Recent advances in the understanding of membrane protein assembly and structure

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1. Introduction

For a variety of reasons – not the least biomedical importance – integral membrane proteins are now very much in focus in many areas of molecular biology, biochemistry, biophysics, and cell biology. Our understanding of the basic processes of membrane protein assembly, folding, and structure has grown significantly in recent times, both as a result of new methodological developments, more high-resolution structure data, and the possibility to analyze membrane proteins on a genome-wide scale.

So what is new in the membrane protein field? Various aspects of membrane protein assembly and structure have been reviewed over the past few years (Cowan & Rosenbusch, 1994; Hegde & Lingappa, 1997; Lanyi, 1997; von Heijne, 1997; Bernstein, 1998); here, I will try to bring together a number of exciting recent developments. Particularly noteworthy are the discoveries related to the mechanisms of membrane protein assembly into the inner membrane of *E. coli*, the inner membrane of mitochondria, and the way transmembrane segments are handled by the ER translocon.

Other advances include detailed studies of the interaction between transmembrane helices and the lipid bilayer, and of helix–helix packing interactions in the membrane environment. The availability of full genomic sequences have made it possible to study membrane proteins on a genome-wide scale. Finally, a handful of new high-resolution 3D structures have appeared.

This review will deal only with helix bundle proteins, i.e. integral membrane proteins where the transmembrane segments form α-helices. For reviews on the other major class of integral membrane proteins – the β-barrel proteins – see Schirmer (1998) and Buchanan (1999). For readers who prefer a more ‘literary’ introduction to the membrane protein field, may I suggest von Heijne (1999).

2. Membrane protein assembly in *E. coli*

Almost all we know about membrane protein assembly is based on studies either in *E. coli* (mainly in vivo studies) or in the mammalian ER (mainly in vitro studies). In both systems, the
knowledge about membrane protein biogenesis has lagged behind studies on secretory proteins, but now that the basics of protein secretion seem to have been worked out more attention is being paid to the membrane proteins. Most membrane proteins use the same translocation machinery as the secretory proteins (Oliver et al. 1995; de Gier et al. 1997; Bernstein, 1998; Bibi, 1998), though certain aspects of this machinery are peculiar to membrane protein biogenesis.

For E. coli, recent major advances in our understanding of membrane protein assembly include a better definition of the role of the E. coli signal recognition particle (SRP), the identification of a new component of the translocation machinery (YidC) that may be particularly important for membrane protein biogenesis, and new results on the assembly of membrane proteins that appear not to require a fully functional translocation machinery for membrane insertion.

2.1 Role of the SRP

The basic protein translocation machinery in the E. coli inner membrane is composed of the SecYEG translocon, and the associated inner membrane proteins SecD, SecF, and YajC. In addition, the peripherally associated SecA ATPase and the cytoplasmic components SecB, SRP (composed of the Ffh protein and a 4-5S RNA), and the SRP receptor FtsY are important participants in the secretory pathway (Ito, 1996; Polschroeder et al. 1997; Fekkes & Driessen, 1999).

While the role of the Sec components is quite well understood, the SRP and its receptor FtsY have come into focus only recently. When the Ffh and 4-5S RNA components of the SRP were initially identified, it came as somewhat of a surprise that their inactivation had only very mild effects on the translocation of many secretory and outer membrane proteins (Luirink et al. 1992). More recent studies on membrane protein assembly have provided at least a partial answer: the E. coli SRP/SRP receptor pathway seems to preferentially recognize the strongly hydrophobic transmembrane segments of inner membrane proteins rather than the normally less hydrophobic signal peptides of secretory proteins (MacFarlane & Müller, 1995; de Gier et al. 1996; Seluanov & Bibi, 1997; Ulbrandt et al. 1997; Valent et al. 1997).

Beyond this, the SRP/SRP receptor pathway leading ultimately to the Sec translocon seems to work according to similar principles in E. coli and in the eukaryotic ER, and the membrane attachment of FtsY has been shown to be absolutely required for its function (Zelazny et al. 1997). However, although most proteins that interact with SRP seem to end up at the Sec translocon (Valent et al. 1998), it appears that this may not be an obligatory delivery station for SRP-targeted proteins (Cristobal et al. 1999b).

2.2 YidC – a translocon component devoted to membrane proteins?

Rather unexpectedly, no mitochondrial Sec-like components were found in the S. cerevisiae genome sequence (Glick & von Heijne, 1996), raising the question of how mitochondrial inner membrane proteins are inserted into the membrane. Genetic studies have so far uncovered two proteins that appear to have a role in the assembly of mitochondrial inner membrane proteins: Oxa1p (Hell et al. 1997, 1998; Herrmann et al. 1997) and Pnt1p (He & Fox, 1999).

Interestingly, Oxa1p homologues are found in a wide variety of bacteria, including E. coli (Hell et al. 1998; Sääf et al. 1998), suggesting a conserved function. Very recent studies have
identified the *E. coli* Oxa1p homologue, YidC, as a component that can be co-purified with the Sec translocon, and have further shown that nascent inner membrane proteins can be crosslinked to YidC as well as to SecY in inner membrane vesicles (Scotti *et al*. 2000). Although no functional data on YidC have been obtained yet, these observations suggest the exciting possibility that YidC may be an important component of the Sec translocon and, even more intriguingly, that Oxa1p may be able to support the assembly of mitochondrial inner membrane proteins even in the absence of the other Sec components.

The role of Pnt1p is not well understood – *pnt1* knockouts only partially prevent cytochrome oxidase assembly in *S. cerevisiae*, although the effect is stronger in *K. lactis* (He & Fox, 1999) – and there are no obvious bacterial homologues.

2.3 The TAT pathway

The so-called ‘twin arginine translocation’ or TAT pathway was initially discovered during studies of protein translocation into thylakoids where it was found that certain proteins with a signal peptide containing a typical Arg–Arg motif were translocated via a ∆pH-dependent but Sec-translocon independent translocation mechanism (Mould & Robinson, 1991; Klösgen *et al*. 1992; Robinson *et al*. 1994; Chaddock *et al*. 1995; Creighton *et al*. 1995; Hynds *et al*. 1998). Similar twin-Arg signal peptides were also noted to be present on bacterial cofactor binding periplasmic proteins that were thought to be translocated in a folded state (Berks, 1996). Genetic studies in maize and *E. coli* recently uncovered a set of *tat* genes encoding components of this machinery (Settles *et al*. 1997; Bogsch *et al*. 1998; Santini *et al*. 1998; Sargent *et al*. 1998).

Since the TAT machinery can translocate folded proteins, it would be very interesting to know how it would handle inner membrane proteins. A first attempt to re-route an *E. coli* inner membrane protein (Lep) to the TAT pathway by the addition of a twin-Arg signal peptide was unsuccessful: although the TAT signal peptide targeted a periplasmic domain from Lep to the TAT pathway, the hydrophobic transmembrane segments in full-length Lep apparently overrode the TAT signal peptide, and the fusion protein was inserted by the normal Sec pathway (Cristobal *et al*. 1999a). Whether inner membrane proteins can be assembled by the TAT machinery thus remains unknown.

2.4 ‘Spontaneous’ membrane protein insertion

The possibility that certain membrane proteins may insert spontaneously into the lipid bilayer, i.e. without the need for a translocon, has been discussed repeatedly over the years (von Heijne, 1994). Obviously, *in vivo* studies can only suggest, but cannot prove, a fully spontaneous insertion mechanism, and bacterial *in vitro* translocation/membrane insertion systems reconstituted from pure components do not yet exist.

Not surprisingly, the availability of new mutant strains with stronger phenotypes have uncovered dependencies on the Sec translocon for proteins that were once thought to insert spontaneously (de Gier *et al*. 1998; Cristobal *et al*. 1999b), and it now appears that the large majority of the inner membrane proteins in *E. coli* are targeted by the SRP/SRP receptor to the SecYEG translocon (de Gier *et al*. 1997). Only the phage M13 procoat and Pf3 coat proteins have so far withstood all attempts to demonstrate SRP or SecAYEG dependence (Cristobal *et al*. 1999b; Kiefer & Kuhn, 1999); time will tell if these proteins indeed insert
spontaneously into the inner membrane in vivo. It should be noted that certain thylakoid membrane proteins are also believed to insert spontaneously (van Wijk et al. 1995; Kim et al. 1998, 1999; Thompson et al. 1998).

3. Membrane protein assembly in the ER

With the ER translocon now rather well characterized, both genetically and biochemically (Rapoport et al. 1996; Johnson, 1997), interest has recently turned to its role in membrane protein assembly (Bibi, 1998; Hegde & Lingappa, 1999). In particular, site-directed chemical crosslinking as well as quenching studies on fluorescent probes introduced into nascent membrane proteins have provided a first glimpse of the process whereby hydrophobic transmembrane segments are moved laterally from the translocation channel into the surrounding lipid bilayer. Studies on the topology of membrane protein constructs containing parts with conflicting topological information have further revealed a previously unsuspected complexity in membrane protein topogenesis. Although no clear understanding of this important process has emerged so far, it is already obvious that the ribosome/ER translocon complex reacts to the presence of hydrophobic segments in the nascent polypeptide chain, and to additional kinds of topogenic information such as flanking charged residues and so-called stop-transfer effector (STE) sequences.

3.1 How TM segments exit the translocon

Since the lumenal parts of integral membrane proteins are translocated through the ER translocation channel while the ribosome is attached to the cytoplasmic channel entrance, it is clear that transmembrane segments in the nascent chain emerging from the ribosome at some point must exit laterally from the channel into the surrounding lipid bilayer.

Different studies have come to different conclusions regarding the timing of this event. Thus, nearly full-length, ribosome-attached nascent opsin molecules can be extracted from microsomal membranes by urea, implying that membrane integration is a late event (Borel & Simon, 1996). More recent studies using site-specific crosslinking or fluorescent probes attached to specific sites in the nascent chain on the contrary suggest that transmembrane segments may exit rapidly from the translocon. Thus, the N-terminal transmembrane segment in the E. coli inner membrane protein Lep was found to efficiently form lipid crosslinks already when the nascent chain was just barely long enough to allow the TM segment entry into the ER translocon (Mothes et al. 1997). In another crosslinking study, a TM segment was found first in the vicinity of Sec61α at short chain lengths, then close to TRAM, and finally in a lipid environment when the chain was released from the ribosome (Do et al. 1996), suggesting an ordered process where a TM segment moves through a ‘lateral exit pathway’ in the translocon.

Studies with fluorescent probes in the TM segment have provided further insights into the exit process. Surprisingly, while quenchers added to the cytosolic side of the microsomal membrane cannot quench probes in the nascent chain during translocation of a secretory protein (Crowley et al. 1993, 1994) – thus suggesting a tight ribosome/translocon junction – the appearance of a hydrophobic TM segment inside the ribosome appears to loosen the ribosome/translocon interaction and allow passage of quenchers from the cytosol into the nascent chain conduit in the large ribosomal subunit (Liao et al. 1997; Hamman et al. 1998).
Fig. 1. Model for membrane protein integration into the ER membrane (Mothes et al. 1997). The appearance of a hydrophobic segment (black) within the ribosome–translocon channel causes the channel to close and the segment moves laterally into the lipid bilayer (I). The nascent chain loops out towards the cytoplasm (II). The appearance of a second hydrophobic segment induces a re-opening of the translocation channel and translocation of the downstream sequence resumes (III). The second segment moves out into the bilayer (IV).

Proteolysis studies of the Lep protein referred to above further show that portions of the nascent chain following a TM segment become exposed outside the ribosome (Mothes et al. 1997). Together, these studies suggest a model for the membrane integration of TM segments where the ribosome/translocon complex alternates between a tightly bound state where portions of the nascent chain are extruded through the translocation channel, and a more loosely bound state where the nascent chain loops out into the cytosol (Fig. 1). Note, however, that it is not known whether all or just some TM segments can exit by themselves, or if pairs or even bundles of TM helices can assemble in the translocon before membrane integration.

3.2 Proteins with multiple topologies

As topology studies have been carried out on more integral membrane proteins, a number of examples of ‘topological heterogeneity’ have been found (Levy, 1996). Perhaps the most dramatic is that of ductin, a protein with four TM helices that can serve both as a component of a connexon channel of gap junctions and as a subunit in a V-type ATPase. The two functions are carried out by identical molecules with opposite membrane orientations, and ductin has indeed been shown to insert with an approximately equimolar ratio of $N_{\text{in}}$–$C_{\text{in}}$ and $N_{\text{out}}$–$C_{\text{out}}$ oriented molecules in microsomes (Dunlop et al. 1995). This ratio is not affected by mutations that alter the number of positively charged residues in the N-terminal tail, and the sequence determinants responsible for the mixed topology have not been identified. Populations with ‘inverted’ membrane orientation have also been seen for epoxide hydrolase (Zhu et al. 1999) and certain members of the cytochrome P450 family (Wu & Cederbaum, 1992; Loeper et al. 1998b; Neve & Ingelman-Sundberg, 2000). In these latter cases, the balance between the two orientations is strongly affected by charged residues flanking the TM segment (Sato et al. 1990; Zhu et al. 1999). There are conflicting views on whether molecules with a luminal or cytosolic orientation of the catalytic domain are differentially exported to the plasma membrane (Loeper et al. 1998a; Zhu et al. 1999).
For the multidrug-resistance protein P-glycoprotein (Pgp) and the cystic fibrosis transmembrane conductance regulator (CFTR), mixed topologies where particular hydrophobic segments are inserted into the membrane only in a fraction of all molecules have been detected (Han & Zhang, 1998; Zhang et al. 1998a; Chen & Zhang, 1999). It is not entirely clear what the topological determinants are in these cases, although charged residues and overall hydrophobicity of the TM segment have been implicated to play a role. Whether the mixed topologies seen for these proteins have any functional relevance is not clear.

In *E. coli*, the SecG component of the Sec translocon, a protein with two TM segments, apparently re-orientates itself across the membrane during the catalytic cycle of the SecA component (Nishiyama et al. 1996), suggesting that the topology of a membrane protein may change in a dynamic fashion.

In another recent study, it was found that a polar N-terminal tail in a multi-spanning membrane protein may initially be translocated across the membrane, only to be returned to the cytosolic side at a later stage of the membrane insertion process (Goder et al. 1999). A related example is the large envelope protein from the hepatitis B virus, where the N-terminal preS domain appears to cross the membrane not when the protein inserts into the ER but at a later stage during virus maturation (Bruss et al. 1994).

What these examples show is both that a single polypeptide chain may adopt multiple topologies and that the topology may not in all cases be totally fixed during the initial membrane insertion step. Nevertheless, the number of examples of such ‘non-classical’ behaviour is still small, and it is likely that the large majority of all membrane proteins have only a single, fixed topology.

### 3.3 Stop-transfer effector sequences

Stop-transfer effector (STE) sequences are short stretches of predominantly acidic residues that affect the efficiency with which a downstream hydrophobic stretch integrates into the ER membrane as a transmembrane helix. So far, STE sequences have been identified in murine IgM (Andrews et al. 1992) and in the prion-related protein PrP (Lopez et al. 1990; De Fea et al. 1994). The mode of action of STE sequences is poorly characterized, though their acidic character seems to be important (Falcone et al. 1999).

### 3.4 Non-hydrophobic TM segments?

Recent topology studies of a chimeric protein containing two TM segments, each of which normally orients itself Nlum–Ccyt, have provided evidence that non-hydrophobic segments may be ‘forced’ into a transmembrane disposition (Ota et al. 1998). Although this study was carried out on a heavily engineered chimeric protein, it nevertheless demonstrates that the view that TM segments are invariably hydrophobic may not be entirely correct. Whether natural examples of this kind exist is still unknown, however.

### 3.5 ‘Frustrated’ topologies

While conflicting topological determinants apparently can force non-hydrophobic segments to cross the membrane, such topological conflicts can also cause hydrophobic segments not to insert across the membrane. This was shown by designing ‘topologically frustrated’
proteins, where the distribution of positively charged residues in different parts of the molecule was such that ‘leave-one-out’ topologies with one hydrophobic stretch left outside the membrane were formed (Gafvelin & von Heijne, 1994; Gafvelin et al. 1997).

3.6 N-tail translocation across the ER

One aspect of membrane protein assembly that has not received much attention is the process whereby in some proteins an N-terminal tail is translocated across the membrane without the aid of an upstream signal peptide (Dalbey et al. 1995). Studies in E. coli have shown that such N-tail translocation is very sensitive to the presence of positively charged residues in the tail, and that it apparently does not necessarily depend on the Sec machinery (Whitley et al. 1994, 1995; Cristobal et al. 1999b).

Recent work using in vitro translation in the presence of mammalian microsomes have provided the first direct evidence that N-tail translocation is initiated from the first downstream TM segment and proceeds in a C-to-N-terminal direction (Monné et al. 1999a; Nilsson et al. 2000). Further, the efficiency of N-tail translocation can be influenced by as many as four downstream TM segments, demonstrating that the topological information in the nascent chain may be ‘non-local’ in the sense that a number of TM segments need to be presented to the translocation machinery before the final localization of the N-tail is decided (Monné et al. 1999a; Nilsson et al. 2000). Whether this is because more N-terminal TM segments may be retracted from the lipid bilayer into the translocon, or that a number of TM segments can be ‘stored’ in the translocon before they are released into the membrane, is not known.

4. Membrane protein assembly in mitochondria

Mitochondria are particularly interesting from the point of view of membrane protein assembly because they not only synthesize some inner membrane proteins themselves but also import a large number of inner membrane proteins from the cytosol (Stuart & Neupert, 1996). Recent developments in this field include the initial discovery of the important role of the Oxa1p protein, and a new import/assembly pathway utilized by inner membrane proteins of the mitochondrial carrier protein family.

4.1 The Oxa1p pathway

A role of the Oxa1p protein for the assembly of certain inner membrane proteins was first suggested in 1997 (He & Fox, 1997; Hell et al. 1997). In yeast oxa1 mutants, the large C-terminal domain of cytochrome oxidase subunit II cannot be translocated across the inner membrane (Hell et al. 1997). Similarly, Oxa1p itself, a protein with five putative transmembrane segments and with the N-tail located in the inter-membrane space (Säätä et al. 1998), is not properly assembled in oxa1 mutant cells (Herrmann et al. 1997). Oxa1p appears to be part of a large protein complex, but no further components of this complex have been identified so far (Hell et al. 1998).

If YidC, the E. coli homologue of Oxa1p, is indeed a functional component of the SecYEG complex as discussed above, the loss of SecYEG but not Oxa1p from S. cerevisiae...
mitochondria suggests that Oxa1p may be able to function as a ‘mini-translocon’, either as a homo- or hetero-oligomeric complex. Interestingly, the positive-inside rule (see below) has been found to apply to mitochondrial inner membrane proteins that need Oxa1p for assembly (Rojo et al. 1999).

4.2 The TIM22/54 pathway

Members of the major mitochondrial carrier protein family in the mitochondrial inner membrane (including the ADP/ATP carrier) all have a canonical topology with six TM segments and the N- and C-termini facing the inter-membrane space. In addition, they seem to have arisen from a threefold duplication event such that the TM1–2, TM3–4, and TM5–6 regions show a clear sequence similarity to each other.

Studies on the import and membrane assembly of \textit{S. cerevisiae} carrier proteins have recently uncovered a new branch of the import pathway, leading from the outer membrane translocation complex utilized by nearly all imported mitochondrial proteins to complexes of inter-membrane space proteins (the TIM9/10/12 and the TIM8/9/13 complexes) and to inner membrane components (the TIM22/54 complex) (Koehler et al. 1998a,b; Sirrenberg et al. 1998; Adam et al. 1999; Endres et al. 1999; Leuenberger et al. 1999). Translocation of the positively charged TM5/6 loop of the ADP/ATP carrier to the matrix side requires an electrochemical gradient (negative inside) across the inner membrane, and is thought to be driven by an electrophoretic mechanism (Endres et al. 1999). Thus, although inserted from the outside of the inner membrane, the mitochondrial carrier proteins also follow the positive-inside rule.

5. Evolution of membrane protein topology

How can Nature evolve membrane proteins with different topologies? So far as is known, the two major topological determinants both in prokaryotic and eukaryotic membrane proteins are the hydrophobic stretches that ultimately form the transmembrane helices (and that often ensure targeting to the bacterial inner membrane or the ER membrane) and the positively charged residues in the regions flanking the hydrophobic stretches (the positive-inside rule). The importance of these two features for proper membrane assembly has been demonstrated beyond doubt, and many ‘topology engineering’ experiments have shown that the topology of model proteins can be manipulated by the insertion or deletion of hydrophobic stretches and by redistribution of positively charged residues between different flanking loops (von Heijne, 1996). Only very recently, however, has it been possible to demonstrate that homologous proteins have evolved different topologies using precisely the same strategies as have been applied in the laboratory.

5.1 RnfA/RnfE – two homologous proteins with opposite topologies

The first clear example of divergent evolution of membrane protein topology was recently discovered (Sääf et al. 1999). In this study, theoretical topology predictions on the RnfA/RnfE family of evolutionarily related proteins suggested that the RnfA homologues differed from the RnfE homologues in their orientation across the membrane (N\textsubscript{out}–C\textsubscript{out} vs N\textsubscript{in}–C\textsubscript{in}), but not in the number of TM segments (six in each). Experimental mapping of the topologies of the \textit{E. coli} homologues of the RnfA and RnfE proteins using PhoA fusions
confirmed this prediction (Fig. 2). Strikingly, in RnfA and its homologues, the TM1/2, TM3/4, and TM5/6 loops are rich in lysine and arginine, whereas in the RnfE family the N- and C-tails as well as the TM2/3 and TM4/5 loops carry more positive charge. In contrast, there is no consistent difference in the distribution of negatively charged residues between the two families. Thus, the evolution of the two opposing topologies apparently has been achieved by a gene duplication followed by selective re-distribution of positively charged amino acids between the polar segments flanking the transmembrane stretches.

5.2 YrbG – duplicating an odd number of TMs

While the RnfA/RnfE pair of homologous proteins show that two proteins can evolve opposite membrane orientations after a gene duplication event, we have also found an example where an internal duplication has led to the evolution of a protein where the N- and C-terminal halves of the protein have opposite topologies. The *E. coli* YrbG protein belongs to a large family of Na⁺/Ca²⁺ transporters with both prokaryotic and eukaryotic members. All members of this family show clear evidence of an internal duplication. Theoretical topology predictions suggest a duplicated topology with 5+5 TM segments, implying that the N- and C-terminal halves have opposite orientations across the membrane.

Again, experimental mapping of the topology of the *E. coli* member of the family using PhoA fusions confirmed the prediction (Baars, Sääf & von Heijne, in preparation), providing evidence that re-distribution of positively charged residues have led to changes in the topology of a protein domain over evolutionary times.
A second example of a protein family with two homologous but oppositely oriented membrane domains is provided by the major intrinsic proteins (MIPs), including the aquaporins. In this case the topology is 3+3 transmembrane helices, with low but detectable sequence similarity between the two halves of the protein (Reizer et al. 1993). The bias in Arg+Lys content between the cytoplasmic and non-cytoplasmic sides of the E. coli MIP protein is not as dramatic as for YrbG, however.

6. Genome-wide analysis of membrane proteins

With the recent explosion in genome sequencing and the rather reliable topology prediction methods now available, it has become possible to collect statistics on membrane proteins on a genome-wide scale. Questions addressed so far include estimates of the total number of integral membrane proteins encoded in a given genome, the conformity to the positive-inside rule, and identification of dominant classes of membrane proteins in different organisms.

6.1 Prediction methods

Three major prediction problems can be identified in the membrane protein field: (i) discrimination between membrane proteins and non-membrane proteins, (ii) prediction of membrane protein topology, and (iii) prediction of membrane protein 3D structure. No general-purpose method for 3D-structure prediction exists today, whereas good methods have been developed both for (i) and (ii).

In principle, most modern topology prediction methods rely on the ‘hydrophobicity-charge’ paradigm, i.e. they try to identify the transmembrane segments while at the same time requiring that the predicted topology conforms to the positive-inside rule. Simple prediction programs such as TOPPRED (von Heijne, 1992; Claros & von Heijne, 1994) have now been superseded by computationally more advanced methods such as PHD (Rost et al. 1996), TMHMM (Sonnhammer et al. 1998) and HMMTOP (Tusnady & Simon, 1998) based on machine learning algorithms (neural networks, hidden Markov models). These methods can be used both for tasks (i) and (ii).

For discrimination between membrane and non-membrane proteins, recent work shows that TMHMM has a very low rate of both false negative and false positive predictions (on the order of a few per cent; Larsson, Sonnhammer, von Heijne & Krogh, in preparation), whereas TOPPRED has a much higher rate of false positive predictions. For topology predictions, the best programs (PHD, TMHMM and HMMTOP) reach ~75% correct predictions of full topologies, and are well over 95% correct in identifying individual transmembrane segments. Genome-wide identification of membrane proteins and prediction of their topologies is thus feasible with current methodology.

6.2 How many membrane proteins are there?

Although different authors have come to somewhat different conclusions, genome-wide predictions of the number of integral membrane proteins of the helix bundle class now seem to be converging at around 20–25% of all ORFs for most organisms (our unpublished data). One difficulty that has not yet been fully solved is to discriminate between secretory proteins
with a cleavable N-terminal signal peptide and proteins anchored in the membrane by an uncleaved N-terminal signal-anchor sequence, but these problematic cases are expected to be rather few, especially in bacteria where not many signal-anchor proteins have been found, and where most multi-spanning proteins lack cleavable signal peptides (Broome-Smith et al. 1994).

6.3 The positive-inside rule

Since the importance of the positive-inside rule has been experimentally verified only for proteins from the E. coli inner membrane, the mammalian secretory pathway, and a few mitochondrial inner membrane proteins, genome-wide statistical studies are the only reasonable way to assess its importance across a wide spectrum of species. By focusing on membrane proteins with clear-cut hydrophobicity profiles for which the number of transmembrane segments can be predicted with very high reliability, it has been possible to show that the positive-inside rule indeed appears to apply to most if not all organisms from all three kingdoms of life (Wallin & von Heijne, 1998). In fact, it has been found to hold even for an acidophilic bacterium with an inverted membrane potential (van de Vossenberg et al. 1998). The prediction methods discussed above thus can be expected to perform well across all species, although minor species-specific differences in membrane protein design may warrant the development of corresponding species-specific, properly fine-tuned prediction methods.

6.4 Dominant classes of membrane proteins

When membrane proteins are grouped according to their predicted topology (number of transmembrane segments, N_in or N_out orientation), pronounced differences are seen between different species. In general, archaea, eubacteria, fungi and plants have large assortments of transporters built on a common 12TM framework (Wallin & von Heijne, 1998; Devoto et al. 1999), whereas the membrane protein population in the worm C. elegans (and most likely other animals, including humans) is dominated by seven TM proteins (mainly G-protein coupled receptors).

Interestingly, about 50% of all membrane proteins have an even number of TM segments and N_in–C_in topology, whereas the other three combinations of N- and C-terminal locations are about equally populated (Larsson, Sonnhammer, von Heijne & Krogh, in preparation). Possibly, this may be a reflection of a preference for inserting pairs of closely spaced TM helices (‘helical hairpins’) into the membrane during assembly.

7. The structure of transmembrane α-helices

To a first approximation, transmembrane α-helices may be viewed as rather dull, regular helices composed mainly of hydrophobic residues, and thus may not evoke much enthusiasm. Perhaps for this reason, isolated transmembrane helices have not been much studied for their own sake. A couple of rather basic questions regarding transmembrane helices have nevertheless been posed recently: what is the minimal design for a ‘helical hairpin’, and how do different amino acids affect the position of a transmembrane helix relative to the lipid bilayer?
7.1 What TM helices look like

Statistical studies of membrane proteins of known 3D structure have defined the ‘typical’ TM helix (Bowie, 1997; Wallin et al. 1997). The typical length varies between 20 and 30 amino acids. The typical amino acid composition is a hydrophobic central segment rich in aliphatic residues and phenylalanine with short ‘border regions’ enriched for Trp and Tyr, followed by polar ‘caps’ often containing N- and C-terminal helix-capping residues such as Asn and Gly. Most TM helices pack against their sequential neighbouring helices at typical ridges-into-grooves packing angles. Most connecting loops are rather short, although large globular domains are sometimes found between two consecutive helices.

7.2 The ‘helical hairpin’

As noted above, the ‘helical hairpin’ – two closely spaced TM helices with a short connecting loop and overall N\textsubscript{in}–C\textsubscript{in} topology – seems to be a preferred structural element in membrane proteins from all organisms. To better define the basic requirements for helical hairpin formation, we have recently carried out a series of studies where the ‘hairpin-inducing potential’ of the 20 naturally occurring amino acids was measured in poly-Leu transmembrane segments of different lengths (Nilsson & von Heijne, 1998; Monné et al. 1999b, c). The shortest possible poly-Leu based helical hairpin was found to have the sequence L\textsubscript{PL}, suggesting that \sim 15 amino acids are required to span the hydrophobic core of a biological membrane. As expected, different amino acids have different abilities to induce the formation of a helical hairpin, with charged residues and proline being the most efficient.

Small but interesting differences in hairpin-induction were found when the loop between the two TM helices was placed either on the cytoplasmic or the extra-cytoplasmic side of the membrane, suggesting that the formation of a cytoplasmic loop requires the presence of a higher number of hairpin-inducing residues than does the formation of an extra-cytoplasmic loop (Sääf, Hermansson & von Heijne, in preparation).

7.3 Prolines in TM helices

Although proline is regarded as the classical helix-breaker, it is not uncommon in transmembrane helices. In multi-spanning proteins, around 5% of the residues in the helices are proline (Wallin et al. 1997). When embedded in the middle of a hydrophobic transmembrane stretch, prolines do not break the helix but rather induce a pronounced kink in the helical backbone (von Heijne, 1991; Sansom, 1992).

To evaluate the conformational effects of Pro residues in more detail, we have used a ‘glycosylation mapping’ approach that makes it possible to detect small changes in the position of a TM helix relative to the ER membrane. This method is based on the fact that the active site of the oligosaccharyl transferase enzyme – itself associated with the ER translocon – is held at a well-defined distance from the membrane (Nilsson & von Heijne, 1993). By using a series of engineered glycosylation acceptor sites placed \sim 10 residues away from a TM helix, small changes in the position of the helix relative to the membrane caused by, for example, point mutations in the TM segment can be detected.

With this approach, it was possible to show that prolines cause a repositioning of a model 23-residues long poly-Leu TM segment relative to the ER membrane when placed as much as 5–6 residues from either end, but not when placed more centrally (Nilsson et al. 1998). The
size of the ‘sensitive region’ was reduced in shorter poly-Leu segments, suggesting that a central stretch of ~12–13 residues in a TM segment is always helical, no matter what its amino acid composition is.

7.4 Charged residues in TM helices: the ‘snorkel’ effect

The same glycosylation mapping approach has also been used to study the effects of charged residues, both in a poly-Leu context and in a natural transmembrane segment (Monné et al. 1998; Armulik et al. 1999). Interestingly, while Asp and Glu affect the position of the poly-Leu segment relative to the membrane in a similar way as does Pro, Lys and Arg have a much weaker effect when placed near the end of the hydrophobic stretch. A possible interpretation is that the long, flexible side-chains of Arg and Lys can ‘snorkel’ up into the lipid headgroup region while the Cα is well within the hydrocarbon tail region of the membrane (Segrest et al. 1990; de Planque et al. 1999).

7.5 The ‘aromatic belt’

As noted above, the polar aromatic residues Trp and Tyr are found frequently near the ends of transmembrane helices. Studies using both biophysical approaches (Yau et al. 1998; de Planque et al. 1999) and glycosylation mapping (Braun & von Heijne, 1999) have demonstrated a clear tendency for Trp to prefer an interfacial location. Phe, in contrast, behaves like the aliphatic residues.

In conclusion, the experimental studies on the effects of proline, charged residues, and aromatic residues on the position of transmembrane helices relative to a membrane, are broadly in agreement with the statistical preferences found in the known 3D structures, and provide a basis for improvements in prediction methods that try to pinpoint the precise ends of transmembrane helices.

8. Helix–helix packing in a membrane environment

The final level of understanding in going from the sequence to the structure of membrane proteins is helix–helix packing (Popot & Engelman, 1990; Bormann & Engelman, 1992; Lemmon & Engelman, 1994). This area has lagged behind, mainly due to a lack of suitable general assays. Detailed studies on the glycoporphin A (GpA) helix–helix dimer and novel genetic assays to probe helix–helix interactions have provided a first glimpse of what the most important driving forces for helix packing in a membrane environment might be.

8.1 Lessons learnt from glycoporphin A

GpA is a homo-dimeric, single-spanning membrane protein found in the plasma membrane of red blood cells. The GpA transmembrane helix has the rather unique ability to form stable dimers in SDS, and the monomer–dimer equilibrium can be easily monitored by SDS-PAGE.

Using saturation mutagenesis, Engelman and co-workers were able to identify seven interface residues in the GpA transmembrane helix: LIXGVXGVXT (Lemmon et al. 1992a, b). Based on molecular modelling, they further suggested that the close helix–helix
contacts made possible by the Gly residues were critical for the interaction (Treutlein et al. 1992; Lemmon et al. 1994).

The model was later confirmed when the structure of the GpA dimer in detergent was solved by NMR (MacKenzie et al. 1997). In addition to the close packing made possible by the Gly residues, it was further noticed that the side-chain rotamers found in the NMR structure were all among those known to be preferred for non-interacting residues, suggesting that the GpA dimer is formed by the association of helices with pre-formed, complementary interfaces. In this way, entropic losses due to the freezing out of side chain rotamers are minimized. These ideas have been incorporated into a simple quantitative model that is able to predict the monomer–dimer equilibrium for a large set of GpA mutants (MacKenzie & Engelman, 1998). Accurate thermodynamic measurements of GpA dimerization have also been performed by analytical ultracentrifugation in various detergents and lipids (Fisher et al. 1999).

8.2 Genetic screens for helix–helix interactions

The work on GpA has been difficult to generalize since most helix–helix interactions are not stable in SDS. Recently, however, a couple of two-hybrid genetic screens for dimer formation between transmembrane helices in the E. coli inner membrane have been described (Langosch et al. 1996; Brosig & Langosch, 1998; Russ & Engelman, 1999). So far, these screens can only be applied to parallel homodimers or to heterodimers.

The GpA results obtained by SDS-PAGE have been largely confirmed by the two-hybrid screens. More interestingly, randomization of poly-Leu based transmembrane helices followed by selection of sequences that form homodimers have shown that various versions of the GXXXG motif appear to promote the formation of particularly stable homodimers (Russ & Engelman, 2000).

8.3 Statistical studies

Although the small number of known 3D membrane protein structures precludes sensitive statistical analyses of helix–helix interfaces, some progress has been made, both by detailed analysis of the 3D structures and by searching for over- and under-represented motifs in sequence databases.

A recent statistical study of a very large collection of transmembrane segments extracted from SwissProt uncovered a number of over-represented pair and triplet motifs that are hypothesized to mediate helix–helix interactions (Senes et al. 2000). In particular, variations of the GXXXG ‘GpA motif’ constitute a large fraction of the over-represented pairs and triplets. Similarly, it has been pointed out that Gly residues are often found at points of close contact between transmembrane helices in the known structures (Javadpour et al. 1999). The GpA dimer may thus be a good representative for a much larger class of helix–helix interactions, giving us some hope that efficient packing algorithms may be within reach.

8.4 Membrane protein folding

Kinetic studies of protein folding within a lipid or detergent environment have so far proved to be very difficult. The best results have been obtained with bacteriorhodopsin (BR), a protein of known 3D structure with seven transmembrane helices and a covalently bound
retinal chromophore (Booth et al. 1995, 1997; Booth, 1997; Riley et al. 1997; Curran et al. 1999). For BR, spectroscopic methods can be used to follow the change in environment of the retinal, and thus allow some aspects of the folding kinetics to be resolved. New methods to measure the thermodynamic stability of integral membrane proteins have also been developed (Lau & Bowie, 1997).

9. Recent 3D structures

Although membrane protein structure determination is still in its infancy, a couple of new high-resolution structures appear each year. The most exciting recent structure is no doubt that of the KcsA ion channel, which disclosed the structural basis for ion selectivity. Other noteworthy structures include the MscL pressure-sensitive ion channel, the cytochrome bc1 complex, and fumarate reductase. For bacteriorhodopsin – the most well-characterized integral membrane protein in terms of its biophysical properties – details of the photocycle have now been resolved by structural studies.

9.1 KcsA – the first ion channel

A major recent breakthrough in the membrane protein structure field was the determination of the structure of the tetrameric K+ channel KcsA from Streptomyces lividans (Doyle et al. 1998). The KcsA channel is thought to be similar in overall architecture to a wide range of K+ channels, both from prokaryotes and eukaryotes.

The most interesting part of the KcsA channel is the so-called selectivity filter region that is formed by a short strand–turn–helix structure that dips into the channel from the periplasmic side. Backbone oxygens from this so-called P-loop region form a constricted passage, just wide enough to efficiently desolvate K+ ions but too wide to desolvate smaller Na+ ions. As a result of this, the permeability for K+ ions is at least four orders of magnitude larger than for Na+ ions. Other ion binding sites in the channel also contribute to the overall permeation rate (Roux & MacKinnon, 1999).

9.2 MscL – sensing lateral pressure changes

MscL is a bacterial, non-selective, mechanosensitive ion channel. It is composed of five identical subunits, each contributing two transmembrane helices (Chang et al. 1998). The pore structure extends into the cytoplasm, where five α-helices, one from each subunit, form a helix bundle closely apposed to the transmembrane helices. The pore is lined by mainly polar amino acids, and has a hydrophobic occlusion near the cytoplasmic side of the membrane thought to form the gate. It has been speculated that increased lateral tension in the membrane will pull the transmembrane helices away from each other, thus opening the gate.

9.3 The cytochrome bc1 complex

The cytochrome bc1 complex from bovine mitochondria consists of 11 subunits with a total of 13 transmembrane helices (Iwata et al. 1998; Zhang et al. 1998b). The most dramatic feature of the structure (obtained from two different crystal forms) is a large movement of the Rieske
iron–sulfur subunit, which suggests that the [2Fe–2S] cluster moves electrons between the cytochrome c₅₅₃ heme and heme b₅ in cytochrome b by a swinging motion of the entire Fe–S binding domain.

9.4 Fumarate reductase

The structures of fumarate reductases from both *E. coli* and *Wolinella succinogenes* were solved almost simultaneously (Iverson et al. 1999; Lancaster et al. 1999). The structures are quite similar, except that the water-soluble subunits are differently rotated relative to the membrane anchor subunits in the two structures. Interestingly, the *E. coli* membrane anchor is composed of two subunits, each with three transmembrane helices, while the *W. succinogenes* membrane anchor is formed by a single subunit with five transmembrane helices. The latter can be thought of as having arisen by fusion of the two separate subunits with a deletion of the third transmembrane helix in one of these. This would retain the overall topology of the molecule, and provides a nice example of how membrane proteins can evolve through gene fusion without changes in topology.

9.5 Bacteriorhodopsin – watching a membrane protein at work

Two landmark studies have recently provided the first glimpse of the structural changes associated with certain steps in the photocycle of the light-driven proton pump bacteriorhodopsin (Edman et al. 1999; Luecke et al. 1999). Although the details of these changes are beyond the scope of this review, it is very instructive to see how changes in a network of hydrogen bonds (including bound water molecules) affect the protonation of key residues, leading ultimately to proton movement across the membrane.

10. Concluding remarks

Although membrane proteins are hard to handle for the biochemist and problematic for the structural biologist, they are no more difficult than globular proteins for the molecular biologist and are, in fact, relatively easy for the bioinformatician. These pros and cons all stem from the fact that membrane proteins are designed to function at the interface between an aqueous and a lipid phase, which on the one hand necessitates the use of troublesome detergents but on the other strongly reduces the structural complexity of membrane proteins compared to globular proteins.

As a consequence, questions that can be addressed by mutagenesis and *in vivo* analysis or by statistical studies have been quite well resolved, and we now have a basic understanding at the level of the individual transmembrane helix, its insertion into the membrane (although many mechanistic details are lacking), and its orientation relative to the membrane. This knowledge has been translated into quite efficient methods for topology prediction that can be applied on a genome-wide scale.

The focus is now shifting to 3D structure, both in terms of membrane protein crystallography (2D and 3D), and in terms of basic experimental and theoretical studies of helix–helix packing. These are difficult problems, and the rate of progress may slow down noticeably unless structural proteomics approaches can step up the rate of structure
determination, and efficient in vitro screens for pairwise homo- and heterodimeric (or oligomeric) interactions can be developed.

In summary, the field of membrane protein assembly and structure has come quite a long way using rather simple methodology. Many of the basic concepts now seem to be in place, although they need to be refined and made more quantitative. The ultimate goal — ab initio 3D structure prediction from the amino acid sequence — remains elusive. Another five years, perhaps?

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12. References


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CROWLEY, K. S. et al. (1993). The signal sequence moves through a ribosomal tunnel into a nonelectrolytic aqueous environment at the ER membrane early in translocation. Cell 73, 1101–1115.


ENDRES, M. et al. (1999). Transport of the ADP-ATP carrier of mitochondria from the TOM complex to the TIM22.54 complex. EMBO J. 18, 3214–3221.


Scott, P. et al. (2000). YidC, the E. coli homologue of mitochondrial Osx1p, is a component of the Sec translocase. *EMBO J.* 19, 542–549.


Zelazny, A. et al. (1997). The NG domain of the

