. coli* SecE is required for a functional Sec-translocon [70]. Viewed from the top the translocon has a clamshell conformation with the two halves of SecY (TM1-5 and TM6-10) hinged at the loop between TM5 and TM6. The opposite (front) side at the intersection of TM2 and TM7 is thought to form a flexible lateral gate to allow the release of TMs during co-translational insertion of an IMP. This intuitive model is supported by biochemical data but also by a recent cryo-electron microscopy (EM) structure of the SecYE complex complexed with a ribosome that is occupied with the nascent IMP FtsQ, which is a bitopic IMP involved in cell division [71,72]. The cryo-EM structure revealed an extra rodlike density near the lateral gate that probably represents the exposed signal anchor sequence of FtsQ.

Viewed from the side the Sec-translocon has an hourglass shape of which the extracellular cavity is filled with the α-helical plug. The narrow constriction in the middle is lined with hydrophobic residues and also contributes to the effective sealing of the pore to prevent leakage of ions in the resting channel. Recent structure based analysis indicates that during both co- and post-translational translocation proper sealing is maintained by the coordinated movement of the constriction, the flexible plug and the translocating peptide itself [73].

It has been proposed that the Sec-translocon 'breathes'; *i.e.*, its lateral gate rapidly alternates between an open and closed state so that the translocating polypeptide can be sampled by the lipid bilayer thereby allowing TMs to move from the translocon into the bilayer [71,74,75]. The aforementioned cryo-EM structure indeed indicates direct protein–lipid interactions to be critical during translocon-mediated membrane insertion. The cryo-EM structure along with molecular dynamics simulations indicates association between lipids and rRNA helix H59 resulting in a disorder in the lipid bilayer in proximity to the lateral gate of the Sec-translocon channel [72]. This in turn may favor membrane insertion of TMs by decreasing the energy barrier for the TM to access the hydrophobic core of the lipid bilayer through the layer of charged head groups. The TMs of a membrane protein must not only be recognized but also correctly oriented before or during insertion into the lipid bilayer. Interestingly, the ribosome–translocon complex can coordinate the topogenesis of signal anchor sequences in the ER [76,77]. The Sec-translocon pore was recently suggested to accommodate structures with an average diameter of at least 22–24 Å, which is much larger than previously assumed [78] but compatible with inversions of signal anchor sequences that can occur in the translocon [77,76].

SecY and SecE are both required for cell viability and together they form the core of the Sec-translocon, whereas the non-essential SecG only facilitates Sec-translocon function [79]. Additional Sec-components are SecA, SecD, SecF and YajC [79]. SecA, which is like SecY and SecE also required for cell viability, is a peripheral membrane ATPase associated with the Sec-translocon that has so far been identified only in bacteria and chloroplasts [36]. Post-translational protein export through the Sec-translocon is energized by the proton motive force and by SecA that pushes a substrate protein across in a series of ATP-dependent insertion and deinsertion steps. SecA is also required for translocation of sizeable

hydrophilic domains of IMPs, *i.e.*, domains with a length of >60 amino acids, across the inner membrane [80,81]. SecA, FtsY and the ribosome use overlapping binding sites on the SecY subunit of the Sec-translocon [82]. It is not understood how SecA and the ribosome operate together during the biogenesis of an IMP with sizeable periplasmic loops.

SecD, SecF, and YajC form an accessory complex that is found associated with the Sec(A)YEG-translocon [83]. SecD and SecF both contain six TMs and a large periplasmic domain, whereas YajC consists of only one TM. SecDFYajC have been implicated in stabilizing the inserted state of SecA and it has been suggested that these proteins play a role in the late stages of protein translocation [84]. Interestingly, recent structural and functional data indicate that the SecDF complex conducts protons and may function as a membrane-integrated chaperone that is powered by the proton motive force, to achieve ATP-independent protein translocation and folding of secreted proteins [85]. Furthermore, an *in vivo* depletion study has provided evidence that the putative chaperone complex SecDFYajC can also play a role in the biogenesis of IMPs [86].

Proteomics studies have shown that YajC is a relatively abundant IMP that not only forms a complex with SecDF [87]. The IMP AcrB, which is part of the AcrB:AcrA:TolC multidrug efflux pump complex [88] was crystalized in complex with YajC [89]. The way YajC clamps around AcrB is reminiscent of how SecE clamps around SecY. Interestingly, YajC expression is significantly upregulated upon YidC depletion [88–90]. Together the data suggest that YajC functions in the assembly or maintenance of the structural integrity of IMP complexes.

The oligomeric state of the Sec-translocon is a matter of ongoing debate [91,92]. Based on the aforementioned *M. jannaschii* translocon structure, it has been proposed that a single SecYEG heterotrimer forms the active translocation channel. A recent biophysical study indicates that the SecYEG monomer is indeed sufficient to constitute a functional translocon [93]. Cryo-EM studies on the Sec61-complex in mammals and yeast, and the *E. coli* Sec-translocon indicate also that it is monomeric when associated with the RNC [72,94]. In an alternative model SecYEG functions as a dimer with one copy of SecY forming the channel [95]. This view is supported by a study in which a SecYEG dimer is shown to be stabilized by antibodies in detergent solution [96]. Furthermore, dimers were also detected using blue-native (BN) polyacrylamide gel electrophoresis (PAGE) [97,98]. It has been suggested that the active channel is formed by two SecYEG heterotrimers arranged with the lateral openings facing one another [99,100]. Another model proposes SecYEG forming a tetramer of heterotrimers [101]. Freeze–fracture analyses indicated that the SecYEG complexes in the membrane are in an equilibrium of monomers, dimers, and tetramers. The formation of dimers and tetramers in the lipid environment was significantly stimulated by the interaction with translocation ligands SecA, preprotein and ATP [102]. The oligomeric state of the active SecYEG complex may also be influenced by the processed substrate [98].

Another debated issue is the oligomeric state of SecA. Most structural studies show SecA as a homo-dimer, although the interface between the dimers varies in these models [103]. The variety of dimerization interfaces could imply that either multiple dimeric conformations are possible, or that some of the observed dimers do not reflect a physiological conformation. For instance, the 4.5 Å crystal structure of the SecA–SecYEG complex from *Thermotoga maritime* shows that one copy of SecA is bound to one copy of the SecY channel and is oriented roughly parallel to the membrane surface [104]. However, it has been argued that the *T. maritime* structure was obtained in detergent and at high salt concentration, both conditions that favor dissociation of the SecA dimer [105]. Structures of the complete Sec-translocon with substrates may end the discussions as to the oligomeric state of SecA in complex with the SecYEG-translocon.

2.4.2. YidC

In 2000, two studies showed that not only the Sec-translocon but also the IMP YidC plays a key role in the biogenesis of IMPs. Samuelson et al. demonstrated that the depletion of YidC in *E. coli* leads to the inhibition of the insertion of both Sec-dependent and independent IMPs. Notably, Sec-independent IMPs were previously thought to insert spontaneously into the inner membrane [106]. Scotti et al. showed that YidC co-purifies with the SecYEG-translocon and can be cross-linked to the signal anchor sequence of nascent Sec-dependent IMPs [107].

The IMP YidC is a member of the conserved YidC/Oxa1/Alb3 family, which comprises evolutionary conserved proteins involved in the biogenesis of membrane proteins in the cytoplasmic membrane of bacteria, the mitochondrial inner membrane, and the thylakoid membrane of chloroplasts [108–110]. Oxa1 was the first member of this family shown to play a role in membrane protein biogenesis in mitochondria [111,112]. Subsequently, homologs of Oxa1 were found in bacteria and chloroplasts: YidC and Alb3, respectively [108–110]. The conservation in this family is probably due to the fact that mitochondria and chloroplasts are derived from bacteria by an endosymbiotic event.

The depletion of YidC in *E. coli* induces a massive phage-shock protein A (PspA) response, which is indicative of dissipation of the proton motive force [113,114]. The dissipation of the proton motive force is probably due to defects in the functional assembly of cytochrome bo_3 oxidase and the F_1F_o-ATPase. The PspA stress response is believed to function in maintaining the energy state of the cell by "patching" the damaged inner membrane [114].

In contrast to Gram-negative bacteria, most Gram-positive bacteria, such as *Bacillus subtilis* and *Streptococcus mutans* have two YidC homologs [109,110]. In *B. subtilis*, the YidC homologs are termed SpoIIIJ and YqjG and deletion of either gene does not result in cell death, but deletion of both is lethal [115]. The expression of either *spoIIJ* or *yqjG* in *E. coli* complements for the defects in membrane insertion due to YidC depletion [116]. In addition, both SpoIIIJ and YqjG are found associated with the F_1F_o-ATPase complex suggesting that they have a role late in the biogenesis of membrane protein complexes [116]. Although the two homologs appear to be functionally exchangeable in membrane protein insertion and complex assembly, only SpoIIIJ is essential for spore formation in *B. subtilis* and cannot be complemented by YqjG for that function [117].

S. mutans also has two YidC homologs: YidC1 and YidC2 [118]. Attempts to simultaneously delete YidC1 and YidC2 failed, indicating that the presence of at least one copy of YidC is required for cell survival. Elimination of YidC2, but not YidC1, resulted in acid sensitivity, decreased levels of membrane-associated F_1F_o-ATPase activity and an inability to initiate growth at low pH or high salt concentrations [118]. Both *S. mutans* YidC1 and YidC2 complement defects of YidC-deficiency in *E. coli* and can mediate insertion of both Sec-dependent and Sec-independent ("YidC-only") substrates. However, the effects of introducing YidC1 or YidC2 into YidC-depleted *E. coli* are not identical, suggesting that the proteins are functionally distinct [119]. YidC2 deletion leads to a similar phenotype as the deletion of components of the SRP-targeting pathway, while double mutants of YidC2 and SRP resulted in a strong growth defect. This synthetic effect suggests that YidC2 can compensate for the absence of the SRP-targeting pathway, and that YidC2 overlaps in function with the SRP-targeting pathway in co-translational protein insertion in *S. mutans*. Interestingly, YidC2 and Oxa1 can be exchanged between *S. mutans* and yeast cells and partially complement each other. Like for Oxa1, the positively charged C-terminal tail of YidC2 binds to ribosomes to mediate co-translational translocation upon expression in yeast mitochondria [120]. This suggests that direct targeting of ribosomes to YidC2 may obviate the absolute need for SRP in *S. mutans*.

All YidC family members, whether in mitochondria, chloroplasts, or bacteria, with the exception of the one YidC-like protein in archaea, have a conserved domain of five TMs [109]. In addition, the Gram-negative *E. coli* YidC protein has an extra TM and a large periplasmic domain separating the first two TMs [121]. At the C-terminus, some YidC homologs in bacteria have an extended C-terminal tail that aids in positioning the ribosome to the cytoplasmic membrane. The structure–function relationships of *E. coli* YidC have been probed in

mutagenesis studies to define the regions of YidC that are critical for function. The N-terminal TM serves merely as targeting sequence and can be replaced by a cleavable signal sequence from maltose binding protein (MBP) [122]. In contrast, the five C-terminal TMs are essential for YidC activity. Systematic mutagenesis of residues in TM2, TM3, and TM6 as well as swapping TM4 and TM5 with unrelated TMs proved to have little effect on YidC activity [122]. Apparently, YidC is rather tolerant toward point mutations, which is more consistent with a role as an insertion platform than as an active insertase. The TMs of YidC may function as a platform to bind hydrophobic segments of the substrates as TMs insert into the membrane. Whether the transfer of TMs from YidC into the lipid bilayer occurs *en bloc,* as shown for mannitol permease (MtlA, six TMs), or sequentially as shown for leaderpeptidase (Lep, two TMs), may depend on the particular substrate (see below) [123,125].

Surprisingly, most of the large periplasmic domain P1 is not essential for the function of YidC [122]. 92% of the periplasmic domain can be deleted without impairing YidC function. Only deletion of the C-terminal region of the periplasmic domain impairs cell viability and membrane insertion of a number of Sec-dependent and Sec-independent substrates [125]. The deletion may cause a structural perturbation, as scanning mutagenesis in this region does not identify residues vital for YidC membrane insertion function [124]. Crystal structures of the periplasmic domain have been presented [126,127]. They show a twisted β-sandwich with an α-helical linker, which orients the sandwich near the core TM domains. A portion of this periplasmic domain mediates an interaction with SecF, although this feature is not essential for IMP biogenesis or cell viability [125]. So far, besides binding to SecF, the function of this large periplasmic domain remains unknown.

The C-terminal tail of YidC in *E. coli* is not critical for *in vivo* YidC activity [122]. However, in a co-sedimentation study, YidC was found to bind ribosomes *via* its C-terminal tail through electrostatic interactions [128]. Deletion of the positively charged C-terminal tail (13 residues long) resulted in loss of ribosome binding. Similar data were previously obtained for the C-terminal tail of Oxa1 [129,130]. Why deletion of the C-terminus of YidC has so little effect *in vivo* remains to be determined.

Several studies indicate that YidC functions as a dimer. Purified YidC migrates as a monomer and dimer in native gels [131]. Furthermore, YidC runs as a dimer in BN-PAGE upon solubilization of inner membranes [4,88,132,133]. A cryo-EM 10 Å projection map of YidC also suggests that it forms dimers in the membrane [134]. Dimers of YidC and Oxa1 form *Saccharomyces cerevisiae* were localized at the exit site of a ribosome stalled in the translation of a YidC substrate [128]. The attachment sites on the ribosome appeared conserved between YidC and Oxa1. Helix 59 of the 23S rRNA is a contact point, as well as the L23/L29 region [128]. YidC has an additional strong anchor point at L24, whereas Oxa1 shows only a weak connection in this area. These are the same contact sites as used by the Sec-translocon [95,99]. In the crystal structure, the large periplasmic domain of YidC is monomeric, suggesting that YidC dimerization determinants reside in the TM region of the protein [126,127]. Taken together, it is clear that YidC has a tendency to dimerize. However, it is currently unknown what the oligomeric state of YidC is when it mediates the biogenesis of an IMP either by itself or in conjunction with the Sec-translocon.

Cross-linking experiments have also revealed interactions between nascent membrane proteins and FtsH, and between FtsH, YidC and HflC/K [135]. FtsH functions as an inner membrane chaperone/protease and HflC and HflK modulate its activity and also may have chaperone activity [136]. These observations imply a linked role for these proteins in the quality control upon insertion of IMPs.

2.4.3. Sec/YidC-pathway

For proper insertion of some IMPs, both the Sec-translocon and YidC are strictly required. The best studied representatives of this pathway are CyoA, subunit II of cytochrome bo_3 oxidase and subunit a of the F_1F_o-ATPase [137–139]. CyoA is a lipoprotein that is synthesized as a precursor, which is processed by signal peptidase II following insertion [140]. Mature CyoA consists of two TMs connected by a cytoplasmic loop and two translocated periplasmic termini: a lipid-modified N-terminus and a large C-terminal domain. After targeting to the membrane *via* the SRP-targeting pathway, insertion takes place in two distinct stages. First, YidC is required and sufficient for membrane insertion of the N-terminal part of the protein consisting of the cleavable signal sequence, short periplasmic loop, and the first TM. Second, translocation of the second TM and the large C-terminal periplasmic domain is mediated by the Sec-translocon. CyoA inserts in a strictly sequential mechanism requiring the insertion of the N-terminal domain prior to the insertion of the C-terminal domain. In this case, the Sec-translocon acts downstream of YidC. In contrast, when the cytoplasmic loop between the two TMs is lengthened considerably, the C-terminal TM inserts *via* the Sec-translocon independently of the YidC-dependent N-terminal domain insertion [141].

The F_o part of the *E. coli* F_1F_o-ATPase consists of three integral membrane protein subunits with a stoichiometry of $a_1b_2c_{10}$[142]. Subunit a of the membrane integral F_o complex consists of five TMs without any large cytoplasmic or periplasmic domains. F_oa is another example of a substrate whose insertion is strictly dependent on both YidC and the Sec-translocon [143–145]. First, F_oa is targeted to the inner membrane *via* the SRP-targeting pathway. Then, the first TM is inserted into the membrane by the Sec-translocon independent of YidC, whereas insertion of the whole protein requires both the Sec-translocon and YidC. This protein serves as an example in which YidC acts downstream of the Sec-translocon, but both components are essential for proper membrane insertion.

E. coli NADH dehydrogenase I consists of 13 subunits, NuoA through to NuoN with NuoC and NuoD fused to form one protein. If any of these subunits is absent, a functional enzyme complex cannot be formed [146]. Using an *in vitro* proteoliposome based insertion assay, it has been shown that NuoK, which contains three TMs, requires both YidC and the SecYEG-translocon for insertion [147]. Specifically, the presence of the negative charges in TM2 and TM3, Glu36 and Glu72, determines the YidC-dependent insertion. Substitution of the glutamates at these positions produced a protein that, like most studied IMPs, requires only the SecYEG-translocon for integration into the membrane. NuoK is one of many integral membrane respiratory proteins that contain membrane-negative charges. NuoA has a structure similar to NuoK with glutamates at positions 81 and 102 (TM2 and 3) and an aspartate residue at position 79 (TM2). It would be of interest if these similar structural features necessitate YidC in the insertion process.

For the substrates discussed above, CyoA, F_oa, and NuoK both YidC and the Sec-translocon are strictly required. In the next section, the second role of YidC in conjunction with the Sec-translocon is discussed. Here, YidC is not essential to obtain sufficient levels of these IMPs in the inner membrane, yet it was found in close proximity to the TMs of the nascent substrate proteins.

Photo-cross-linking studies have shown that nascent chains of FtsQ insert into the membrane close to SecY and lipids and move to a combined YidC/lipid environment upon peptide elongation. Here, YidC probably aids in the lateral transfer of the TM of FtsQ from the translocase into the lipid bilayer [131,148]. Lep is another example of a Sec-dependent protein which could be cross-linked *in vitro* to both YidC and the Sec-translocon during insertion into the inner membrane [123,131,149,150]. A detailed view emerges from these cross-linking studies using nascent Lep: TM1 in short nascent Lep contacts both SecY and YidC depending on the position of the cross-linking probe in the TM. Longer nascent chains have more contact with YidC, whereas in even longer nascent Lep, TM1 moves away from the Sec/YidC complex to become lipid embedded. Further translation results in exposure of TM2, which contacts first SecY, then shifts to YidC to eventually move into the lipid phase. These observations are consistent with a linear insertion mechanism of TMs into the membrane. This linear insertion model has been challenged by a study that used MtlA as model for TM insertion [124]. The observation that the first

three TMs of MtlA were simultaneously cross-linked to YidC resulted in a model in which YidC assists in the assembly of TMs prior to their concerted release *en bloc* into the lipid phase. It remains unclear whether these observed differences in the contacts of TMs with YidC are due to the different model proteins (two TMs in Lep *versus* six TMs in MltA) or to differences in the experimental set-up.

Irrespective of the exact TM release model, the tested Sec-dependent IMPs FtsQ and Lep interact with YidC *in vitro* in cross-linking experiments, but their membrane insertion *in vivo* is only mildly affected by depletion of YidC (*e.g.*, [106,148,82]). The role of YidC for the insertion of these Sec-dependent IMPs has not been fully defined. The next section deals with the third way in which YidC plays a role in membrane biogenesis of Sec-dependent IMPs, *i.e.*, in (late) folding and complex formation.

YidC is not required for insertion of the twelve TMs of LacY, whereas the Sec-translocon is essential [151]. However, by using an *in vitro* system and two monoclonal antibodies directed against conformational epitopes, it was shown that LacY is only immuno-precipitated in the presence of YidC. Moreover, LacY only folds properly in proteoliposomes containing also YidC [151]. These results indicate that YidC plays a key role in folding of LacY into its final tertiary conformation *via* an interaction that likely occurs transiently during insertion into the lipid phase of the membrane. On a similar note, YidC acts in the biogenesis of MalF, which functions in maltose transport as a complex with the integral IMP MalG and the peripheral IMP MalK. MalF consists of eight TMs with a large periplasmic domain between TM3 and the TM4 [152]. YidC is not required for the insertion of MalF into the membrane whereas it is essential for the stability of MalF and the formation of the $MalFGK_2$ maltose transport complex [153]. Probably, YidC supports the folding of MalF into a stable conformation before it is incorporated into the maltose transport complex.

In summary, the data discussed above indicate that YidC can act both downstream and upstream of the Sec-translocon. For a subset of proteins, both YidC and the Sec-translocon are strictly required for insertion. For other IMPs, YidC seems to be close and may facilitate lipid partitioning and assembly of TMs rather than being absolutely critical. For yet another subset of IMPs, YidC seems essential for assembly of TMs to trigger proper folding and oligomerization of IMPs.

2.4.4. YidC only pathway

YidC can also act as an insertase, independent of the Sec-translocon. YidC is essential for the biogenesis of Sec-independent IMPs, such as the Pf3 and M13 phage coat proteins [106]. Based on *in vitro* experiments using liposomes these IMPs were previously thought to require only the proton motive force for insertion. However, depletion of YidC results in complete inhibition of the insertion of the Pf3 and M13 phage coat proteins, the first "YidC-only" substrates that were identified [106]. Pf3 coat protein (44 residues) has one TM with a translocated N-terminus, while M13 procoat protein (79 residues) has one TM and a cleavable signal sequence. Additional evidence that YidC plays a role in membrane insertion of these small IMPs is provided by the demonstration that YidC physically interacts with the TM of Pf3 coat protein during insertion and that proteoliposomes that contain only YidC are sufficient to insert the Pf3 coat protein [154,155]. Recent evidence suggests that the *in vitro* insertion of M13 coat protein into diacylglycerol (DAG)-containing liposomes is strictly dependent on YidC [156]. Even though DAG occurs in the inner membrane of *E. coli*, only liposomes without any DAG had been used for studying the biogenesis of M13 procoat protein so far. This explains why its insertion into the inner membrane was considered to be YidC independent for a long time.

Subunit c of the F_1F_o-ATPase was the first identified endogenous substrate that solely depends on YidC for insertion into the inner membrane. Similar to the phage coat proteins M13 and Pf3, F_oc is small and hydrophobic, consisting of two TMs, with small translocated N- and C-termini. F_oc forms an oligomeric ring of 10 subunits in the inner membrane. The first indication that F_oc is a YidC substrate came from the study in which YidC depletion led to decreased amounts of F_oc in purified inner membranes [113]. Additional *in vitro* and *in vivo* data unambiguously showed that YidC alone is essential and sufficient to insert F_oc into the inner membrane [144,157,158]. In mitochondria, Oxa1 forms a stable complex with the F_oc oligomers, and Oxa1 deficient mitochondria have reduced levels of F_1F_o-ATPase activity [159]. Similarly, in *B. subtilis* the YidC homologs SpoIIIJ and YqjG are co-purified with the F_1F_o-ATPase complex, suggesting that YidC is in contact with F_oc at late stage the biogenesis of the F_1F_o-ATPase complex [116]. These data together suggest that YidC, in addition to its role as the insertase for F_oc, may also play a part in assembling subunit c into the oligomeric complex.

Thus far, the mechanosensitive channel with large conductance (MscL) has been described as the second endogenous YidC substrate [160], although conflicting data on the role of YidC have been reported. The MscL protein plays a role in protecting cells exposed to osmotic shock [161]. The crystal structure of the MscL homolog from *Mycobacterium tuberculosis* shows a homopentameric channel [162]. Each MscL subunit spans the inner membrane twice and has a small periplasmic domain between the two TMs. MscL is inserted into the membrane independently of the Sec-translocon, whereas YidC appeared essential for efficient membrane insertion [160]. In contrast, Pop et al. reported that inner membrane vesicles (IMVs) produced from YidC depleted cells had the same level of MscL protein as IMVs from wild type cells, indicating that insertion was not impaired in these cells [163]. However, in the absence of YidC assembly of the homopentameric MscL complex was strongly reduced as shown by BN-PAGE. These results indicate a late role for YidC in formation of an oligomeric complex than rather involvement in insertion [164]. A third study on MscL biogenesis reported that MscL, produced in a cell-free system, is able to insert directly into a pure lipid bilayer in an active conformation as judged by patch-clamp experiments [165]. Also its subsequent oligomerization into homopentamers is reported to be a spontaneous event and results in active channels [166]. These findings do, however, not exclude that *in vivo* chaperones such as YidC may be needed to make the process of insertion and complex assembly more efficient. Indeed, MscL inserts more efficiently into proteoliposomes containing YidC.

The number of IMPs identified to require YidC for their biogenesis remains limited. Thus far, the substrates identified to require only YidC for insertion include the M13 and Pf3 phage coat proteins, subunit c of F_1F_o-ATPase, and possibly MscL. In addition, a subset of Sec-dependent proteins requires YidC for proper insertion. These substrates include CyoA, NuoK, and F_oa. A third set of substrates needs YidC for efficient folding and/or complex formation. These include MalF and LacY and probably MscL. It is currently not clear which features determine if an IMP is directed to the YidC-only pathway *versus* the Sec or Sec/YidC pathway.

2.4.5. Intrinsic biogenetic information of IMPs

Interestingly, the TMs of single-spanning membrane proteins are more hydrophobic than TMs of multi-spanning membrane proteins [75]. This suggests that a relatively large fraction of the TMs in multi-spanning membrane proteins depends on interactions with neighboring TMs for proper partitioning into the membrane. Indeed, several such cases have been described [167].

It has been known for a long time that positively charged residues flanking TMs are important topological determinants [12]. Recently, it was shown that a single positively charged residue placed in different locations throughout the protein, including the very C-terminus can control the final topology of an IMP [168]. This observation points to an unanticipated plasticity in IMP insertion/folding mechanisms. It is not known if any involvement of chaperones is required for this plasticity.

If a membrane protein has more than one TM, the TMs can interact during or after membrane insertion is completed, thereby forming a helical bundle. Helix–helix interactions are modulated by the sequence context and by lipid bilayer properties [10,169]. It is generally assumed that after helix packing the extramembranous domains are folded. Interestingly, it has been shown recently that integrin TM complex formation depends on the conformational state of soluble domains [170]. Thus, it appears that folding of soluble domains can also mediate the organization of TMs and thereby folding of a membrane protein as a whole and its incorporation into complexes.

2.4.6. Factors assisting IMP folding

In addition to YidC, SecDF and YajC, SecY has been implicated in the folding of IMPs (see Sections 2.4.1–2.4.4). More specifically, SecY mutants have been isolated that impair IMP folding [171]. These mutants induce, just like the depletion of YidC, cell envelope stress responses. Thus, SecY is not merely part of a protein-conducting channel but also involved in assisting IMP folding. In the ER, it has been shown that TMs remain temporarily and specifically associated with Sec-components after their release from the protein channel component of the Sec-translocon, and in *E. coli* TMs remain associated with the auxiliary Sec-translocon component YidC (see Sections 2.4.2–2.4.3). This sequential triage appears to be required for the proper folding of a polytopic membrane protein as a whole. Cytoplasmic chaperones and periplasmic chaperones may also be involved in mediating folding of extra membraneous loops/domains either before, during or after helix bundle formation. Finally, other partners in an oligomeric setting may mediate folding and affect stability.

It has been shown that the biogenesis of the cytochrome cbb_3 oxydase from *Rhodobacter capsulatus* requires specialized chaperones [172,173]. So far, such specialized chaperones involved in the biogenesis of IMPs/IMP complexes have not yet been identified in *E. coli*.

The orientation of TMs (see Section 2.4.5) also depends on the phospholipid composition of the membrane during initial assembly as well as on changes in lipid composition after assembly [11]. The membrane translocation potential of negative amino acids working opposite to the positive-inside rule is largely dampened by the presence of phosphatidylethanolamine (PE), thus explaining the dominance of positive residues as retention signals. PE provides the appropriate charge density that permits the membrane surface to maintain a charge balance between membrane translocation and retention signals and also allows for the presence of negative residues in the cytoplasmic face of proteins for other purposes. The lipid composition of a membrane can also affect other aspects of membrane protein folding like helix-packing and as a consequence the activity of a membrane protein [10].

The presence/absence of a substrate can affect the folding and stability of an IMP as has recently been shown for the *E. coli* uracil transporter UraA [174]. In the presence of uracil UraA is stable whereas in the absence of uracil it is rapidly degraded. Thus, a substrate can conceptually function as a folding modulator. In addition, for cytochrome bo_3 it has been shown that the binding of a co-factor (heme *b*) is necessary for folding and complex formation [175].

2.4.7. Involvement of YidD in the biogenesis of IMPs

A very recent study suggested a novel factor, YidD, to function in the biogenesis of IMPs [176]. The gene encoding YidD is located upstream of *yidC* in a gene cluster that is highly conserved in Gram-negative bacteria, the gene order being *rpmH*, *rnpA*, *yidD*, *yidC*, and *trmE*. In *E. coli yidD* overlaps with *rnpA* and has only 2 bp spacing to *yidC*. YidD is expressed in *E. coli* under standard laboratory conditions and it localizes to the inner membrane probably through an amphipathic helix. Inactivation of *yidD* does not affect cell growth and viability. However, compared to control cells, *ΔyidD* cells were affected in the insertion and processing of three YidC-dependent IMPs, *i.e.*, CyoA, Foc and the SRP/YidC dependent artificial IMP M13P2. Furthermore, *in vitro* crosslinking showed that YidD is in proximity of nascent FtsQ during its localization in the Sec–YidC translocon, suggesting that YidD is involved in the insertion process.

2.5. Degradation of IMPs

In the inner membrane, the recognition and disposal of malfolded, misassembled or damaged IMPs are performed by quality control factors/proteases.

The best known and characterized protease in the inner membrane of *E. coli* is probably FtsH [136,177]. It consists of two N-terminally located TMs and a main cytosolic region consisting of AAA-ATPase and Zn^{2+}-metalloprotease domains and forms a homo-hexamer, which is further complexed with an oligomer of the membrane-bound modulating factor HflKC. FtsH is known to degrade *e.g.*, subunit a of the ATP synthase, SecY when it is overexpressed in the absence of interacting subunits as well as some cytoplasmic proteins. FtsH uses ATP hydrolysis to dislocate the IMPs in a processive manner from the membrane for further degradation. To initiate degradation of an IMP, FtsH requires a hydrophilic tail of 20 residues or longer. In mitochondria, the AAA proteases that are homologous to FtsH play important roles in the quality control and degradation of nonassembled subunits from the mitochondrial inner membrane [178]. Recently, it was shown that FtsH degrades SecY when the Sec-translocon is jammed by a LamB–LacZ fusion protein leading to cell death [179]. The IMP YccA inhibits the function of FtsH and regulates its activity, and it can counteract the suicidal activity of destructing jammed Sec-translocons [179]. The aforementioned protein YidC has also been found in a large complex with FtsH [135]. Possibly, YidC not only assists the biogenesis of IMPs but also selects (malfolded) IMPs and delivers them to FtsH [135].

Besides FtsH there are other proteases in the inner membrane, such as the IMPs HtpX, YaeL and GlpG, which play a role in quality control of IMPs [180–185]. The metalloprotease HtpX has a function that overlaps with FtsH and degrades malfolded mutant IMPs as well as some cytoplasmic proteins. YaeL is the homolog of the eukaryotic S2P protease and can catalyze intramembrane proteolysis. GlpG is a protease with its catalytic groups within the membrane and was shown to degrade some artificial IMPs. The exact role these proteases play in inner membrane proteome homeostasis needs further study.

When the targeting of IMPs to the inner membrane is impaired by the depletion of SRP, the heat shock response is induced and the cytoplasmic proteases Lon and ClpQ become essential for maintaining viability [186]. These proteases appear to play an important role in the degradation of mislocalized IMPs and proteins that aggregate as a consequence of the presence of mistargeted (hydrophobic) IMPs in the cytoplasm [90,186].

Upon YidC depletion, the CpxA–CpxR envelope stress response system is induced [172]. When IMP folding is impaired in the aforementioned SecY mutants (Section 2.4.6) and under YidC depletion conditions, both the Cpx and the alternative sigma factor σ^E envelope stress response systems are activated, inducing the expression of some proteases that are probably involved in clearing the cell of malfolded IMPs [171].

3. Assembly of inner membrane protein complexes

Almost all IMPs are part of complexes. Cytochrome bo_3, which is the major respiratory oxidase located in the cytoplasmic membrane of *E. coli* when grown under high oxygen tension, and the maltose transporter complex are two of the few examples of *E. coli* inner membrane protein complexes of which the assembly has been studied in some detail.

Cytochrome bo_3 consists of four membrane subunits [187]. Subunit 1 (CyoB) spans the membrane 15 times and contains the heme *b*, heme o_3, and the copper B site. Subunit 2 (CyoA) spans the membrane with two TMs and has a large domain protruding into the periplasm. The

requirements for biogenesis of CyoA have been studied extensively (see Section 2.4.3). Subunits 3 (CyoC) and 4 (CyoD) have five and three TMs, respectively. Using a combination of BN-PAGE and 2D BN/SDS-PAGE the biogenesis of cytochrome bo_3 has been studied in great detail [175] (Fig. 2). The operon encoding the four subunits was expressed from a T7 promoter based plasmid. This allowed for the radiolabeling of the subunits using the rifampicin-blocking technique in which the synthesis of chromosomally encoded proteins is suppressed. In BN-gels the complex subunits 3–4, subunits 3–4–1, and subunits 3–4–1–2 could be detected suggesting an ordered assembly of the complex. Furthermore, it was shown that blocking the binding of the cofactor heme *b* to subunit 1 disturbs the formation of the oxidase complex because mutations of the histidines that coordinate the heme *b* to subunit 1 led to the accumulation of subunit 1 and some of the subcomplexes. Taken together, the biogenesis of cytochrome bo_3 seems to be an ordered process and binding of cofactors is critical for the formation of the complex.

The maltose transporter complex contains the IMPs MalF and MalG as well as a dimer of the ATP-binding cassette MalK in the cytoplasm [152]. The complex transports maltose from the periplasm across the inner membrane. MalF and MalG contain eight and six TMs, respectively. The IMPs MalF and MalG interact directly and form a complex with the ATPase $MalK_2$ forming the maltose transporter complex, which can interact with the periplasmic maltose-binding protein (MBP). Recently, the structure of the maltose transporter ($MalK_2MalFMalG$) with MBP bound has been solved [152]. The biogenesis of the maltose transporter complex has been studied extensively using different approaches. Based on *in vitro* studies using isolated subunits it has been proposed that subunits within the complex can assemble randomly [188,189]. MalK can form a dimer in the absence of MalF or MalG. In experiments studying only two of the three protein components at a time, all combinations (K_2, FG, FK_2 and GK_2) of the subunits can be stably isolated, whereas expression of F, K, and G gives rise to the tetrameric complex FGK_2. Thus, based upon *in vitro* studies using isolated subunits assembly of the maltose transporter complex seems to occur in a rather disordered, random fashion. The biogenesis of MalF has also been studied in quite some detail using a combination of *in vitro* cross-linking and *in vivo* approaches [153]. MalF requires the SecAYEG-translocon; the depletion of SecE led to a block in the insertion of MalF across the membrane and the addition of sodium azide, an inhibitor of SecA, negatively affected the membrane insertion of MalF. As mentioned above, depletion of YidC did not affect the insertion of MalF, but prevented the folding of the protein. Upon YidC depletion MalF was degraded and the amounts of the $MalFGK_2$ complex were reduced in YidC-depleted cells. These observations suggest that the biogenesis of the maltose transport complex may not be as disordered as the *in vitro* studies using isolated subunits indicated.

The characterization of the composition and dynamic organization of the inner membrane proteome of *E. coli* will make it possible to study the assembly of more inner membrane protein complexes (see Section 4). This is needed to better understand the rules of engagement during their assembly.

4. Toward a comprehensive description of the biogenesis of the inner membrane proteome of *E. coli*

The ultimate goal is to understand the biogenesis of the inner membrane proteome as a system. It is commonplace knowledge in biology that the whole is greater than the sum of its parts and this holds also true for the biogenesis of membrane proteomes. The IMP-biogenetic components do not function in isolation but rather influence each other in synergistic and complementary ways. Further, the dynamics of the biogenesis, quality control, and turn-over of the membrane proteome adjust in response to environmental stimuli and changing needs of the bacterial cell. In the same way, the components of membrane proteomes do not work in isolation but are organized in functional (super-)complexes depending on the needs. The dynamic assembly and de-assembly of these complexes needs to be precisely coordinated and changes over time and space. At present, we are still far from grasping these dynamics of the biogenesis of the membrane proteome.

How do we move toward a comprehensive description of the biogenesis of the inner membrane proteome of *E. coli*? Firstly, we need to get a better picture of the composition and dynamic organization of the inner membrane proteome. Non-gel based approaches have successfully been used for the large scale and relative quantitative analysis of the *E. coli* inner membrane proteome [3,190]. Also 1D and 2D iso-electrofocussing (IEF)/SDS-PAGE combined with mass spectrometry have been used to study the constituents of the *E. coli* inner membrane proteome and their abundance [3,190]. Unfortunately, 2D IEF/SDS-PAGE is not compatible with most proteins containing TMs [191,192]. The 2D BN/SDS-PAGE technique is very suitable for studying the organization of the constituents of a membrane proteome in complexes [97]. For the 1st dimension of 2D BN/SDS-PAGE, membrane protein complexes are separated according to size in a gradient gel. In the 2nd dimension, the subunits of the separated complexes are resolved by SDS-PAGE. 2D BN/SDS-PAGE has successfully been used to characterize the inner membrane proteome of *E. coli*[4,5,8,87,193,194]. Techniques like sucrose gradient centrifugation, chemical cross-linking, X-ray crystallography, electron microscopy, fluorescence microscopy and mass spectrometry have also been used to study the composition and organization of the *E. coli* inner membrane proteome although on a limited scale [7,9,135,197–198].

Secondly, further factors playing a role in IMP biogenesis have to be identified and characterized. It is highly likely that we are still missing substantial quality control factors and chaperones involved, as many IMPs have no assigned function yet. For example, recently it could be shown that the so far uncharacterized protein YidD plays a role in the biogenesis of IMPs (Section 2.4.7).

Thirdly, the biogenesis of the inner membrane proteome of *E. coli* has to be described in a more quantitative manner. In a 'normal' *E. coli* cell, there are around 20,000–30,000 ribosomes, 40 SRP, 10,000 FtsY, 500 SecY/E/G, 2000 SecA, 30–40 SecD/F, and 2500 YidC molecules [199]. A recent study nicely explains the at first sight maybe low number of SRP molecules: the narrow window for the recruitment of the SRP to short nascent peptides allows efficient use and quick recycling of the limited number of SRPs available (see Section 2.2). However, further investigation is needed to link the quantities and the localization of the components involved in the biogenesis of the inner membrane, and the dynamic organization of the inner membrane proteome to enable a quantitative description of its biogenesis. Silhavy and co-workers have pioneered describing the biogenesis of a membrane protein in such a way using the outer membrane protein LamB [200].

Thus far, the use of global approaches to study the biogenesis of the inner membrane proteome of *E. coli* has been explored on a very limited scale. Various proteomic techniques have been used to study the effects of SecE, SRP and YidC depletion on the biogenesis of the *E. coli* inner membrane proteome [56,201–204]. These studies have led to the identification of many potential SecE, SRP and YidC substrates and insights in the role of these components for protein homeostasis in the bacterial envelope. However, even these studies were merely providing snap-shots of the biogenesis of the inner membrane proteome. Further advances in proteomics, microscopy and bioinformatics will push the comprehensive understanding of the dynamics of the biogenesis of the inner membrane proteome through generating and integrating quantitative information of its components in time and space.

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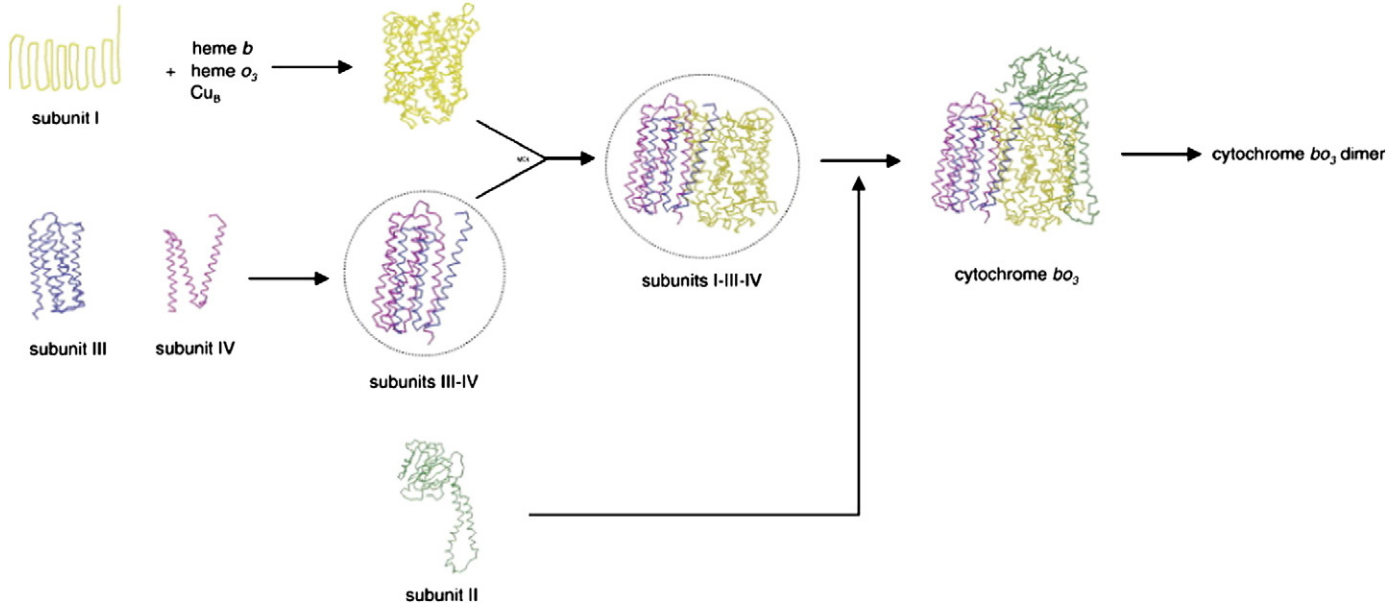

Fig. 2. Model of the assembly of cytochrome bo_3 following insertion of subunits into the membrane. Assembly intermediates are circled. See Section 3 for an explanation. Taken from reference [177] with permission.

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