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Mechanisms of integral membrane protein insertion and folding

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Abstract

The biogenesis, folding, and structure of α-helical membrane proteins (MPs) are important to understand because they underlie virtually all physiological processes in cells including key metabolic pathways, such as the respiratory chain and the photosystems, and the transport of solutes and signals across membranes. Nearly all MPs require translocons—often referred to as protein-conducting channels—for proper insertion into their target membrane. Remarkable progress toward understanding the structure and functioning of translocons has been made during the past decade. Here we review and assess this progress critically. All available evidence indicates that MPs are equilibrium structures that achieve their final structural states by folding along thermodynamically controlled pathways. The main challenge for cells is the targeting and membrane insertion of highly hydrophobic amino acid sequences. Targeting and insertion are managed in cells principally by interactions between ribosomes and membrane-embedded translocons. Our review examines the biophysical and biological boundaries of membrane protein insertion and the folding of polytopic membrane proteins *in vivo*. A theme of the review is the under-appreciated role of basic thermodynamic principles in MP folding and assembly. Thermodynamics not only dictates the final folded structure, it is the driving force for the evolution of the ribosome-translocon system of assembly. We conclude the review with a perspective suggesting a new view of translocon-guided MP insertion.

Keywords

membrane protein folding; membrane protein biogenesis; transmembrane helix; lipid-protein interactions

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Introduction

Membrane proteins occur in all living cells and form part of key metabolic pathways, such as the respiratory chain and the photosystems. Membrane proteins also mediate the transport of solutes and the transduction of signals across membranes. For proper insertion into their target membrane, nearly all membrane proteins require the aid of translocons, so-called protein-conducting channel proteins. In the past decade, structures of key translocon complexes that mediate the translocation or insertion of membrane proteins have been solved and biochemical studies have yielded important insights into key aspects of membrane protein insertion and folding. The cartoon of Fig. 1 illustrates a general scheme for the assembly of multi-span membrane proteins. We will add many details to this scheme in the course of this review, but many questions will still remain about the membrane insertion of proteins, how they fold into their final tertiary structure, and how they assemble into homo- and hetero-oligomeric complexes.

Our aim in this review is not to provide a comprehensive overview of the literature, but rather to appraise critically current models of membrane protein insertion and folding *in vivo* and to identify the most important gaps in our understanding of these processes. We start with a discussion of the basic biophysics of protein-lipid interactions and then turn our attention to how the biophysical principles are played out in the *in vivo* context. We suggest in the concluding section some outstanding questions that the field needs to address. We close with some general thoughts on how translocons may facilitate insertion and folding. Throughout, our focus is on helix-bundle integral membrane proteins and SecYEG/Sec61-type translocons (found in the inner membrane of bacteria and the endoplasmic reticulum (ER) membrane of eukaryotic cells); β-barrel and peripheral membrane proteins will not be discussed.

Biophysical boundaries of membrane protein insertion and folding

Membrane proteins and equilibrium

Molecular dynamics (MD) simulations of membrane proteins in fluid lipid bilayers reveal graphically the complex environment of membrane proteins. The environment of the SecYEG translocon is especially complex as a result of its large hour glass-shaped interior filled with about 400 water molecules (Fig. 2). The structural stability of SecYEG and other membrane proteins depends upon numerous physicochemical interactions, summarized in a simplistic way in Fig. 3. Despite the complexity, the equilibrium structures of MPs are in principle calculable given a complete quantitative description of the interactions. These interactions are understood in broad terms [1-3], but many essential details are lacking. Even though the physical chemistry of soluble proteins is also complex, we at least have a simple measure of their stability defined by their free energies of folding, ΔG_{fold}. These free energies, typically obtained using calorimetric or chemical denaturation methods [4], are typically −5 to −10 kcal mol^{-1} regardless of the number of amino acid residues [5].

Even defining what ‘unfolding’ of an α-helical MP means is problematic [6]. For example, bacteriorhodopsin (bR), a protein with 7 transmembrane helices (TMHs), can be unfolded by heating, but unfolding is irreversible [7]. Other MPs can be unfolded reversibly with

detergents, but the structure of the unfolded detergent-protein complex is unknown [8]. All we know for sure in either case is that the denatured proteins remain about 50% helical, which means that α-helices are extremely stable in lipids and detergents; this is expected from simple thermodynamic considerations (below). Despite the unsolved challenges of MP folding, understanding the thermodynamic stability of MPs is important, because it defines the energetic framework within which the translocon apparatus must operate.

Thermodynamic measurements of MP stability are of little value unless native membrane proteins are equilibrium structures. Several lines of evidence suggest that MPs are in fact equilibrium structures. Perhaps the single most important test of equilibrium is whether the MP structure is independent of the assembly pathway. Several experimental approaches, albeit for a limited number of MPs, are consistent with equilibrium. Bacteriorhodopsin (bR) can be proteolytically cleaved into two membrane-spanning fragments and reconstituted separately into lipid vesicles. Fusion of the vesicles by multiple freeze-thaw cycles results in reconstitution of bR with a retinal absorption spectrum indistinguishable from wild-type spectrum [9]. Furthermore, low-resolution diffraction patterns of the reassembled protein are indistinguishable from those of wild-type protein [10]. If the final stages of folding MPs are guided by equilibrium thermodynamics, then co-expression of contiguous segments of a MP should lead *in vivo* to properly folded and functional proteins. This hypothesis has been confirmed for several α-helical proteins, including rhodopsin [11], lactose permease [12], the red cell anion exchanger protein [13]. Interestingly, even the β-barrel outer membrane protein OmpA can be assembled from fragments [14]. Additional strong support for equilibrium comes from a recent study of the topology of lactose permease (LacY) in lipid bilayers [15], which shows that *in vivo* LacY topology changes induced by manipulation of *E. coli* inner membrane lipid composition can be replicated in lipid vesicles.

Given that MPs are equilibrium structures and ignoring possible kinetic barriers, we should, in principle, be able to reconstitute them into lipid vesicles or nanodiscs [16] without the intervention of a translocon under extremely dilute conditions: Imagine dropping a single MP into an aqueous phase containing lipid bilayer vesicles with an appropriate lipid composition. That single protein must eventually find its way to the vesicles and there fold spontaneously into the bilayer and eventually reach its native equilibrium state. This thought experiment is impractical to implement, of course, but an equivalent experiment would be to use a cell-free expression system containing only the essential components of protein synthesis. In essence, proteins emerging from the ribosome would find their way to lipid vesicles or nanodiscs and fold into the membrane in the absence of translocons. This approach seems to have been successful in several cases [17-21]. The main uncertainty is the exact composition of the cell-free extracts. It is not clear, for example, if the extracts are completely free of translocon components or chaperone components such as trigger factor [22].

Membrane protein intrinsic interactions: TM helix folding and stability

In the absence of methods for folding and refolding α-helical MPs reversibly in a structural context, the next best approach is to consider the interactions of fragments of MPs, such as α-helices, with lipid bilayers. Because MPs are equilibrium structures, their intrinsic

interactions with lipid bilayers can be described by any convenient set of experimentally accessible thermodynamic pathways, irrespective of the biological synthetic pathway. One particularly useful set of pathways is the so-called four-step model [1] (Fig. 4). Although these pathways do not necessarily mirror the actual biological assembly process of MPs, they can help us understand the thermodynamic constraints on MP structure formation and possibly give insights into the functioning of translocons.

Each of the steps in the 4-step model have been intensively studied by several laboratories during the past twenty years [1-3, 23]. Extensive measurements of water-to-bilayer and water-to-octanol partitioning free energies of model peptides [24-26] show that structure formation is dominated by the unfavorable free energy cost ΔG_{CONH} of partitioning non-hydrogen bonded peptide bonds and the favorable free energy of the hydrophobic effect, ΔG_{HF}. The latter free energy is readily calculated from the accessible surface area of non-polar amino acids, as shown long ago by Reynolds and Tanford [27]. They found that a plot of transfer free energies against accessible surface areas A_{acc} of a large number of non-polar compounds yielded a linear curve with a slope of 20 to 25 cal mol^{-1} $Å^{-2}$, referred to as the atomic solvation parameter, σ, so that $\Delta G_{HF} = \sigma A_{acc}$. Measurements of the partitioning free energy of hydrophobic pentapeptides into *n*-octanol from water and careful accounting of their non-polar accessible surface areas yielded a value of −23 cal mol^{-1} $Å^{-2}$ [24]. Interestingly, however, measurements of the partitioning free energies of the pentapeptides into the interface of phosphatidylcholine bilayers yielded a value of σ of about −11 cal mol^{-1} $Å^{-2}$ [25], about one-half the octanol value. Why should the value be smaller? The hydrophobic effect depends upon complete dehydration of the non-polar surface when a solute is moved from bulk water to a bulk non-polar phase. The bilayer interface, however, contains waters with restricted motions due to headgroup hydration as well as the interfacially exposed bilayer hydrocarbon core. A non-polar solute that partitions into the interface is never fully immersed in hydrocarbon and never fully dehydrated, causing σ to be reduced by half.

The favorable hydrophobic partitioning free energy of a non-polar amino acid is opposed by the unfavorable cost ΔG_{CONH} of partitioning the peptide bond. But if the peptide bonds can form hydrogen bonds (H-bonds) through secondary structure formation, the partitioning cost can be dramatically reduced [28, 29]. The reduction occurs because ΔG_{Hbond} of partitioning hydrogen-bonded peptide bonds is considerably lower than ΔG_{CONH} of partitioning peptide bonds alone. For example, computational studies [30, 31] of peptides in bulk alkanes suggest that ΔG_{CONH} for water-to-alkane transfer is +6.4 kcal mol^{-1}, compared to only +2.1 kcal mol-1 for ΔG_{Hbond}. The per-residue free energy cost of disrupting H-bonds in an alkane is therefore about 4 kcal mol^{-1}, which means that a 20 amino acid TM helix would cost in excess of 80 kcal mol^{-1} to unfold within the membrane hydrocarbon. This explains why unfolded polypeptide chains cannot exist in a transmembrane configuration.

The energetic cost of partitioning a peptide chain into the membrane interface is also reduced by H-bonding to form secondary structure [28, 29], the best current estimate being about −0.4 kcal mol^{-1} per residue [32]. Although a modest number, the driving free energy for secondary structure formation by a 15-residue hydrophobic peptide would be about −6 kcal mol^{-1} (ΔG_{if} in Fig. 4). Because of this phenomenon, a modestly hydrophobic peptide

such as 26-residue melittin from bee venom, which is largely unfolded in free solution, avidly folds into membrane interfaces [29].

The value of ΔG_{Hbond} also sets the threshold for transmembrane helix stability. Even though hydrogen bonded, the cost of dehydrating peptide bonds upon insertion of a helix into the hydrocarbon core of a bilayer is still considerable, the best estimate being about +1.2 kcal mol^{-1} per residue [33]. This explains why TMHs must be very hydrophobic: the unfavorable cost of partitioning the helical backbone must be compensated by the favorable cost of partitioning hydrophobic sidechains. Figure 5 shows a free energy accounting for the insertion the TM segment of glycophorin A (GpA), one of the first membrane proteins to be sequenced [34]. The cost of partitioning the H-bonded helical backbone ($\Delta Gbb(h)$) of +24 kcal mol^{-1} is well compensated by the favorable hydrophobic free energy of −36 kcal mol^{-1} for partitioning the sidechains (ΔG_{sc}). The net free energy ΔG_{TM} favoring insertion is thus −12 kcal mol^{-1}. As is common in so many biological equilibria, the free energy minimum is the small difference of two relatively large opposing energetic terms. This simple example emphasizes that the balance between the unfavorable cost of dehydrating the H-bonded peptide bond and the favorable gain from dehydrating non-polar sidechains determines whether or not an α-helix is independently stable in a TM configuration. To emphasize again the importance of H-bonds in determining structure, the cost of partitioning the unfolded GpA peptide backbone would exceed 100 kcal mol^{-1}! This huge cost explains why heat or chemical denaturation of membrane proteins always results in ‘unfolded’ states that are rich in α-helical structure.

But is the cost realistic? Atomic force microscopy provides an answer. TMHs can be pulled out of membranes one by one using atomic force microscopy (AFM) methods [35]. In a classic AFM study of bacteriorhodopsin [36], for example, a long, flexible cantilever with an atomically sharp probe attached to one end was used to scan the surface of a native bacteriorhodopsin membrane. The deflection of the cantilever as it moved across the surface was recorded, producing a topographic map of the surface. The AFM was then used to pull single molecules out of the surface while recording the force exerted on the cantilever. Because both the force *F* on the cantilever and the distance *d* between the tip and the membrane can be measured simultaneously, the unfolding of the protein can be visualized as a force-distance (*F*-*d*) curve. Importantly, the unfolded state is an extended polypeptide chain in aqueous buffer. This means that the underlying thermodynamic states are the folded protein in the bilayer and the unfolded protein in water. The unfolding is a non-equilibrium process and is not reversible, but the data are nevertheless useful, because the non-equilibrium problem can be worked around using Jarzynski's identity [37]. Careful application of the identity to *F*-*d* curves collected over wide range of temperatures and pulling rates yielded [38], for the first five helices of bR, a free energy difference between membrane-folded and water-unfolded states of 230±40 kcal mol^{-1}, or an average free energy change of about 1.3 kcal mol^{-1} per residue. This value agrees well with the value of about 200 kcal mol^{-1} computed from octanol-water partitioning free energies [1, 24], which underlie the calculations of Fig. 5.

Apart from these AFM measurements that apply in special cases, essential thermodynamic information is generally difficult to obtain through direct measurements, because greasy

peptides are very insoluble and have a strong tendency to aggregate in water. An alternative for understanding in atomic detail the partitioning of highly hydrophobic peptides is to use microsecond-scale equilibrium MD simulations [39]. Simulation technology has not yet advanced sufficiently to examine the folding of multi-span MPs, but the technology is good enough to examine the folding of single-span polyleucine segments. Such simulations are initiated by placing a single unfolded polyleucine peptide into a simulation cell containing a lipid bilayer and allowing the simulation to run until all accessible states have been sampled many times and cataloged. In simulations lasting several microseconds, polyleucine peptides are observed to adsorb into the membrane interface where they fold into a helical conformation and make numerous excursions into the membrane as a TM helix. Figure 6 shows a representation of the folding and insertion of a Leu_{10} peptide in which the insertion depth and helicity of the peptide are sampled during the course of the simulation. For each sampled time point, the insertion depth (relative the bilayer center) is plotted against the helicity, and the points connected sequentially by lines.

Two observations are significant. First, the peptide rapidly adsorbs at the bilayer interface. Second, after the first 50 ns the peptide becomes α-helical and moves repeatedly between a helical surface configuration and a helical transmembrane configuration. This behavior is exactly what one would expect from considerations of the cost of peptide bond partitioning described above (Figs. 4 and 5). During the time course of the simulation, the peptide is never seen to leave the membrane and return to the aqueous phase, which is the behavior expected from extensive studies of the interactions of melittin with bilayers (above). Indeed, microsecond-scale simulations of melittin folding at the membrane interface are in quantitative agreement with experiments [40, 41].

These considerations of the partitioning of peptides into membranes provide an important perspective on how translocons may manage the MP assembly reaction pathway. They lead inexorably to the conclusion that the membrane interface in the vicinity of the translocon likely participates in assembly.

Membrane protein intrinsic interactions: Helix-helix interactions

There is much more to intrinsic interactions than just the folding and insertion single TMHs into lipid bilayers, because so many biologically important MPs are multi-spanning proteins. The other part of the MP folding problem is therefore the energetics of helix-helix interactions that cause single helices to come together to form functional three-dimensional structures in the lipid bilayer. Popot and Engelman first focused attention on this problem through their “2-stage model” of folding [42], which was an outgrowth of their work on the reassembly of functional bR from proteolytically cleaved fragments separately reconstituted into lipid vesicles. Our clearest understanding helix-helix interactions comes from studies of glycophorin A (GpA) that forms dimers when solubilized in sodium dodecyl sulfate (SDS) [43] and lipid bilayers [44]. Engelman and co-workers took advantage of this observation to discover the location and properties of the dimerization domain, $-L_{75}IxxG_{79}V_{80}xxG_{83}V_{84}xxT_{87}-$, within the GpA TMH [45, 46] and to determine the structure of the dimer in detergent micelles using NMR [47] (Fig. 7). Langosch et al. [48] extended GpA dimerization measurements to *Escherichia coli* inner membranes.

The GpA dimer is held together by van der Waals interactions and tight shape complementarity, called “knobs-into-holes packing” [49, 50]. The GpA data led to the identification of the first widespread interaction motif for TMHs, the so-called GXXXG motif [51, 52]. The interaction free energies of GpA dimer formation are readily determined for detergent-solubilized helices using analytical ultracentrifugation to measure the equilibrium amounts of monomers and dimers [53], but the relationship between interaction free energies in detergents and in lipid bilayers have been unclear. A recent study shows that helix-dimer stability in lipid bilayers depends strongly on the type of lipid, ranging from −12 kcal mol^{-1} in pure phosphatidylcholine bilayers to about −3 kcal mol^{-1} in *E. coli* lipids [54], the latter value being similar to that observed in natural membrane ‘blebs’ [55].

H-bonds between TMHs can also be important for stabilization. For example, TM segments composed only of leucine have relatively little tendency to dimerize *in vivo* in *E. coli* unless a polar residue, especially Asn, Asp, or Glu, is also present in the sequence to form H-bonds [56]. However, a double-mutant thermodynamic cycle analysis of bacteriorhodopsin has shown that the stabilization free energy of single sidechain H-bonds contribute only about 0.6 kcal mol^{-1}. Of course, if multiple H-bonds are present between sidechains in a MP, the additive stabilization could become significant.

Because the simple thermodynamic considerations discussed earlier, MPs are absolutely constrained to have their native helical structure in bilayer membranes. The first stage of the two-stage model [42]—the establishment of TMHs across membranes—is accomplished through co-translational insertion and the catalytic activity of the translocon (below). Cells thus avoid the thermodynamic challenges associated with managing highly non-polar proteins in the aqueous environment. The second stage of folding—helix association within the bilayer environment—should be the primary focus of MP stability rather than complete denaturation of MPs using heat or detergents, which are problematic. Just as we measure the stability of soluble proteins in their native aqueous environment, we should really measure the stability of MPs within their native membrane environment. The question is then simply the energetic cost of separating the TMHs from one another within a lipid bilayer. This challenging task has now been accomplished for bR in a bicelle environment using so-called steric trapping [57]. Steric trapping is based upon engineering two strepavidin binding sites into the protein that are close together in the native state. For folded proteins, only a single monovalent streptavidin can bind due to steric overlap of the binding sites. But when the protein is unfolded in the membrane, then streptavidin can bind to both sites. The free energy of unfolding can be determined by measuring of the amount of doubly bound protein during unfolding. In this way, Chang et al. determined a value of approximately −11 kcal mol^{-1} for the unfolding of bR [58], which is within range of values observed for water-soluble proteins. This is not a marginal value of stability. If other MPs are eventually found to have values similar to those of water-soluble proteins and membrane-bound bR, then one can safely assume that once TMHs are inserted into membranes, native three-dimensional structure will follow.

Biological boundaries of membrane protein biogenesis

How does the cell exploit and control the physical interactions that underlie the spontaneous insertion of hydrophobic peptides into lipid bilayers in order to produce properly folded helix-bundle membrane proteins, while avoiding the ever-present risk of protein aggregation? The basic solution to this problem that nature has come up with is co-translational insertion: with few exceptions [59], helix-bundle membrane proteins are inserted directly into a target membrane as they come out of the ribosome. Co-translational insertion is mediated by one or other type of translocon, i.e., a protein-conduction channel in the membrane that is constructed such that it can both shield polar parts of the polypeptide from lipid contact as they are translocated across the membrane, and at the same time expose hydrophobic segments in the protein to the surrounding lipid bilayer, facilitating the formation of TMHs.

In this section, we first review recent advances in our understanding of how ribosomes from different kinds of organisms handle nascent polypeptide chains, and how ribosomes synthesizing membrane proteins are specifically targeted to translocons. We then discuss current models of translocon-mediated insertion and folding of membrane proteins.

Ribosomes

Ribosomes are highly conserved RNA-protein complexes that translate mRNA into protein in all living cells. For our present purposes, the most important parts of the ribosome are the polypeptide-transferase center (PTC) where the incoming tRNA delivers its amino acid to the growing polypeptide, the tunnel in the large ribosomal subunit through which the nascent chain exits the ribosome, and the polypeptide exit site on the “backside” of the large subunit (Fig. 8). Peptide-bond formation at the PTC is catalyzed by ribosomal RNA (“The ribosome is a ribozyme” [60, 61]). Large sections of the tunnel walls are also formed by ribosomal RNA, creating a polar, negatively charged environment for the nascent chain. There is a higher density of ribosomal proteins around the exit site, and many accessory proteins such as chaperones and targeting factors bind there. The area around the exit site is also involved in docking the ribosome to the translocon, such that the nascent chain can pass directly from the ribosomal tunnel into the translocon channel.

The chief differences between prokaryotic and eukaryotic ribosomes are found in their protein composition, with eukaryotic ribosomes having a larger complement of proteins than prokaryotic ones. There are also some extra “expansion loops” in the RNA present in eukaryotic ribosomes [62-64]}. The core ribosomal structure is highly conserved, however, and the basic operational principles—including the biosynthesis of integral membrane proteins—are very similar from bacteria to man.

The key player in the early steps of membrane protein biosynthesis is the signal recognition particle (SRP). In eubacteria such as *E. coli*, SRP is composed of a small 4.5S RNA and a single protein subunit, Ffh [65, 66]. Ffh contains both a signal-peptide binding domain and a GTPase domain, and can bind near the polypeptide exit site on the ribosome. When a sufficiently hydrophobic polypeptide segment, typically a signal peptide or a segment that will eventually form a TMH, appears at the exit site of the ribosome-nascent chain complex,

it binds to SRP with sub-nM affinity [67]. Binding will cause a major structural rearrangement of the SRP [66, 68], priming the ribosome-nascent chain-SRP complex for binding to the SRP receptor FtsY located on the surface of the inner membrane. The ribosome-SRP-FtsY complex then binds to the SecYEG translocon (see next section). FtsY also has a GTPase domain, and proper docking of SRP to FtsY brings the SRP and FtsY GTPase domains into juxtaposition such that two complete active sites are formed and two GTPs can be hydrolyzed. This process, which serves as a quality control mechanism by assuring that the ribosome is correctly docked to SecYEG [69], leads to the release of the nascent chain from SRP, allowing it to insert into the translocon.

The mammalian SRP is more complex, with a larger 7S RNA and five additional proteins. The Ffh homolog is called SRP54, and has a similar signal-peptide binding groove [65, 66, 70]. A major difference between the bacterial and mammalian SRP is that the latter not only binds hydrophobic segments in the nascent chain and mediates targeting to the translocon, but also slows down or halts translation when bound to a ribosome-nascent chain complex [65]. Presumably, this gives the ribosome more time to find and dock to a translocon in the complex cytosolic environment of a eukaryotic cell, ensuring the tight coupling between translation and membrane insertion.

Residues in the nascent polypeptide chain can interact with the tunnel wall, especially in its upper parts. Such interactions can induce translational stalling, presumably by subtly altering the geometry of the PTC. Specific peptide sequences (translational arrest peptides: APs) can stall translation on their own (*intrinsic* APs), or in combination with a small molecule such as an amino acid or an antibiotic (*inducible* APs) [71]. The latter are important in regulation of antibiotic resistance genes, which have arrest peptides that are activated in the presence of the antibiotic [72]. As will be explained below, translational APs have recently been employed as “force sensors” to detect dynamic interactions between a growing nascent chain and its immediate surroundings.

Translocon-mediated membrane insertion

In bacteria, membrane insertion of nearly all helix-bundle membrane proteins is mediated by two translocons: SecYEG and the structurally unrelated YidC. Some proteins require only one or the other, and some require both. YidC is about five-fold more abundant in *E. coli* than SecYEG [73, 74]. SecYEG and YidC can form a 1:1:1:1 super-complex (the holo-translocon) together with SecDF (four-fold lower cellular abundance than SecYEG) and YajC (three-fold higher cellular abundance than SecYEG) [75].

The eukaryotic Sec61 translocon in the ER is homologous to SecYEG, but there are no YidC, SecDF or YajC homologues in the ER. YidC homologues are, however, found in the inner mitochondrial membrane (Oxa1) and the chloroplast thylakoid membrane (Alb3/Alb4) [74]. On the other hand, the Sec61 super-complex involves additional membrane proteins such as TRAM, TRAP, and the oligosaccharyl transferase that have no counterparts in *E. coli*. Eukaryotic Sec complexes also include Sec62 and Sec63, which facilitate post-translational transport (reviewed in [75]). Mitochondria in most organisms do not have an equivalent to Sec61 in the inner membrane; the only translocase there is Oxa1. Because of

the absence of a Sec-type translocon, mitochondrial ribosomes are specialized to bind directly to the inner membrane [76].

The SecYEG/Sec61 translocon can mediate both co- and post-translational translocation of proteins; in bacteria, most secreted proteins are translocated post-translationally with the help of the SecA motor protein, whereas membrane proteins are nearly always inserted into the membrane co-translationally [77]. In contrast, YidC mediates post-translational membrane insertion, except in mitochondria where a C-terminal domain in Oxa1 can bind to mitochondrial ribosomes in a co-translational fashion [74]. In eukaryotic cells, insertion of proteins into the ER membrane is nearly always co-translational, while translocation of secretory proteins into the lumen of the ER can be both co- and post-translational [77].

High-resolution three-dimensional structures (Fig. 9) are known for several SecYEG translocons from different prokaryotic organisms [78-80] and for the Sec61 complex from pig [64]. The central translocation channel is formed by the SecY subunit (Sec61α in eukaryotes), a protein with 10 TMHs. A small plug domain closes the channel from the periplasmic side, presumably in order to prevent ion leakage through the inactive translocon. The SecY subunit is buttressed by SecE that partly encircles SecY and enhances its stability [81]. SecG is more peripherally located, and its precise function is unclear [82].

A lateral gate located between TMH2b and TMH7 in SecY can open up the channel towards the surrounding membrane, as seen in Fig. 9. The simplest conceptual model for how TMHs in the nascent polypeptide are inserted into the membrane is that they first enter the central SecYEG channel, then exit it via the lateral gate and insert into the lipid bilayer one by one (Fig. 1). In essence, they partition between the translocon and the membrane. As will be shown below, this is certainly an over-simplification, but it serves as a good starting point for the discussion.

The idea of translocon/membrane partitioning can be examined experimentally by suitably designed test-proteins in which a hydrophobic segment is placed far away from any potentially interfering TMHs. Assays to measure the efficiency of membrane insertion of such model hydrophobic segments (H-segments) have been developed, both for Sec61-mediated insertion the mammalian ER [83, 84] and SecYEG-mediated insertion into the inner membrane of *E. coli* [85]. Analysis of hundreds of H-segments with systematically varied amino acid sequences led to the establishment of a ‘biological’ hydrophobicity scale, in which the contribution from each of the 20 natural amino acids to the overall apparent free energy of membrane insertion of the H-segment (ΔG_{app}) is given as a function of the position of the residue in the H-segment [83]. Further studies in which an amino acid with a non-natural side chain—either a linear alkyl chain with 1 to 8 carbons, or aromatic groups as large as biphenyl—was included in different position in the H-segment made it possible to measure the contribution of apolar surface area (i.e hydrophobic effect with solvation parameter σ) to ΔG_{app} : For an aliphatic surface this came out as −10 cal mol^{-1} Å^{-2}, and for aromatic surface as −7 cal mol^{-1} Å^{-2} [86] when at the center of the H-segment. These solvation parameter values, measured for the translocon-mediated insertion process, are a factor 2-3 smaller than expected for simple water-to-hydrocarbon transfer free energies. Intriguingly, however, these values are similar to solvation parameters determined for the

partitioning of hydrophobic peptides into bilayer interfaces [25] (see Membrane protein intrinsic interactions: TM helix folding and stability, above). The similarity suggests that the state of water in the translocon may be similar to the state in hydrated bilayer headgroups [87]. The smaller solvation parameter obtained in the translocon partitioning experiments [86] thus raises the possibility that the nascent chain may be exposed to the surface of the lipid bilayer surrounding the translocon at an early stage of membrane insertion. This possibility has recently received support from equilibrium molecular dynamics simulations of the water-to-bilayer partitioning of H-segments whose values of ΔG_{app} were determined experimentally [88].

Recently, a structure of a ribosome-nascent chain-translocon complex has been obtained at 3.4 Å resolution [64] (Fig. 8), and two more at 8-15Å resolution [89, 90], all by single-particle cryo-electron microscopy. The interpretation of the structures is complicated, however, because they represent equilibrium configurations—established using various experimental protocols—that may or may not represent way points on the physiological insertion pathway. A particular problem is that the translocons in the structures are invariably in detergent micelles rather than lipid bilayers.

The first structure [64] provides detailed views of both an idle ribosome-translocon complex with no nascent chain, and an active complex in which the nascent chain can be followed through the ribosome but is not visible within the translocon. The cytoplasmic end of the lateral gate is partly open in the active complex, and the plug domain is displaced from its original location. In the second structure [89], an H-segment engineered into a host protein has been caught in an open lateral gate, party exposed to the surrounding detergent, and in the third [90] the hydrophobic signal peptide of the periplasmic protein DsbA is similarly located. These structures show that a TMH can occupy the lateral gate, but do not address the question of whether such H-segments first enter the central channel in the translocon and then exit into the lipid bilayer via the lateral gate, slide progressively into the lateral gate from the top—always being partly exposed to lipid (Fig. 10), or perhaps first come into direct contact with the lipid bilayer. If the energetic barrier to opening the lateral gate is not too high, the ‘sliding’ possibility seems more likely to represent the lowest-energy trajectory. Indeed, this is how nascent chains behave in a coarse-grained molecular dynamics model of translocon-mediated protein translocation and insertion developed by Zhang and Miller [91]. A recent crosslinking study has identified a possible initial binding site for TMHs at the top of the lateral gate and in close proximity to surrounding lipid [92], from which the TMH could conceivably slide deeper into the membrane within a progressively expanding lateral gate. In the sliding model, a TMH would be partially exposed to lipid already at an early stage during insertion, possibly explaining the coincidence between insertion free energies calculated from the biological hydrophobicity scale and those obtained by molecular dynamics for peptide insertion into a bilayer alluded to above. Further, the sliding model would predict that highly hydrophobic TMHs may insert directly into the membrane while making little contact with the lateral gate, amphiphilic TMHs insert with their more polar face buried in the lateral gate, and marginally hydrophobic TMHS—i.e., TMHs that insert efficiently only by making specific

interactions with already inserted TMHs [93-95]—would insert largely through the translocon channel and only expose a limited surface through the lateral gate.

A high-resolution structure of the YidC translocon has just been published [96], as has an accurate structural model [97]. Somewhat surprisingly, YidC is a monomer in the crystal, while a previous projection structure obtained by electron crystallography of two-dimensional crystals showed it as a dimer [98], and the density attributed to YidC in a single-particle EM study of ribosome-YidC complexes was also interpreted as representing a YidC dimer [99]. On the other hand, and consistent with the X-ray structure, binding studies using YidC reconstituted into nanodiscs suggested that ribosome-nascent chain complexes bind only a single copy of YidC [100]. Very recent cryo-EM structures of YidC bound to a ribosome is also consistent with a monomer [97, 101].

The X-ray structure and accompanying molecular dynamics simulations show a deep, hydrated cleft within YidC that extends halfway across the membrane and is open towards both the cytoplasm and the lipid bilayer, Fig. 11a. The cleft is capped on the periplasmic side by what appears to be a tightly packed, stable structural domain. When Trp^{244} at the top of the cleft is substituted by the photo-activatable amino acid analog *p*-benzoyl-L-phenylalanine, it can be crosslinked *in vivo* to a co-expressed substrate protein, showing that substrate has access to the deep cleft [96]. There is also a rather mobile part, composed of two α-helices that lie flat on the cytoplasmic face of the membrane, in front of the entrance to the cleft. The role of this part is unclear; perhaps it facilitates membrane insertion of substrate TMHs by locally distorting the lipid bilayer.

The structure [97, 101] is suggestive of an insertion mechanism in which a short polar tail or loop in the substrate protein penetrates at least halfway across the membrane within the water-filled cleft, leaving the adjoining hydrophobic TMH(s) in the lipid bilayer, similar to the sliding model proposed for SecYEG above. The resulting intermediate state would be of lower free energy than an initial state where the TMH is embedded in the lipid headgroup region, but of higher free energy than a final state where the TMH completely spans the membrane. According to this model, YidC is designed to lower the energy barrier for the initial, partial insertion of the TMH. A speculative possibility is that the outer lipid monolayer becomes sufficiently perturbed in the vicinity of the intermediate YidC-substrate complex to make it possible for the polar tail or loop to translocate fully across the membrane at a reasonable rate.

A comparative analysis of the insertion of model TMHs into the inner membrane of *E. coli* of YidC- and SecYEG-dependent membrane proteins showed that the threshold hydrophobicity required for 50% insertion of a hydrophobic segment of composition *n*L/(19-*n*)A is similar ($n_{50\%} \approx$ 1-2) for the two translocons [85, 102]. Likewise, the individual contributions to the overall ΔG_{app} for YidC- and SecYEG-dependent insertion are similar for the non-polar residues, whereas polar and charged residues have roughly two-fold larger ΔG_{app} values when membrane insertion is mediated by the YidC translocon, Fig. 12. Possibly, a TMH is more lipid-exposed during YidC-mediated insertion than during insertion mediated by the SecYEG translocon (where polar residues may be sequestered into the lateral gate region, away from lipid contact).

Because some fraction of SecYEG and YidC are bound together in the holo-translocon, they can conceivably act sequentially on membrane protein substrates, or may even act simultaneously on different parts of a multi-spanning membrane protein. Within the context of the holo-translocon, where the lateral gates in SecYEG and YidC conceivably are not far apart, one can imagine that an incoming N-terminal TMH, or an internal 'helical hairpin' composed of two TMHs with a short connection loop, can preferentially partition into the membrane via YidC, while TMHs flanked by longer polar segments that cannot fit into the YidC cavity will preferentially be inserted via SecYEG.

Ribosome-translocon-membrane insertion pathways

The progression of a hydrophobic transmembrane segment along the ribosome–translocon–membrane insertion pathway has been followed mainly by chemical crosslinking experiments and, more recently, by using translational APs as *in vivo* force sensors. Many crosslinking experiments have been carried out by *in vitro* generation of stalled ribosome-nascent chain-translocon complexes using dog pancreas rough microsomes, i,e, ER-derived membrane vesicles that contain Sec61 translocons. Early studies showed that signal peptides in stalled nascent chains could be crosslinked both to translocon components and to lipid [103], suggesting that the signal peptide is transiently held in an interfacial location between the translocon and surrounding lipid. This is in full agreement with the electron microscopy structures discussed above. As noted above, a recent crosslinking study has further identified an early interaction between an N-terminal TMH in a substrate protein with residues located at the cytoplasmic tip of the lateral gate in Sec61 [92], as would be expected if hydrophobic segments start to partition into the lateral gate region immediately upon entering the translocon, rather than first moving into the central channel and exiting through the lateral gate only at a later stage (c.f., Fig. 10b).

The development of a novel technique where translational APs are used to measure forces acting on a nascent chain during co-translational processes such as membrane insertion now makes it possible to study the kinetics of membrane protein insertion and folding *in vivo*. APs are short stretches of polypeptide, typically ~10-15 residues long, that bind in the upper parts of the ribosomal tunnel and induce ribosomal stalling at a specific codon in the mRNA [71]. Stalling can be prevented if a sufficiently strong 'pulling force' acts on the nascent chain at the precise point when the ribosome reaches the critical codon [104]; presumably, pulling on the nascent chain breaks the interactions between the AP and the ribosome that control stalling.

APs from the SecM protein have proven particularly useful. The AP from *E. coli* SecM is 17 residues long and rather weak, but stronger APs have been found in SecM proteins from other bacteria [105]. The strongest AP known to date is a mutated version of the SecM AP from *Mannheimia succiniciproducens* with sequence HPPIRGSP (called *Ms*-Sup1) [106]. This short AP can be introduced into any protein, and will report on the tension in the nascent chain at the precise point during translation when the ribosome reaches the proline codon at the 3' end of the AP.

The SecYEG-mediated insertion of polypeptide segments (H-segments) of varying hydrophobicity into the inner membrane of live *E. coli* cells was recently analyzed using the

approach detailed in Fig. 13a [106]. Strong pulling forces proportional to the hydrophobicity of the H-segment were recorded at tether lengths $L \approx 30$ and $L \approx 40$ residues, Fig. 13b. From the known dimensions of the ribosome-translocon complex, the peak in the force profile at $L \approx 40$ residues most likely corresponds to the final insertion of the H-segment into the membrane. The interaction responsible for the peak at $L \approx 30$ residues is more difficult to pin-point: it depends only on the hydrophobicity of the N-terminal end of the H-segment and could represent an interaction between the H-segment and the cytoplasmic face of the lipid bilayer, or possibly an interaction akin to the one identified between an N-terminal TMH and the tip of the lateral gate in Sec61 discussed above [92]. Regardless, the data show that APs can be used to measure forces acting co-translationally on a nascent chain with high sensitivity and high spatial precision (a force profile recorded with single-residue precision such as the one shown in Fig. 13b has a spatial resolution corresponding to one residue in an extended conformation, i.e. ~3Å).

Segments that flank the TMHs can also affect membrane insertion. In particular, positively charged residues at the ends of a hydrophobic segment can either increase or decrease its insertion propensity, in accordance with the ‘positive-inside’ rule [107-110].

Folding of polytopic membrane proteins *in vivo*

Translocon complexes mediate the insertion of TM segments into membranes. Although this process is guided by the amino acid composition and structure of the segment, its interaction with the translocon and translocon accessory components can be critical. As the polypeptide chain moves through the translocon, it is continuously scanned. A ring of hydrophobic residues is present in the middle of the channel in both SecYEG and Sec61 translocons [78, 111] (Fig. 10). The interaction of the nascent chain with this ring of hydrophobic residues might be involved in the opening of the lateral gate and the transfer of TM segments between the translocon and membrane [112, 113], where they can be exposed to the lipid environment. If a segment is hydrophobic enough, it will insert into the membrane, and current models assume that the transmembrane segments in polytopic membrane proteins are inserted sequentially [114]. One would expect that replacement of the hydrophobic residues in the ring would destabilize the translocon, but that is not the case. Replacement with polar, even charged, residues, were not destabilizing [111]. The replacement does, however, affect ΔG_{app} for membrane insertion of H-segments, consistent with the ring playing a role in translocon/membrane partitioning [112].

As discussed earlier in *Membrane protein intrinsic interactions*, the α-helical structure of a nascent TMH forms at the latest during the exposure of the segment to the lipid bilayer, driven by the necessity to shield the polar backbone from an unfavorable exposure to the hydrocarbon core of the membrane. In case of interactions with the translocon channel, a helical structure apparently can be induced even before the exposure to a lipid bilayer [92]. Helical structure may even be formed in ribosome exit tunnel [115-118]. The stage at which an α-helical structure develops can be important for the formation of tertiary structure, because amino acid sidechains are positioned on different faces of an α-helix, thereby allowing favorable alignment with residues in other transmembrane segments to form tertiary structure.

In polytopic membrane proteins, individual TMHs can form interactions that determine the final fold and function of the mature protein. As discussed earlier, the interactions between transmembrane segments can consist of distinct interaction motifs, such as the GxxxG motif of GpA discussed above (Fig. 7). More generally, small residues spaced four residues apart (small-XXX-small) represent a prominent motif that allows transmembrane helices to approach each other closely and pack tightly due to van der Waals attraction and steric constraints. Extensions of this motif (small-XXX-small-XXX-small) allows for flexibilities of two helices as multiple small residues, each spaced in the distance of four, can form a groove in the surface of α-helices [119]. While any two helices containing such a motif could in principle approach each other, residues surrounding these motifs can provide certain specificity to a given interaction [120-122].

Although these motifs are highly abundant in membrane proteins [123], many other types of interactions can be formed between TMHs that require more specificity, interactions with multiple TMHs at the same time, or a high degree of flexibility. However, many interactions between TMHs cannot be explained by these simple interaction motifs [124]. Highly specific interaction networks between multiple TMHs that determine the final structure can be formed, and the flexibility between these interactions allows for tertiary structure changes that are critical for function. It is therefore not surprising that simple interaction motifs occur mainly in rather simple function contexts such as the on/off function of some receptors [125].

Many membrane proteins have more complex transmembrane domains, e.g., transmembrane channels. Here, the TMHs must form an aqueous pore, lined by hydrophilic residues within the channel. Based upon the biological hydrophobicity scale, a significant number of these segments are predicted not to insert efficiently into a membrane due to their low hydrophobicity [126]. It was therefore a long-standing question how such H-segments insert [127, 128]. One answer is that charged residues, especially arginine, have very favorable interactions with the phosphates of phospholipids [87, 129].

Charged and polar residues can face a high energetic barrier when inserting into a lipid bilayer. Nevertheless, positive and negative charges within the same [130] and different TMHs [131] can interact with each other, thereby drastically reducing this barrier. This concept can be extended to polar side chains, which are partially masked from the lipid bilayer by interacting with each other [56, 132]. In fact, it was proposed early on that membrane proteins are “inside-out” proteins, in the sense of having a polar core and a hydrophobic exterior, opposite to water-soluble proteins [133, 134]. Although this idea is oversimplified, it suggests how the energy barrier for membrane insertion of hydrophilic side chains might be overcome and specific tertiary structure interactions formed at the same time. It has been suggested that polar residues within TMHs can be used to predict the tertiary structure of polytopic membrane proteins [126, 135]. Individual TMHs can associate well after insertion into a membrane to form a functionally folded protein [11, 12]. This implies that the specific interactions between TMHs that determine the final fold do not strictly require the action of a complex folding apparatus, as expected for equilibrium folding. This indicates, that membrane protein insertion and folding might be more complex than the simplistic one

When, then, are specific interactions between TMHs formed? The simplistic and sequential insertion of TMHs one by one as discussed earlier implies that these interactions form after the TMHs have been inserted. While this might be the case for sufficiently hydrophobic TMHs, marginally hydrophobic segments would not insert into a membrane efficiently, so more complex models of membrane insertion and folding are required. One could imagine that these interactions between TMHs occur early, before insertion into a membrane, either once a TMH exits the ribosome or once it enters the translocon. It has been shown that helical hairpins composed of two closely spaced TMHs can form in the ribosome exit vestibule as the TMHs of a voltage sensor domain exit the ribosome [136] (1 in Fig. 14). Although many TMHs risk aggregation when associating in an aqueous environment, the exit vestibule of the ribosome could form a confined compartment that promotes tertiary structure formation [137]. It is presently unclear whether the helix-helix interactions observed by Deutsch and coworkers [136] in the ribosome exit vestibule are native contacts or whether they represent a folding intermediate, although initial results suggest native contacts. These and other results provide a new perspective on co-translational folding of TMHs. It will be interesting to learn if other proteins use this early folding space.

Remarkably, the translocon can accommodate more than two nascent chain segments, so specific interactions could also form before the segments insert into a membrane [138]. In this case, helical hairpins could form as a minimal (pre)folding unit and bury hydrophilic interactions within the helix-helix interface before entering the lipid bilayer (2a and 2b in Fig. 14). It is unclear how the formation of such interactions are induced within the translocon, how water is expelled from the helix-helix interface, or if such early interactions are merely the result of tertiary interactions already formed in the ribosome's exit vestibule.

Currently, a widely discussed mode of the co-translational formation of helix-helix interactions is the formation during the sequential exit of successive TMHs through the lateral gate, where previously inserted TMHs that are in close vicinity of the lateral gate can conceivably mediate membrane insertion and tertiary structure folding at the same time (3 in Fig. 14). Skach and coworkers have shown that multiple TMHs of the same polypeptide chain can be associated with the eukaryotic translocon and mediate the efficient insertion of more C-terminally located TMHs [114]. This has also been confirmed for other membrane proteins [139], suggesting that the translocon itself might be a foldase. However, it is unclear how and where TMHs associate with the exterior of the translocon complex. Although a site has been identified by crosslinking studies, it is unclear if the majority of TMHs occupy the same site [114]. Any specific interactions with a translocon complex that manages thousands of different transmembrane segments would have to be replaced by stronger interactions with the protein's own TMHs as the newly inserted segments emerge from the complex. In such a scheme, a strict hierarchy of interaction strengths in the form of an interaction and folding blueprint would have to be in place for all membrane proteins using the pathway. This might be achieved by the strict coupling of correct tertiary structure formation and membrane insertion. However, this blueprint would have to be at least partially encoded in the nascent chain itself, possibly in the form of conserved polar residues within TMHs [140]. If this hierarchical mechanism of tertiary structure formation is a common one, it will be interesting in the future to decipher the individual strengths of these

interactions and thereby come closer to an *ab initio* understanding of membrane protein folding. As pointed out above, other factors like the point of secondary structure formation, tertiary structure formation and retention within the translocon are critical factors as well.

An interesting special case is the folding of membrane channels that are composed of evolutionarily related halves [141, 142]. Here, each half-channel is (or once was), a separate folding entity, otherwise the high similarity between the halves could create interchangeability between helix-helix interactions from one half with the other that subsequently would result in protein misfolding.

In bacteria, the formation of specific interactions between sidechains from interacting TMHs have been observed by means of APs (see above) [143] for several polytopic membrane proteins during their insertion by the bacterial translocon. Mutating interacting residues in either a membrane inserting segment or an already inserted segment in the same polypeptide chain decreased the pulling force detected by a C-terminally located AP. APs thus offer a highly sensitive *in vivo* tool for studying the co-translational formation of tertiary structure contacts, similar to *in vitro* AFM (see Membrane protein intrinsic interactions: TM helix folding and stability).

A recent publication offers unprecedented structural insight into the biogenesis of a polytopic membrane protein in bacteria [144]. The structure of a ribosome-nascent chain-translocon complex shows the first two TMHs of proteorhodopsin (PR) inserted into the membrane just outside the lateral gate. Although the resolution of the structure is too low to reveal side-chain interactions between the PR TMHs themselves or with the translocon, an interaction of positively charged residues in the PR-loop between TMH1 and TMH2 with the ribosomal RNA helix 59 could be observed. This interaction was verified by mutagenesis of the charged residues, which reduced the amount of co-purified SecY/nascent-chain complex. Interestingly, the lateral gate was in between the fully open and closed conformation, which would allow a translocated hydrophilic segment to probe for tertiary interactions with previously inserted transmembrane segments. It is tempting to speculate about possible mechanisms of membrane insertion and folding based on these results. Nevertheless, the structure represents, at best, a snapshot of the biogenesis of PR. Future investigations and more structural snapshots of the biogenesis of polytopic membrane proteins might yield a more generalized and conclusive picture.

The insertion of more hydrophilic TMHs can occur at a later stage of assembly as well, which requires a larger structural reorganization of the maturing protein (4 in Fig. 14). This could be observed for several membrane proteins [93, 145-147]. In these cases, TMH3 is inserted in the opposite topology and TMH2 and TMH4 are initially not inserted into the membrane. Upon the reorientation of TMH3 at a later stage, TMH2 and TMH4 are inserted into the membrane and the water channel folds into its final functional structure. In order to form the tertiary structure of the protein, large-scale rearrangements involve induction of large tilts during folding as well as topology reversals [148]. In a particularly impressive case, the topology of a bacterial membrane protein was found to be in a flexible state until a specific C-terminal charged residue was synthesized [109].

These examples demonstrate that the formation of the tertiary structure that allows more hydrophilic TMHs to insert into a membrane can occur before, during, and after membrane insertion of the newly synthesized protein. It is currently unknown which is the preferred mode of folding in polytopic membrane proteins, because only a few examples have been investigated to date. In order to achieve a broader understanding and gain a more general view, it is necessary to follow closely the folding of many more polytopic membrane proteins in a high throughput manner.

The concept of specific interactions between TMHs allowing more hydrophilic parts of a protein to insert into a membrane can also be extended to the quarternary structure, when it comes to the homo-oligomerization of the same subunit. Furthermore, interactions between different subunits of a newly synthesized membrane protein complex can form during membrane insertion (hetero-oligomerization; 5 in Fig. 14) as was recently demonstrated for the subunits of the T-cell receptor [149]. Feige & Hendershot found that a marginally hydrophobic TMH that is not assembled into a hetero-oligomeric T cell receptor is translocated into the ER-lumen, rapidly recognized by the ER quality control machinery, and subsequently degraded. However, polar residues in already inserted, interacting subunits guided the membrane insertion and assembly of individual T cell receptor subunits and thereby prevented degradation. This marks an important step towards a mechanistic understanding how membrane protein complexes are assembled in cells. It will be both very challenging and interesting to understand mechanistically the assembly of complexes located in the ER-membrane, complexes located in the mitochondrial inner membrane, and complexes located in the thylakoid membrane.

Membrane protein secondary and tertiary structure can form at different stages: in the ribosome exit tunnel , the ribosome exit vestibule [136], within the translocon channel [115-118], during membrane insertion [39, 88], and within a membrane. Each of these environments is characterized by unique properties and thereby jointly offer an extended folding space for the many different membrane proteins that are chaperoned by the co-translational membrane protein synthesis and insertion complexes. After exploring basic principles of membrane protein synthesis, membrane insertion and folding using model membrane proteins, it is now important to venture into the widely unexplored folding space of cellular membrane proteins, and to follow and understand mechanistically their synthesis within a cell. Not only are these (mis)folding pathways likely to offer new insights into the biogenesis of membrane proteins, but they will provide us with a more quantitative view of which modes of folding are the major ones and which represent the exceptions.

Perspectives & Outlook

Where do we stand in our understanding of membrane protein biogenesis in mid-2014? First, it is clear that our knowledge is much more advanced for the SecYEG/Sec61 translocons than for systems such as the mitochondrial TOM-TIM translocons, the peroxisomal PEX translocons, and the chloroplast TIC-TOC translocons (see e.g. [150-152]). The SecYEG-associated YidC translocon is also slowly yielding its secrets. In recent years, the field has moved forward in a major way thanks to structural studies of the Sec and YidC translocons, and there are now many structures of Sec-translocases in

complex with ribosomes. In parallel, a biophysical description of the basic insertion and folding processes is emerging through the confrontation of thermodynamic and kinetic studies of highly simplified systems (peptide-bilayer interactions, *in vitro* folding of purified proteins, molecular dynamics calculations) with quantitative *in vivo* studies of the kinds described above ('biological' hydrophobicity scales, co-translational force measurements). It is satisfying to see that we are finally moving beyond the simple cartoons (Fig. 1) to more detailed structure-function descriptions.

Still, many open questions remain, even for the Sec-type translocons. How are holo-translocons—both prokaryotic and eukaryotic—put together and how do the different components cooperate? It is currently unclear how the numerous accessory components of the holo-translocons are recruited and what the dynamics of complex assembly/disassembly are. It is also unknown how the bacterial translocons, YidC and SecYEG, cooperate to insert membrane proteins. What are the precise roles of the lateral gate and the plug domain in SecYEG, what controls their movements, and how do they interact with polar and non-polar segments in a nascent chain? How strong is the functional coupling between the ribosome and the translocon, i.e., can conformational changes resulting from interactions between a nascent chain and the ribosome be transmitted to the translocon?

Lipids appear to play an important role protein translocation, but possible mechanisms are largely speculative (reviewed in [153]). From the point of view of a substrate protein, it is not clear to what extent a nascent chain can interact with membrane lipids as it passes from the ribosome into the translocon, or at which point during translation that a TMH first starts to integrate into the bilayer. It seems clear that an incoming TMH can make specific interactions with already inserted TMHs at very early stages of membrane insertion, but how are the upstream TMHs "chaperoned" by the (holo-) translocon while waiting for downstream TMHs to appear? Further, what is the physical basis for the late re-orientation of TMHs that has been seen with e.g., AQP1 [154], EmrE [109], Band III [147], and when the membrane lipid composition is drastically changed [155]?

We tried to capture in Fig. 1 the idea deeply embedded in the literature that the nascent chain, powered by the GTP-based elongation energy of the ribosome, enters the translocon whereupon the hydrophobic ring opens, the plug domain moves out of the way, and the nascent chain passes through. If a suitable hydrophobic segment arrives, it is diverted into the membrane by simple partitioning between translocon and bilayer [83, 84, 87, 156]. Reflecting on the literature we have reviewed here, we wonder if this scheme is entirely correct. To be sure, the translocon is absolutely essential for the assembly of most multi-span membrane proteins. But there is a notable exception, the transmembrane protein KdpD.

KdpD, which acts as a potassium sensor in *E. coli* [59, 157], has several features that are intriguing: The protein is tethered to the cytoplasmic surface of the inner membrane by four TM segments (residues 401-498) with very short interhelical loops that occur far downstream from the amino terminus. It is targeted to the inner membrane by SRP-recognition of a cytoplasmic amphipathic helix (residues 22–48) [158], and membrane insertion does not require SecA, SecE, or YidC [59]. Because KdpD does not apparently engage SecA or the SecYEG channel, a reasonable explanation is spontaneous insertion

following targeting by the SRP to, presumably, the SRP receptor FtsY. In *E. coli*, FtsY is not permanently anchored to the inner membrane, but rather partitions between membrane and cytoplasm via an amphipathic helix [159]. This suggests that SRP recognizes the TMHs of KdpD and targets them to FtsY on the membrane, which allows the TMHs to partition spontaneously. Such a scheme, while highly unusual, is completely consistent with what we know about lipid-protein interactions described at the outset in Biophysical boundaries of membrane protein insertion and folding.

In order to be provocative, which was our charge from the editors of this issue, we propose an alternative view of the translocon-aided insertion of multi-span membrane proteins and the secretion of soluble proteins (Fig. 15). Keeping in mind the huge thermodynamic driving forces for membrane protein folding and assembly, we suggest that a TMH initially contacts the cytoplasmic membrane interface in the vicinity of the translocon, and that it never fully enters the translocon channel but rather slides into the membrane along the lateral gate. This is not to say that the translocon cannot form a proper channel through the membrane, but we suggest that it does this only for secreted proteins and soluble domains or loop regions in membrane proteins, as shown in Fig. 15b. The view is based upon four important observations. First, most hydrophobic and amphipathic helices have a very strong affinity for the interface region of lipid bilayers; it would be thermodynamically surprising if nascent peptide chains of MPs did not interact with the membrane interface at some point during insertion. Second, so far no structural data of active ribosome-translocon complexes reveal TM segments within the translocon channel; they are only seen at lateral-gate exit site. Third, ribosome-translocon complexes are not apparently shielded from the cytoplasm and inner membrane surface; consistent with earlier observations, the recent high-resolution structure of a mammalian ribosome-Sec61 complex shows significant gap between the ribosome and the surface of the membrane [64]. Fourth, mitochondrial inner membranes in general do not have a SecY apparatus, only the YidC-equivalent Oxa1, suggesting that direct contact with the membrane is an essential feature of MP insertion, and that nascent mitochondrial proteins insert and fold spontaneously into the membrane aided by Oxa1 to move charged and or highly polar residues across the membrane.

An important consideration is that we do not know when or how nascent MP chains make initial contact with translocons or in what manner. Do they move directly from the exit tunnel straight into the translocon without intervening steps, as current cartoons suggest (Fig. 1)? Given relative target sizes, it seems possible that nascent chains could make first contact with the membrane in the vicinity of the translocon. This provides a logical explanation for the membrane insertion of the potassium channel S3b–S4 helices that apparently assemble in the ribosome vestibule [136]. Furthermore, it was shown recently that the ribosome detached from the translocon when a hydrophobic segment was placed in the ribosome exit tunnel and only hydrophilic stretches within the tunnel provided a strong attachment of the ribosome to the translocon [144]. The scheme would also explain why changes in *E. coli* inner membrane lipid composition affect the topology of LacY [155]. Consistent with the importance of thermodynamic driving forces in MP folding, Vitrac et al. [15] have shown that so-called topology switching of LacY can occur *in vitro* in lipid vesicles.

We are attracted to the scheme depicted in Fig.10b and Fig. 15a because of its simplicity: A TM segment initially associates with the top of the lateral gate and the surrounding membrane interface and strong thermodynamic forces then drive membrane partitioning. Such a process would be consistent with both crosslinking results and the AP force measurements (Fig. 13). It is not necessary in this scheme for the elongating chain to be threaded back and forth across the membrane as in Fig. 1. For the 4-TMH protein shown in the Fig. 15a schematic, the translocon is mainly required for only two steps: the passage of the TM1-TM2 and TM3-TM4 connecting loops across the membrane, reminiscent of the helical-hairpin insertion hypothesis [23]. Thermodynamic forces are sufficient for membrane partitioning, with the translocon acting as a catalyst.

Why do we need translocons at all, if insertion and folding are driven by thermodynamics? The answer, of course, is that targeting and coordinated assembly of membrane proteins are essential in the extraordinarily complicated environment of cells where thousands of biologically important reactions occur simultaneously in a crowded environment. Without translocons and all the other members of the membrane protein assembly apparatus, chaos would prevail.

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Abbreviations

TM	transmembrane
TMH	transmembrane helix
GpA	glycophorin A
AP	arrest peptide
bR	bacteriorhodopsin

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Highlights

- Helical MPs are inserted co-translationally by ribosomes docked to translocons
- Recent ribosome-translocon structures provide dramatic insights into MP assembly
- Folding of inserted MPs is driven by strong thermodynamic forces
- A new view of translocon-guided MP folding is presented

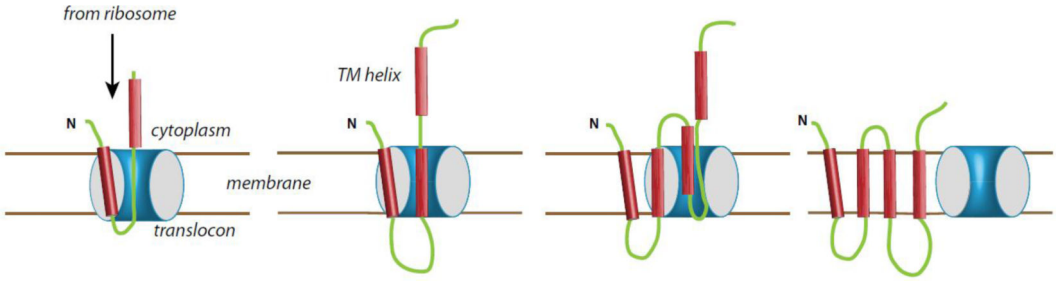

Figure 1.

This schematic cartoon represents in broad terms current thinking about the insertion of multi-span proteins into membranes. Two ideas are captured in the cartoon. First, TM segments (red) emerge from the ribosome and pass into the translocon (blue). Second, the nascent segments partition into the membrane from the translocon. As a starting point for discussion, we present alternative views of the membrane protein insertion pathway in Fig. 15.

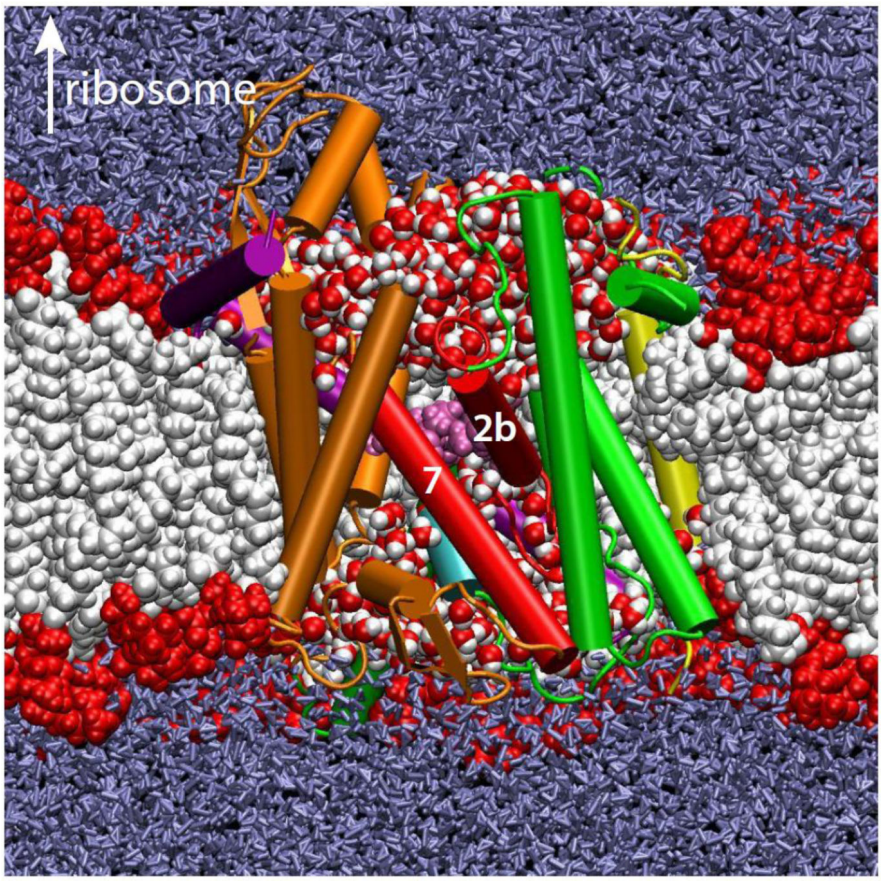

Figure 2.

Alpha-helical MPs exist in their native state in highly thermally disordered lipid bilayers, as illustrated here for the SecYEG translocon [78] from *Methanococcus jannaschii*. The image is from a molecular dynamics simulation of the translocon (PDB code 1RHZ) executed in a phospholipid bilayer. In this view, parallel to the membrane plane, the so-called gate helices 2b and 7 (red cylinders) were exposed by cutting away the lipid bilayer. Water molecules within the translocon are shown as van der Waals spheres in red (oxygen) and white (hydrogen). Waters surrounding the bilayer are shown as H-O-H bonds in blue-gray. Lipid acyl chains are white and the phospholipid headgroups are red. Image provided courtesy of J. Alfredo Frietes and Stephen H. White.

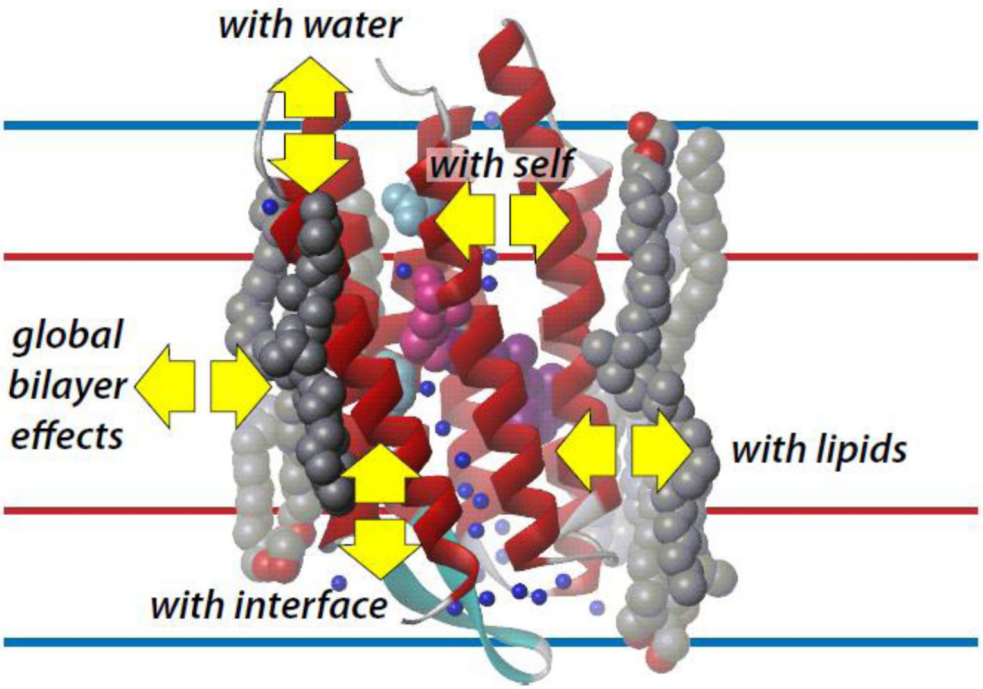

Figure 3.

Summary of the various interactions that stabilize MPs stably folded in fluid lipid bilayers (blue lines are interface boundaries, red lines represent boundaries of the lipid hydrocarbon core). “Global bilayer effects” accounts for changes in the structure and stability of the lipid bilayer when perturbed by the protein [160], emphasizing that the bilayer itself sits in a free energy minimum determined by the tendency of the system to minimize exposure of the acyl chains (grey) to water (blue) on the one hand, and the tendency to maximize the distance between headgroups on the other . Both the bilayer and protein must adjust structurally to minimize the free energy of the protein plus bilayer system. The protein shown (red helices) is bacteriorhodopsin determined to a resolution of 1.55 Å, PDB Code 1C3W [161]. Image modified from [162].

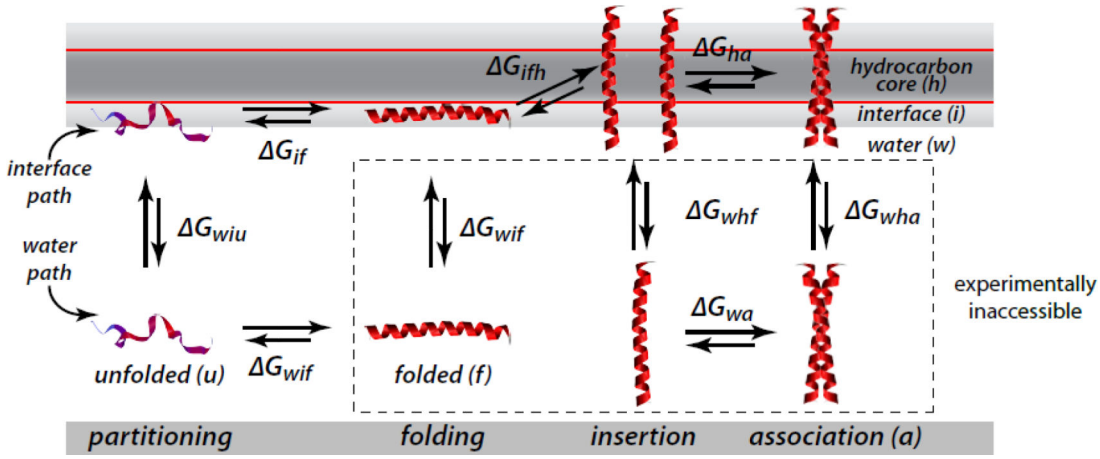

Figure 4.

A four-step thermodynamic cycle for describing the energetics of the partitioning, folding, insertion, and association of an α-helix (red helices) in a lipid bilayer (grey). The process can follow an interfacial path, a water path, or a combination of the two. Studies of folding along the interfacial path are experimentally more tractable [163]. The Δ*G* symbols indicate standard transfer free energies. The subscript terminology indicates a specific step in the cycle. The subscript letters are defined as follows: *w* = water, *i* = interface, *h* = hydrocarbon core, *u* = unfolded, *f* = folded, and *a* = association. With these definitions, for example, the standard free energy of transfer from water to interface of an unfolded peptide would by ΔG_{wiu}. However, some parts of the cycle (dashed box) are generally inaccessible experimentally. Image modified from [1].

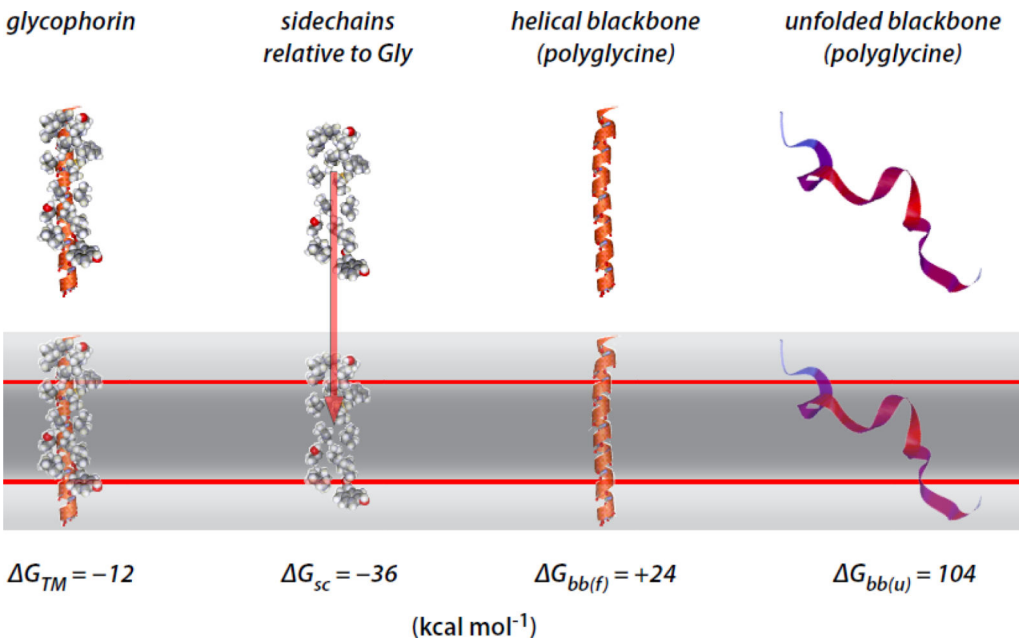

Figure 5.

The energetics of inserting an α-helix into lipid bilayers (grey) is dominated by the peptide bonds, as illustrated here for the glycophorin A (GpA) TM helix. Even with the helical backbone internally H-bonded, it is costly to dehydrate the H-bonded peptide bonds ($\Delta G_{bb(f)}$) upon insertion into the bilayer. For the helix to be stable, the favorable free energy of transfer of the sidechains (ΔG_{sc}), determined by the hydrophobic effect, must compensate for the unfavorable $\Delta G_{bb(f)}$. In the case GpA, the net stability of the helix ΔG_{TM} is −12 kcal mol^{-1}. The energetic cost of unfolding the polyglycine helix within the bilayer is immense: $\Delta G_{bb(u)}$ is greater than 100 kcal mol^{-1}! Modified from [162].

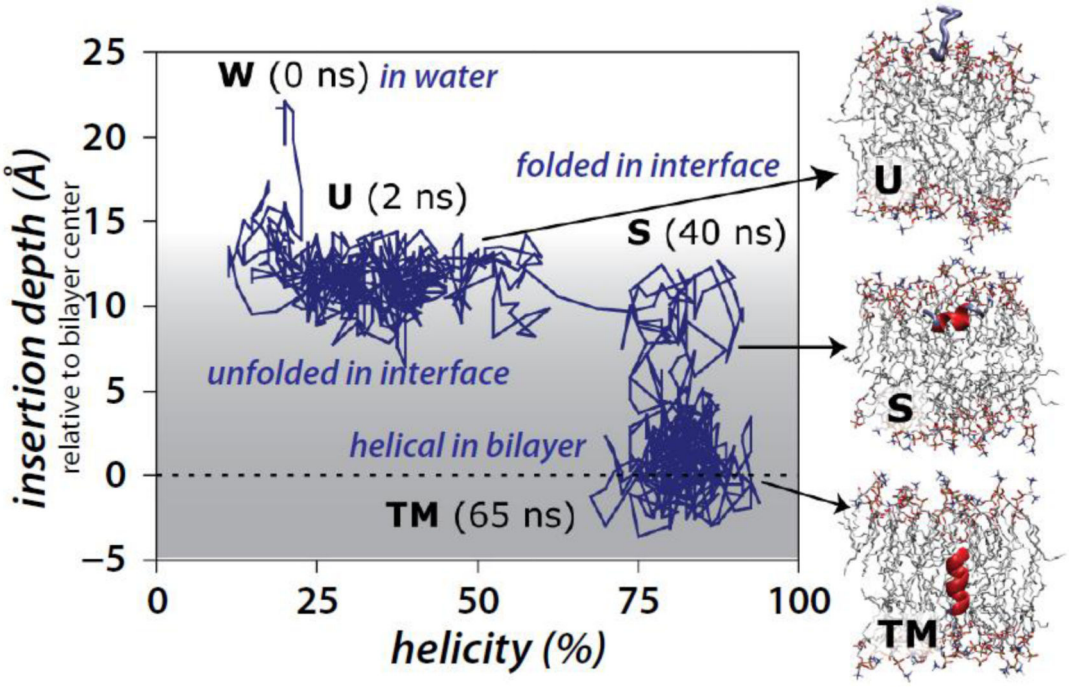

Figure 6.

Equilibrium microsecond-scale simulations of the folding and membrane insertion of polyleucine sequences (here 10 leucines) reveal only two states [39]. The simulation shown here begins with the unfolded peptide (U) in water (W) about 10 Å from the phosphatidylcholine bilayer (grey). Within a few nanoseconds (ns) it absorbs to the membrane interface and never returns to the bulk water phase. After the next 40 ns, the peptide becomes α-helical and fluctuates between being on the surface (S) and across the membrane (TM) during the ensuing several microseconds. The trajectory of the simulation is represented as a plot of the insertion depth of the peptide's center-of-mass against its helicity. The sampled time points are connected sequentially by blue lines. Modified from [39]. This simulation reveals the importance of the membrane interface in membrane protein folding, and suggests that the interface may play a role in translocon-guided insertion of TM helices.

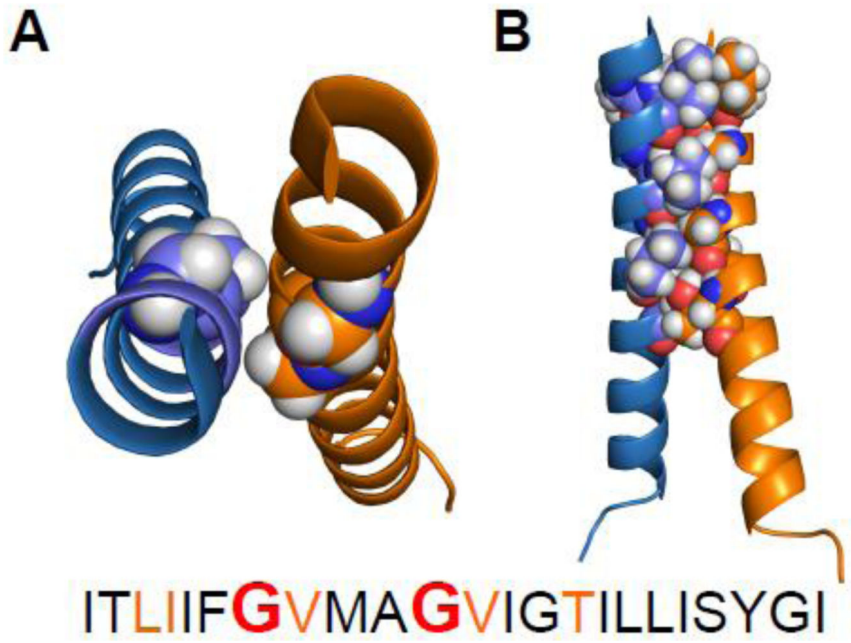

Figure 7.

Helix-helix interactions of the glycophorin A (GpA) dimer (blue and orange helices) based upon the structure of GpA in SDS [47]. A. Glycine (or other small residues) separated by three residues allow the helices to pack tightly. B. Other amino acids in the structural vicinity of the GXXXG motif can facilitate or inhibit specific binding of complementary surfaces [120].

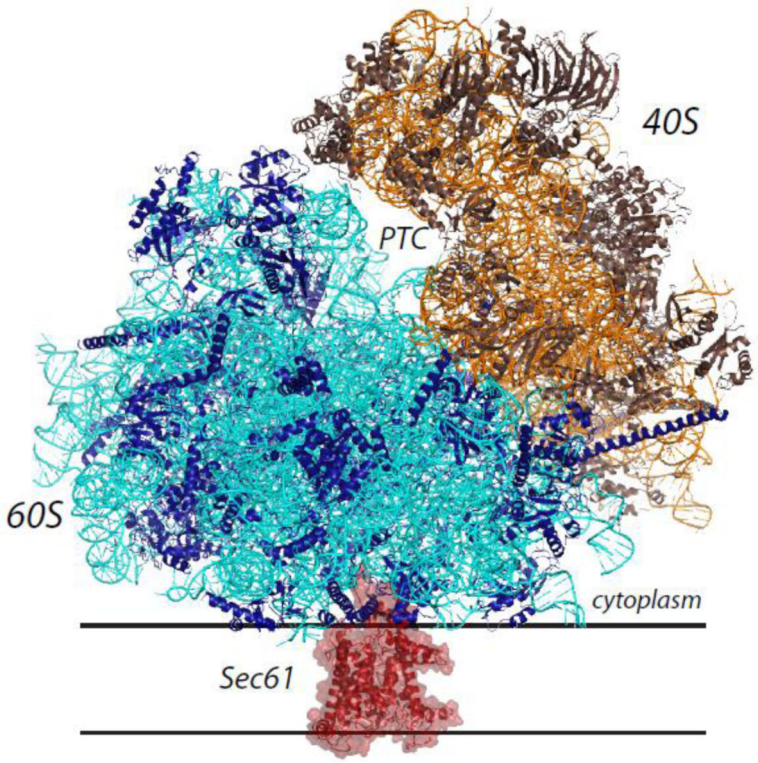

Figure 8.

High-resolution structure of a mammalian ribosome-Sec61 complex. The structure was obtained using advanced cryo-EM methods. PTC indicates the peptidyl transferase center. Image from Voorhees et al. [164].

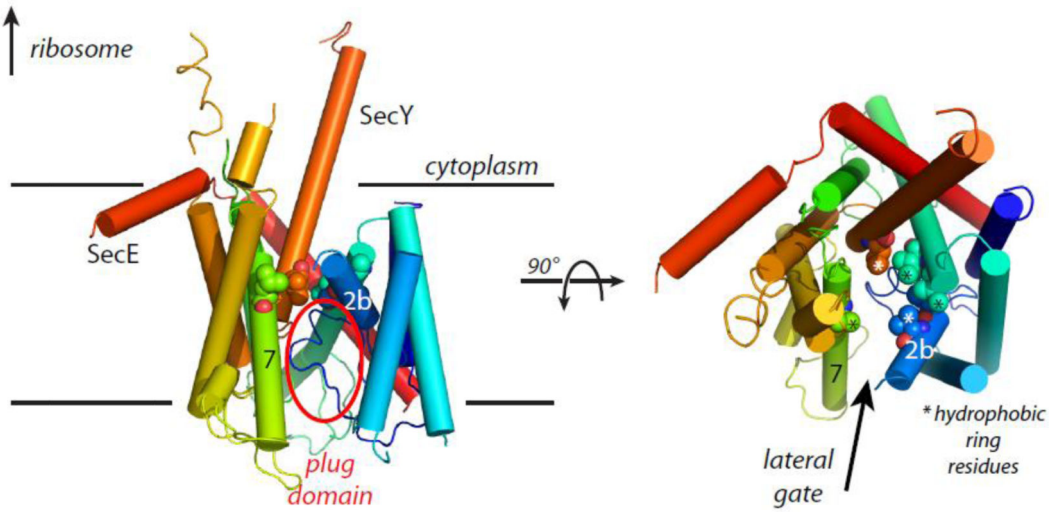

Figure 9.

Structure of the SecYE translocon from *Pyrococcus furiosus* [80]. The left panel shows a view in the plane of the membrane, the right panel shows a view from the cytoplasm. The plug domain is circled, and the residues in the hydrophobic ring are shown as van der Waals spheres, indicated by *. The arrow points into the lateral gate between TMH2b and TMH7. SecE is shown in red, SecY is color coded from N-terminus (blue) to C-terminus (orange).

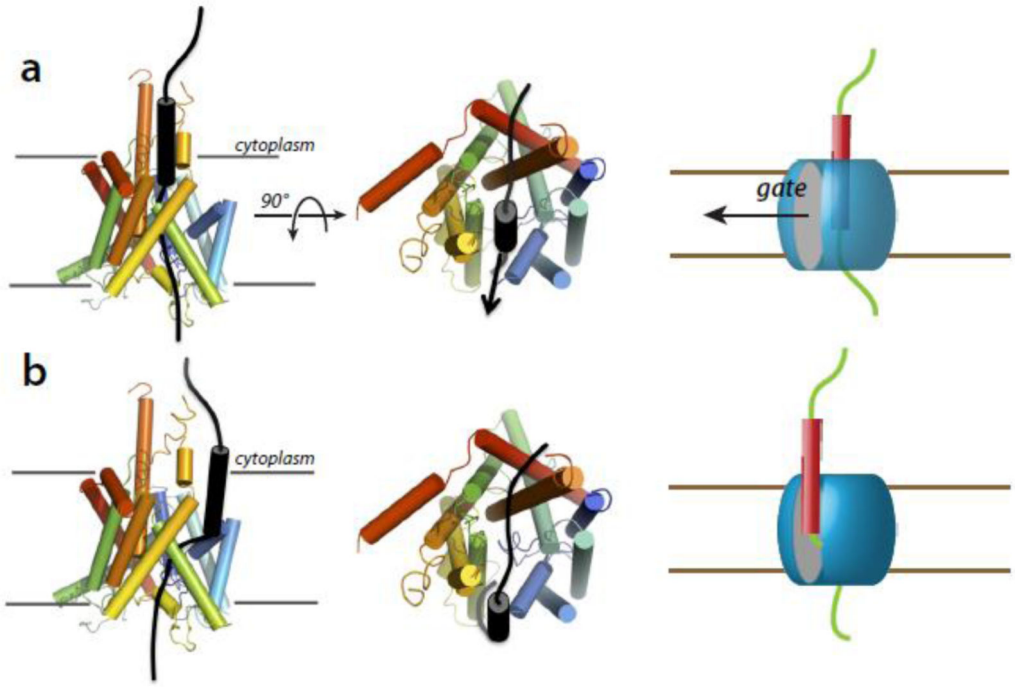

Figure 10.

Two models for translocon-mediated insertion of a N_{out}-C_{in} orientated TMH in a single-span (type I) membrane protein. (a) The “In-out” model”. The TMH (in black) first moves all the way into the central translocon channel, and then exits sideways through the lateral gate. (**b**) The “sliding” model. The TMH slides along the lateral gate into the membrane, with one side exposed to lipid at all times. The leading polar segment penetrates through the lateral gate and is shielded from lipid contact. SecE is shown in red, SecY is color coded from N-terminus (blue) to C-terminus (orange). On the right-hand side a schematic of the translocon (blue) interacting with a membrane protein (green) and a membrane inserting TMH (red) are shown.

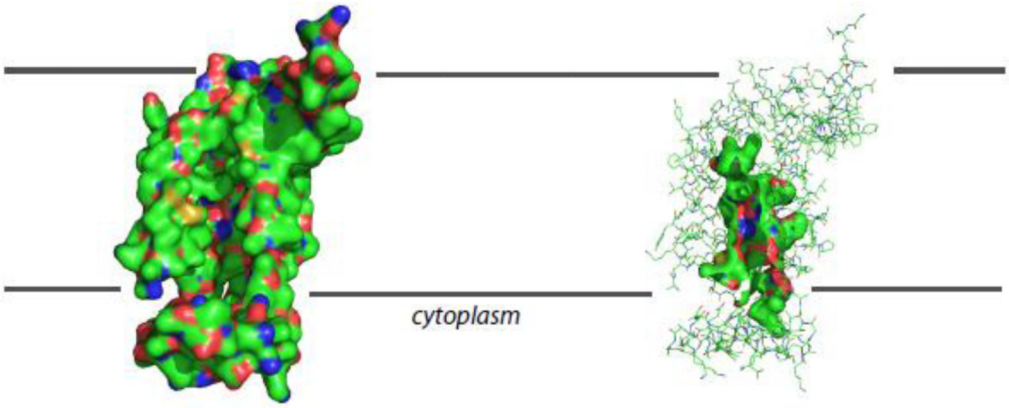

Figure 11.

Structure of the YidC translocon from *Bacillus halodurans* [96] viewed in the plane of the membrane, looking into the hydrated cleft in the center of the protein. The left panel shows a surface representation; the right panel shows the walls of the cavity, with the rest of the protein in stick representation.

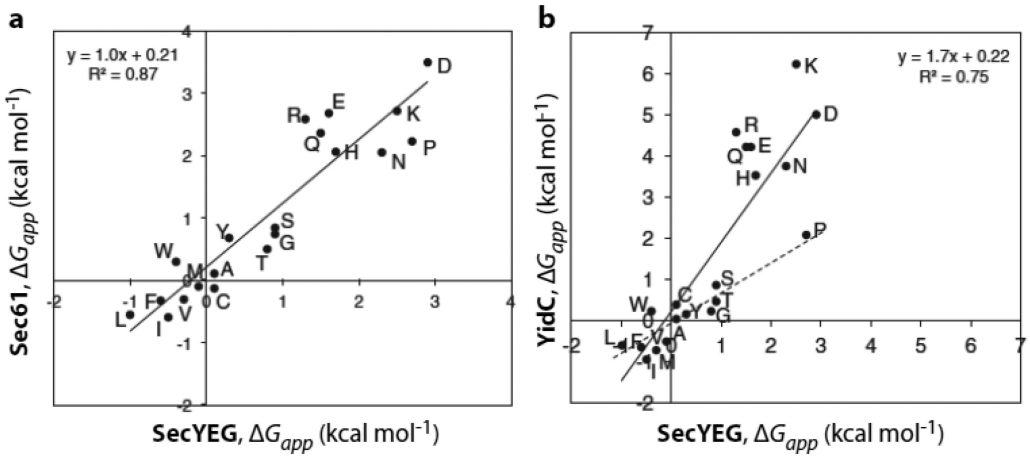

Figure 12.

Amino acid ΔG_{app} values for Sec61-, SecYEG-, and YidC-dependent membrane insertion of a model TMH [84, 85, 102]. The SecYEG (panel a) and YidC (panel b) data are plotted against the Sec61 data. Full lines indicate linear fits to the data (with the equations given in the panels), while the broken line in panel b is the linear fit obtained when including only non-polar and weakly polar residues (slope = 0.8) [85].

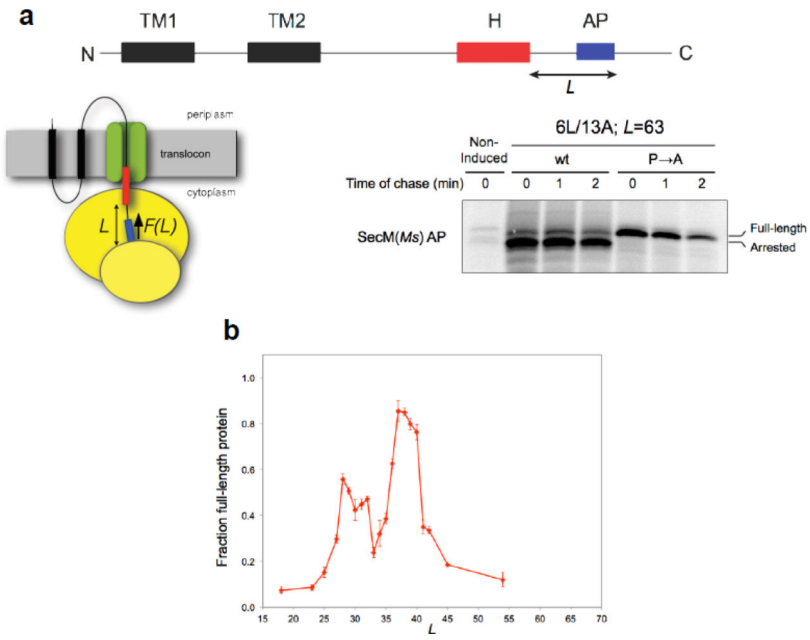

Figure 13.

Using APs as *in vivo* force sensors [106]. (**a**) An AP (AP; in blue) is inserted into a membrane protein with two natural TMHs (TM1, TM2; in black) and a model TMH composed of six leucines and 13 alanines (H; in red). Depending on the length *L* of the tether between the H-segment and the AP, the H-segment will be in different locations relative to the translocon at the time when the ribosome reaches the last codon in the AP, as shown in the cartoon. The pulling force *F*(*L*) on the nascent chain will determine the fraction of stalled vs. full-length protein produced. The fraction full-length protein can be determined by [^{35}S]-Met pulse-labeling of growing *E. coli*, followed by immunoprecipitation of the protein construct and analysis by SDS-PAGE, as shown on the right for a construct with *L*=63 residues (a control construct in which a critical proline in the AP has been mutated to alanine, preventing stalling, is also shown). (**b**) Fraction full-length protein as a function of *L* for a set of constructs designed as shown in panel **a** (top).

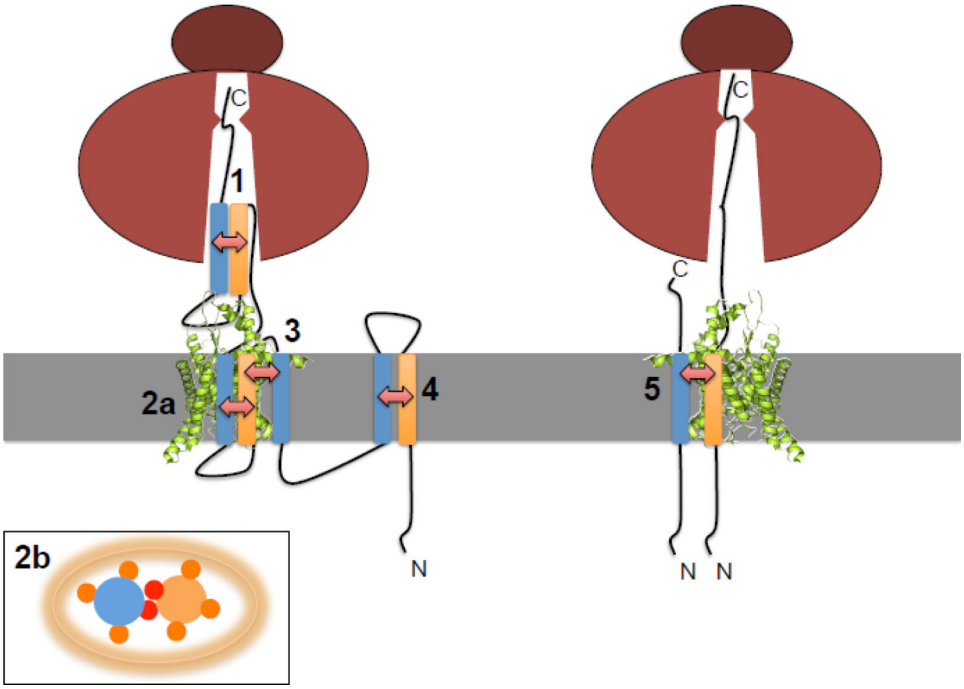

Figure 14.

Folding spaces during membrane protein structure formation. During nascent chain synthesis by the ribosome (brown), helical segments (shown in blue and orange) can associate already in the ribosomal vestibule (1) [136] or within the translocon channel (2a, green) [138], where polar residues can be shielded within the helix-helix interface (2b, red spheres) and hydrophobic side-chains (orange spheres) form a membrane insertion competent surface. Helices can furthermore interact during insertion into a membrane (3, grey) whereas one segment is already inserted into the membrane and drives the insertion of a less hydrophobic segment by shielding hydrophilic residues within the interaction interface [165]. Interactions of helices within different proteins can also occur co-translationally (5) and was shown do drive membrane protein complex assembly [149].

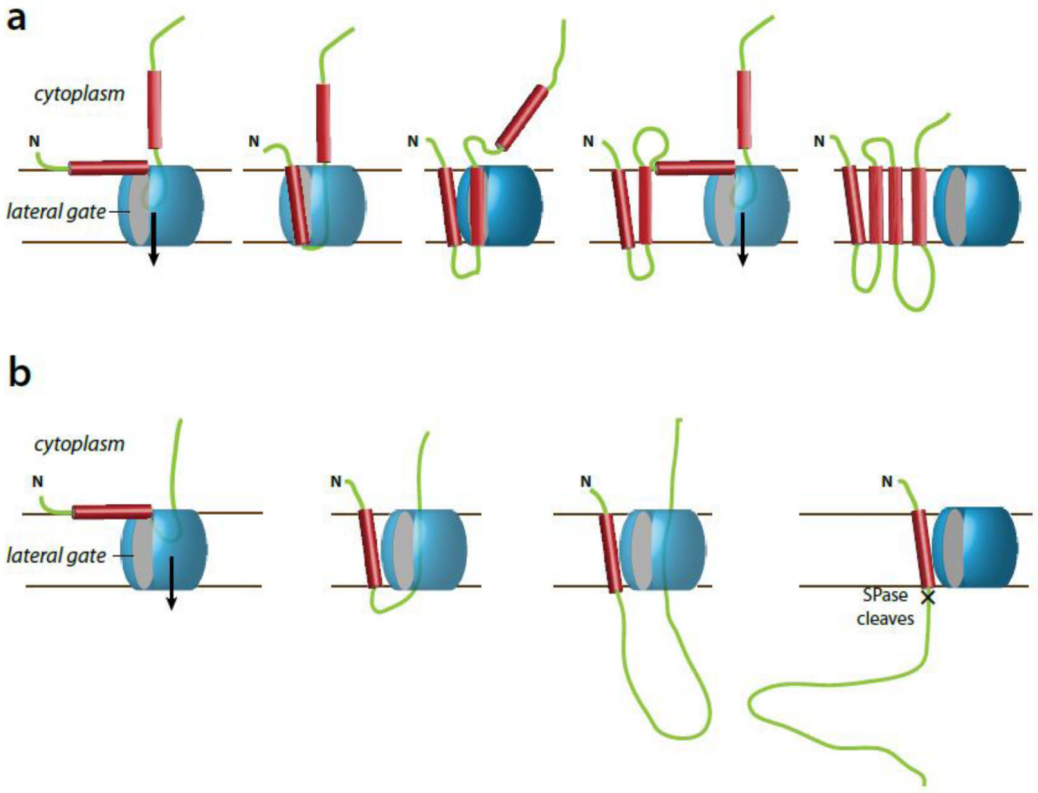

Figure 15.

An alternative view of translocon-aided insertion of multi-span membrane proteins and the secretion of soluble proteins. The idea of this alternative view is shown in a cartoon fashion in panel **a** (also see Fig. 10). We suggest that initial contact of the nascent chain (green, with red TMHs) is with the membrane interface in the vicinity of the translocon (blue), and that the chain does not immediately thread into the translocon. Rather, the translocon has YidC-like behavior; it provides a pathway for polar components of membrane proteins to cross the membrane. This is not to say that it cannot form a passageway through the membrane, but we suggest that it does this only for polar polypeptide segments in membrane proteins and for secreted proteins, as shown in panel **b**.