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Review

The role of lipids in membrane insertion and translocation of bacterial proteins

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Abstract

Phospholipids are essential building blocks of membranes and maintain the membrane permeability barrier of cells and organelles. They provide not only the bilayer matrix in which the functional membrane proteins reside, but they also can play direct roles in many essential cellular processes. In this review, we give an overview of the lipid involvement in protein translocation across and insertion into the *Escherichia coli* inner membrane. We describe the key and general roles that lipids play in these processes in conjunction with the protein components involved. We focus on the Sec-mediated insertion of leader peptidase. We describe as well the more direct roles that lipids play in insertion of the small coat proteins Pf3 and M13. Finally, we focus on the role of lipids in membrane assembly of oligomeric membrane proteins, using the potassium channel KcsA as model protein. In all cases, the anionic lipids and lipids with small headgroups play important roles in either determining the efficiency of the insertion and assembly process or contributing to the directionality of the insertion process.

Keywords: Lipid–protein interaction; Protein secretion; Protein insertion; Membrane protein assembly; Membrane phospholipid

1. Introduction

Synthesis of most proteins is initiated in the cytoplasm of a cell. Proteins that fulfill their function at a different location, in an organelle, a membrane or outside the cell, have to be correctly targeted, inserted and/or translocated. In eukaryotes, many compartments exist and consequently many different transport processes take place, some involving vesicular transport carriers. Prokaryotes are much simpler and only contain one or two membranes, which greatly limits the number of compartments. Moreover, they appear to lack vesicular transport mechanisms, which make them attractive organisms to use in studies on protein transport and membrane biogenesis. *Escherichia coli* has been extensively used as model organism in this respect, because it is easily genetically modified, its entire genome is known [1] and it is amenable to biochemical analysis. The inner membrane of *E. coli* plays a determining role not only as barrier to proteins to be exported out of the cytoplasm, but also as receiving station of the many proteins it harbors. Many aspects of transport and integration of proteins in the inner membrane have similarities with analogous processes at the ER membrane. Therefore, knowledge of inner membrane protein transport and integration provides insight into membrane biogenesis in general.

The insertion into or translocation across the inner membrane of most newly synthesized proteins is protein-mediated. Several pathways exist, but the majority of proteins make use of the Sec-translocon. The components involved in this process are well described and many studies are dedicated to the elucidation of the mechanism of protein translocation and insertion. Essential components in this process include the protein subunits within the integral membrane protein complex SecYEG, which constitutes the core channel across the membrane. The signal sequence directs targeting to the membrane of most secretory proteins. Nascent inner membrane proteins are recognised by the signal recognition particle (SRP) when their first hydrophobic transmembrane segment emerges from the ribosome.

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After the SRP has bound to the ribosome-nascent membrane protein chain, this complex interacts with the SRP receptor FtsY and is docked to the membrane ensuring direct membrane insertion of the protein via the translocase. These prokaryotic SRP-pathway components are highly homologous to the well-studied eukaryotic targeting pathway. The peripheral ATPase protein SecA plays an essential role in protein transport and integration because it mediates translocation of hydrophilic protein stretches through the translocon. In addition, other proteins are known to play roles in the *E. coli* secretory pathway, including the cytosolic chaperone SecB, leader peptidase (Lep), the enzyme that removes the signal sequence during or shortly after translocation, and other integral membrane proteins like SecD, SecF and YajC. More recently, the inner membrane protein YidC was identified to play a role in integration of newly synthesized membrane proteins (for reviews, see Refs. [2–4]). Protein translocation is driven by ATP hydrolysis via the SecA protein and the proton motive force (pmf). In addition to proteins, lipids also have been shown to play important roles in protein transport. This applies both to the anionic lipid phosphatidylglycerol (PG) as well as to the most abundant lipid phosphatidylethanolamine (PE). These lipids appear to act at the level of the translocase as well as the signal sequence. (for a recent review, see Ref. [5]; some elements will be rereviewed here). Much less is known about the roles lipids play in membrane protein integration. This is the main topic of this review. We first briefly summarize the general properties of the lipids in the *E. coli* inner membrane and then describe the main lipid involvements in protein insertion and translocation processes. Subsequently, we will focus on direct influences of lipids on membrane protein integration and assembly.

2. Phospholipids of the *E. coli* inner membrane

The *E. coli* inner membrane phospholipids are organized in a highly impermeable bilayer. The most abundant phospholipid is PE, which accounts for 75–80% of the total phospholipids. The other main group of phospholipids in *E. coli* consists of the negatively charged lipids PG and cardiolipin, ~20% and ~5%, respectively [6]. The PE headgroup is small, zwitterionic and has no net charge at physiological pH. PE by itself prefers to assemble into inverted non-bilayer structures, such as the H_{II} phase. This is the result of the smaller cross-sectional area of its headgroup compared to the acyl chain region. For this reason, it is called a non-bilayer lipid. In the inner membrane, PE is forced into a bilayer organization, which results in curvature stress [7]. Curvature stress causes an increased lateral pressure in the bilayer interior and reduced lateral pressure in the interface. These conditions have been proposed to play essential roles in protein function [8]. In general, the lateral pressure profile of a bilayer has been correlated to membrane protein conformational changes and functioning [9,10]. Consistent with an important functional role of PE, analyses of the phospholipids of the *E. coli* inner membrane grown under different circumstances revealed that the lipid composition is tightly regulated, such that there is a balance between the maintenance of bilayer structure and the presence of a large amount of non-bilayer-preferring lipids [11,12].

Synthesis of phospholipids occurs at the cytoplasmic side of the inner membrane. This biosynthetic pathway is well studied and the genes coding for the enzymes involved in headgroup diversification are characterized (reviewed in Ref. [13] and schematically depicted in Fig. 1). The phospholipid precursor is phosphatidic acid (PA) which is rapidly converted into CDP-diacylglycerol. The enzyme PS synthetase (product of the *pssA* gene) exchanges CDP for serine, resulting in phosphatidylserine (PS) which is subsequently rapidly decarboxylated resulting in PE. Synthesis of the negatively charged lipids in *E. coli* is initiated by the action of the *pgsA* gene product which replaces CDP for glycerol-3-phosphate. After a rapid dephosphorylation step, PG is formed. Two PG molecules are condensed by cardiolipin synthetase (*cls*) to yield cardiolipin. How newly synthesized phospholipids are subsequently transported to the other side of the membrane and to the outer membrane is much less clear. The available data on these topics have recently been reviewed in Refs. [14,15].

Several knockout mutants of the lipid biosynthesis enzymes are available, which provides an important tool to analyze the function of specific lipid classes. Disruption of the *pss* gene blocks PS synthesis and consequently lacks

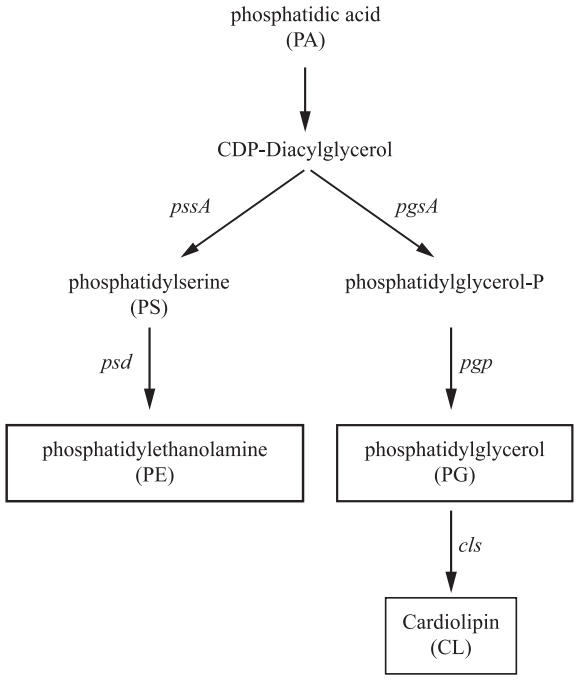

Fig. 1. Schematic overview of the final part of the phospholipids biosynthesis route with the genes encoding for the enzymes involved indicated.

PS conversion to PE, resulting in a membrane which is devoid of PE and contains only anionic lipids, with elevated levels of cardiolipin [16]. These cells require divalent cations for viability. The presence of these cations together with cardiolipin has been shown to be crucial since it restores the essential curvature stress in the bilayer [11,17]. Disruption of the *pgsA* gene blocks synthesis of PG, and consequently cardiolipin, and was initially found to be lethal since the major lipoprotein of *E. coli* requires PG as a precursor. A lipoprotein-deficient strain was able to survive when the *pgsA* gene was placed under control of a lactose operator promotor. In this strain, the PG level could be varied from a limiting 2% to wild-type levels [18]. Due to the accumulation of the negatively charged phospholipid precursor phosphatidic acid (PA), 5–10% anionic lipids are still present in the membrane of this mutant. More recently, an *E. coli pgsA* and lipoprotein-deficient mutant was isolated which lacks detectable PG and CL levels [19], demonstrating that these lipids are nonessential for cell viability. It was suggested that PA can substitute for PG and CL in essential processes for the cell.

3. Lipid involvement in protein translocation

3.1. Influences of PE

The phospholipid mutant strains described above have provided direct in vivo evidence for the importance of a correct phospholipid composition in protein translocation. In the in vivo situation, protein translocation is fully functional in the strain that is defective in PE synthesis, demonstrating that the structure of PE itself is not essential. However, using isolated inner membranes deficient of PE, in vitro protein translocation was very low. This could be restored by reintroducing the property to form non-bilayer structures, either by divalent cations or by non-bilayer-preferring lipids [20].

That non-bilayer lipids act at the level of the translocon was demonstrated in reconstitution experiments. In vitro translocation activity of SecYEG reconstituted into liposomes of defined phospholipid composition could be stimulated by the presence of non-bilayer-preferring lipids, such as PE. Derivatives of PE that adopt bilayer structures did not stimulate protein translocation across a membrane composed of only bilayer promoting lipids [21]. SecYEG forms the actual translocation channel through the membrane and is therefore likely to undergo conformational changes to open and close its channel without disturbing the impermeability of the membrane. In addition, there are indications that the active translocation pore is formed by SecYEG oligomers [22–24]. Possibly, the curvature stress associated with the presence of non-bilayer lipids is required to facilitate the rearrangement of SecYEG to form the active translocase. However, the presence of non-bilayer lipids in pure lipid vesicles also stimulated the ATPase activity of SecA [25], which could be an alternative explanation for the observed stimulatory effect on preprotein translocation through SecYEG.

3.2. Influences of PG

The second group of *E. coli* phospholipids constitutes the negatively charged lipids PG and cardiolipin. Protein translocation in a mutant strain with strongly reduced PG levels was severely impaired, indicating an essential role for wild-type levels of PG [26]. Not only reintroduction of PG could restore in vitro protein translocation across PG depleted inner membrane vesicles, also other anionic phospholipids promoted translocation. This demonstrated that specifically the negative charge at the headgroup of PG is involved in the translocation process [27]. In the reconstituted system with only SecYEG, SecA and a precursor protein, the presence of anionic lipids was essential for translocation [21]. The peripheral interaction of SecA to the membrane has been extensively studied and anionic lipids have been found to be essential for SecA activity. SecA inserts into the membrane in the presence of anionic phospholipids. This insertion is deep into the hydrophobic part of a pure lipid bilayer and is related to conformational changes of the protein [28–30]. Negatively charged lipids are essential for the high affinity binding to SecY and they stimulate the hydrolysis of ATP which coincides with preprotein translocation [31–33]. Remarkably, SecA bound to SecYEG is shielded from the acyl chains of the phospholipids, irrespective of the presence of translocation substrates [30,34]. Recently, a new effect of negatively charged lipids on SecA was revealed. SecA has always been found as a dimer in solution which was also assumed to be the functional state [35–37]. By fluorescent measurements it has now been shown that the SecA dimer is in equilibrium with a small fraction of monomers and that this equilibrium is largely shifted to the monomeric state when liposomes containing anionic phospholipids [38] or lipid derivatives with long acyl chains [39] are added. Moreover, a monomeric SecA derivative could be created by mutating a small number of residues. This mutant could still bind with high affinity to SecYEG in the membrane and displayed significant translocation activity, although lower than wild-type SecA [38]. These data suggest that before insertion into the translocon, when SecA is shielded from the lipids, the presence of anionic lipids induces a large change in the SecA conformation, which probably results in dissociation of the SecA dimer into its monomeric state.

The presence of SecG in the translocon is not essential for translocation and a deletion of the *secG* gene is not lethal but causes a growth defect [40,41]. This defect could be relieved by increasing the anionic lipid content of the membrane [42,43]. Since SecG has been reported to change its topology upon SecA interaction and thereby possibly stimulates SecA activity [44,45], the SecG deletion defect is probably restored by lipid effects via SecA.

In addition to influences of negatively charged lipids on the proteins of the Sec-machinery, another likely candidate to interact with these lipids is the signal sequence of the precursor protein. Signal sequences have been shown to be able to insert into lipid bilayers composed of isolated or synthetic phospholipids (e.g. Refs. [46,47]). Signal sequence–lipid interactions have also been demonstrated in the biological membrane [48]. Strong evidence for a lipid environment of the signal sequence during translocation came from cross-linking experiments in the homologous yeast and mammalian systems. The signal sequence and not the mature part of the protein could be cross-linked to lipid molecules and a Sec-protein, demonstrating a transmembrane insertion of the signal sequence at the translocon protein–lipid interface [49]. The anionic lipids in the membrane are important for effective interaction of the signal sequence with the membrane by binding the positively charged N-terminus and stimulating the folding into the functional helical conformation [48,50–53]. Moreover, synthetic signal peptides were shown to induce clustering of PG, as measured after incorporation into liposomes containing fluorescent labeled PG. The ability to induce lateral segregation of lipids was correlated to the translocation competency of the sequences in vivo [54]. The transmembrane orientation of the signal sequence in the membrane is similar to that of transmembrane segments of membrane proteins. The distribution of charged residues flanking the transmembrane helices of signal sequences and transmembrane segments is comparable. However, the length of the hydrophobic part of the signal sequence is generally too small to span the entire hydrophobic part of the membrane. This hydrophobic mismatch has been shown to result in the formation of non-bilayer structures [55,56], suggesting an involvement of signal sequence-induced curvature stress at the start of protein insertion. Moreover, the small length of the signal sequence and consequently burial of the cleavage site within the bilayer might indicate that Lep has to partially insert into the membrane to cleave the signal sequence (see also in a forthcoming section).

4. Membrane protein insertion

Information on biosynthetic membrane integration of newly synthesized membrane proteins lags behind that of soluble proteins as a result of problems in the handling of these hydrophobic proteins. With the increasing knowledge of techniques to overcome these problems and increasing interest in membrane proteins generated by their abundance as witnessed from genome sequencing [57], much focus is now turned to understanding membrane integration of membrane proteins. Before addressing the functional significance of lipid–protein interactions for membrane protein insertion and assembly, it is important to realize that for those proteins that make use of the translocon, similar lipid dependencies are expected as for secretory proteins. Moreover, since membrane proteins function within a lipid environment, at some stage of the biogenesis of the proteins, interactions with the lipids will have to take place.

After briefly discussing the influences of lipids on the targeting of membrane proteins, we will first focus in detail on the insertion of transmembrane segments into the lipid phase of the bilayer, starting with the relatively simple phage proteins, Pf3 coat protein and M13 major coat protein. Lep will serve as an example, of which lipid influences are known both during the process of its insertion and for its optimal functioning. Finally, the interactions of lipids with the oligomeric channel protein KcsA will be discussed.

4.1. Targeting of membrane proteins

After recognition of a nascent membrane protein chain emerging from the ribosome by SRP, this complex interacts with the GTPase FtsY that is found in the cytosol and at the membrane [58,59]. FtsY, GTP and the inner membrane are needed for the release of SRP from the nascent chain [60]. Interestingly, this FtsY-mediated release of SRP also occurred in the presence of liposomes derived from *E. coli* lipid extracts, indicating a functional interaction of FtsY with lipids [61]. Using model membrane systems, a direct interaction of FtsY with lipids was demonstrated with affinity for both PG and PE [62,63]. Since the interaction with liposomes induced a conformational change in FtsY and enhanced its GTPase activity and because no membrane bound protein receptor has so far been found in *E. coli*, these FtsY–lipid interactions are probably crucial in the SRP-mediated protein targeting.

After targeting by the SRP–pathway, the newly synthesized proteins are mostly delivered to the Sec-translocon, although possibly different sites of the translocon are used for protein translocation and membrane insertion [64]. Translocation of long hydrophilic periplasmic regions requires the activity of SecA [65], which is dependent on the correct lipid composition of the membrane as described above. For the insertion of transmembrane segments, different mechanisms may exist. The hydrophobic segments inserted via the translocon will probably laterally move out the translocon into the lipid phase. Also a Sec-independent pathway is described, in which the transmembrane segments directly have to interact with the lipids of the membrane.

4.2. Direct lipid insertion of small proteins

The bacteriophage major coat proteins Pf3 and M13 are extensively studied proteins to obtain insight in how proteins can ‘spontaneously’ insert into the membrane, i.e. independent of Sec proteins [66,67]. M13 procoat has also been shown to be insensitive to SRP depletion [68]. Both proteins contain one membrane spanning segment and have their N-termini located in the periplasm and the C-terminus

in the cytoplasm. In contrast to Pf3, M13 coat protein is synthesized with a signal sequence that is cleaved by Lep after insertion. Both proteins contain an overall negative charge in the translocated regions and positively charged residues are located in the termini that stay at the cytoplasmic site of the membrane. Membrane insertion is driven by hydrophobic forces and the pmf, with the electrical potential positive on the periplasmic site of the membrane. When the hydrophobicity of the membrane spanning segments of M13 procoat is reduced, insertion becomes more dependent on the pmf and after replacement of the charged residues in the periplasmic loop for neutral residues, insertion is independent of the pmf. When the charged residues of Pf3 are mutated into neutral residues, the hydrophobicity of the transmembrane segment should be increased to maintain membrane insertion, which in addition showed no preferential topology. Reversal of the charge distribution around the membrane spanning segment of Pf3 results in complete conversion of the protein topology [69–72]. These studies have clearly demonstrated the complementary action of hydrophobic and electrophoretic forces in the Sec-independent integration of these small coat proteins.

The mechanism of M13 procoat insertion is proposed to start with electrostatic interactions with the membrane. Constructs of M13 procoat with the positive charges at both termini mutated to negatively charged residues could not bind to the membrane, but accumulated in the cytoplasm [73]. Also, processing of M13 procoat was retarded in the presence of low PG concentrations in the membrane of the *pgsA* conditional strain, which could be restored again by increasing the PG level [74]. This indicated a direct interaction of the protein with the membrane lipids. For insertion into the membrane, both transmembrane segments (the signal sequence and the membrane anchor) are required and the hydrophobic interactions with the lipid acyl chains are sufficient to drive transmembrane insertion [75,76]. In vitro synthesized procoat could even be inserted into protein-free liposomes [77]. It is proposed that the two hydrophobic segments pair up and insert as a loop structure directly into the membrane [78–80].

Since the Pf3 major coat protein is not synthesized with a signal sequence [81], it most likely uses a different mechanism for insertion of its single transmembrane segment and thereby transport of its small periplasmic region across the membrane. However, making use of Pf3 mutants containing no charged residues, or negatively charged residues at the position with normally positively charged side chains, insertion into pure lipid vesicles is possible [82]. The development of an in vitro translocation system, in which the translocation of the N-terminus of Pf3 could be monitored, resulted in a very versatile system in which the properties of the protein and role of the lipid bilayer on direct protein insertion could be analysed. Biophysical studies have shown that amino acid residues with aromatic side chains have high affinity for the membrane water interface and statistical analysis indeed showed interfacial abundance of these residues, although with preference for the *trans*-side of the membrane [83–85]. With Pf3 constructs containing lysine or tryptophan residues flanking the transmembrane segment, the anchoring by the positively charged residues on the *cis*-side of the membrane according to the positive inside rule [86–88] was confirmed, but it was shown that the tryptophans do not influence direct bilayer insertion nor act as topological determinant [82]. This indicated that the positively charged residues direct the topology of a transmembrane segment by anchoring on the *cis*-side, possibly by interacting with the negatively charged lipids of the membrane [89], and the tryptophans may provide additional stabilization of the helix on the trans-membrane side, after insertion is completed.

In contrast to the stimulating effect of the presence of non-bilayer lipids on the Sec-mediated protein translocation, no effect of non-bilayer lipids on the direct membrane insertion of Pf3 constructs was found. Interestingly, however, an increased N-terminal translocation of a Pf3 mutant without any charged amino acid residue was observed upon increasing the amount of negatively charged lipids, up until a certain optimum. The stimulating effect could be correlated to the charge density of the membrane surface, suggesting the importance of electrostatic effects [90]. A possible explanation for these effects on this peptide without charged amino acid side chains might be a small charge contribution from the helix dipole moment, which results in a slightly positive charge at the N-terminal end of a helix and is slightly negative at the C-terminal part. Due to this charge distribution, the N-terminus of the Pf3 mutant will to some extent be attracted to a negatively charged membrane, while the C-terminus will be repulsed. Consequently, translocation of the attracted N-terminus is facilitated. Apparently, negatively charged lipids can stimulate translocation of a small charge on a protein segment. A high concentration of anionic lipids will cause stronger electrostatic interactions with the positively charged side of the helix, resulting in retention of this part of the protein, according to the observed results and the positive inside rule.

Finally, this Pf3 in vitro system is perfectly suited to investigate the effect of length of the hydrophobic transmembrane segment and bilayer thickness on membrane protein insertion. Using constructs that are able to insert into pure lipid vesicles, a combination of two factors plays a role in membrane insertion: translocation of the hydrophilic residues and hydrophobic matching of the membrane segment with the bilayer. Increasing bilayer thickness either by incorporation of cholesterol or by using lipids with longer acyl chains resulted in an effect on Pf3 insertion efficiency as expected based on hydrophobic matching. In addition, by varying the length of the transmembrane segment of Pf3, a higher efficiency of insertion was observed in bilayers that are slightly thinner than expected based on hydrophobic matching, although increasing the hydrophobicity also increased insertion efficiency [91]. These data nicely support the hypothesis of hydrophobic matching during sorting of

membrane proteins in the eukaryotic secretion pathway, in which cholesterol plays an important role.

All the above described studies point to a spontaneous insertion directly into the lipids of the membrane. However, recently, in vivo experiments indicated that both M13 procoat and Pf3 membrane assembly are hampered in cells depleted of YidC or containing mutated YidC [92–95], suggesting a role of YidC in interaction of these proteins with the membrane. Nevertheless, the lipid dependency of the phage coat proteins insertion has also been found in vivo, demonstrating a role of lipids in the Sec-independent insertion of these proteins. What the precise function of YidC is in membrane protein insertion is still unclear. Recently, YidC has been found to be also important during membrane assembly of subunits of pmf generating protein complexes [96]. This might suggest that YidC could participate in the membrane assembly of a selected group of proteins which have to assemble into larger structures to reach their functional conformation. In that sense, YidC could play a chaperone type of function facilitating functional assembly.

4.3. The role of lipids in leader peptidase insertion and function

Lep has been extensively used as a model protein to study membrane protein insertion and topology. It contains two transmembrane spanning helices with its short N-terminus and large C-terminal domain (P2) in the periplasm. Lep is targeted to the membrane via the SRP pathway and membrane integration and translocation of the large P2 domain requires the Sec-translocon and SecA. Nascent chains of Lep can be cross-linked to YidC, but in vivo depletion or mutant YidC strains show that Lep insertion is not absolutely dependent on the presence of functional YidC [66,68,92,95,97–99]. Efficient integration of Lep requires the presence of anionic lipids, which could partially originate from the SecA requirement for negatively charged lipids for optimal activity [98]. Moreover, it has been demonstrated using Lep and Lep constructs that the topology of membrane proteins is regulated by the presence of anionic lipids [89]. For accurate functioning of membrane proteins, it is essential that they are correctly oriented in the membrane. Statistical analysis revealed that in membrane proteins positively charged residues are four times more abundant in the cytoplasmic domains than in translocated parts of the protein (the positive-inside rule [86–88]). Mutagenesis studies with Lep indeed demonstrated that altering the basic residues or inserting extra basic residues in the domains flanking a transmembrane segment results in an inverted topological orientation or inhibition of translocation, e.g. Refs. [100,101]. An explanation for this phenomenon is a role of the pmf acting on positively and negatively charged amino acids during membrane integration [72,102]. However, since also in acidophiles, which have a reversed pmf, the positive-inside rule is valid [103], the effect of the pmf on charged residues cannot be the main determinant in membrane protein topology. Possibly the interaction with negatively charged lipids is dominant in retaining the positively charged residues on the *cis*-side.

The catalytic active site of leader peptidase is located in the P2 domain and cleaves most signal peptides from translocated precursor proteins. The activity of purified Lep in detergent could be stimulated by the addition of phospholipids, with a slightly higher effect for PG compared to PC and PE [104]. The transmembrane segments are not required for activity and in the absence of these segments the large periplasmic domain is still bound to the inner membrane and outer membrane in vivo. Moreover, a purified construct lacking the two transmembrane segments (Δ2–75) can bind to vesicles composed of purified *E. coli* lipids, excluding the need for a proteinaceous receptor. This binding did not seem to depend on electrostatic interactions and the isolated periplasmic domain inserted best into DOPE monolayers as compared to DOPC and DOPG layers, in which Δ2–75 inserted equally well. A mildly hydrophobic segment around the catalytic center was identified to mediate membrane binding by hydrophobic interactions [105]. The subsequent outcome of the crystal structure of the P2 domain revealed a large exposed hydrophobic surface around the catalytic center, confirming the possible association site with the membrane [106].

Most often, membrane affinity of proteins is driven by electrostatic interactions with the negatively charged lipids. The interesting finding of PE- and not PG-stimulated binding was further explored and it was determined that non-bilayer-preferring lipids in general facilitate membrane binding and insertion of Δ2–75 [107]. An increase in binding and insertion was observed when the headgroup size was gradually decreased going from DOPC to derivatives of DOPE with two and one methyl group and finally DOPE. The importance of the presence of a small headgroup was not only established in lipid monolayers, but also using pure lipid vesicles. These data suggest that the presence of small headgroups in a lipid bilayer creates insertion sites for hydrophobic regions on proteins that have to (partially) insert into the membrane. The monolayer results of the insertion of Δ2–75 into layers composed of lipids with different headgroup sizes enabled an estimation of the size of these insertion sites. Largest insertion area was determined to be in the DOPE layer ($\sim$ 15 Å^2, compared to DOPC set at 0 Å^2) [107]. PE is the most abundant lipid in *E. coli*. Since Lep cleaves off signal sequences that in general contain a too small hydrophobic region to span the bilayer, it probably needs to penetrate the membrane to reach the signal sequence cleavage site. The facilitated membrane insertion due to the presence of the PE might be necessary for optimal activity of Lep. Similarly, the existence of membrane insertion sites could explain the observed stimulation of SecA activity by non-bilayer inducing lipids in the membrane [25].

4.4. Membrane assembly of KcsA

Many essential processes in the cell are found to be carried out at the membrane by large protein complexes. Little is known about how these oligomeric proteins are integrated into the membrane and correctly assembled. A relatively simple oligomeric protein is the potassium channel from the gram-positive soil bacterium *Streptomyces lividans*. Although eukaryotic channels are larger and more complex than the bacterial KcsA, sequence alignment revealed a significant homology of KcsA with a large class of eukaryotic K^+ channels [108]. KcsA is an oligomeric protein composed of four identical subunits, with two transmembrane segments per subunit. A fascinating question is how these segments are inserted and assembled to form the channel protein and at the same time where and how the lipids contact and influence the subunits in this process.

KcsA could be expressed in and purified from the *E. coli* inner membrane, which is functional after reconstitution into lipid bilayers [108]. Detailed structural information is available from its crystal structure and spin-labeling results [109–112]. The first transmembrane helix of each subunit is clearly in contact with its membrane environment, while the second transmembrane segments line the wall of the pore through which the potassium ions flow. The N-termini point away from the center of the protein and are at the membrane–water interface at the *cis*-side of the membrane. The periplasmic region contains ~38 amino acids which mostly fall back into the membrane and form a pore helix followed by the selectivity filter.

The KcsA tetramer is symmetrically arranged around the central pore and is highly stable in a wide range of detergents. Only extreme pH values (pH <2 or >12) or heat treatment (>60 °C) result in disassembly of the tetramer. This stability and the structural knowledge make KcsA an attractive protein to use as a model protein in both functional and biogenesis studies to obtain insight in the working mechanisms of channel proteins (for recent reviews, see, e.g. Refs. [113,114]) and in the integration and assembly of oligomeric membrane proteins. In an in vivo approach, short pulse-labelling of KcsA expressing *E. coli* cells revealed the existence of KcsA monomers and formation of tetrameric KcsA [115]. Both forms were carbonate extraction-resistant, which is indicative of integral membrane association and indicates that oligomerisation occurs in the membrane. Dimers or trimers were never observed in these biogenesis experiments, although they could be detected during disassembly of tetramers by mild heat treatment. This suggests that assembly occurs from monomers directly into tetramers. The appearance of a considerable amount of tetramers already after a short pulse (10–30 s) demonstrated that this is a very efficient process in vivo. However, when the pmf across the inner membrane was dissipated, tetramerization was largely reduced and much more monomers were detected in the membrane. Protease treatment from the periplasmic region demonstrated that either in the absence or presence of the pmf, monomers were sensitive for the protease, which implies that monomer insertion was not affected by pmf dissipation, suggesting that oligomerization in the membrane is highly dependent on the pmf. The protease sensitivity of the monomer at the *trans*-site of the membrane was recently supported by experiments of in vitro synthesized KcsA integrating into the membrane of ER microsomes [116]. An engineered glycosylation site in the periplasmic loop was used to detect exposure of this loop in the microsomal lumen. Since glycosylation takes place out of the plane of the membrane [117], the observed high amount of glycosylated KcsA monomer indicated that the periplasmic loop is exposed in the lumen during or directly after monomer insertion. Since from the crystal structure data it is known that in the final tetramer conformation this loop is folded back into the plane of the membrane, one can speculate that the inserted monomer is not yet in its final conformation (Fig. 2). The periplasmic loop, specifically the pore region, and the cytosolic C-terminus have been shown to be important in tetramer stability [118–120]. It is therefore possible that these regions are also important in tetramer formation. The presence of the pmf might facilitate the rearrangement of these protein parts into their final conformation to reach the oligomeric state.

In an in vitro assay, further requirements for KcsA tetramer membrane insertion and tetramerization were investigated. In vitro synthesis of KcsA in aqueous medium resulted in the monomeric protein and tetramers could be detected only in the co-translational presence of mem-

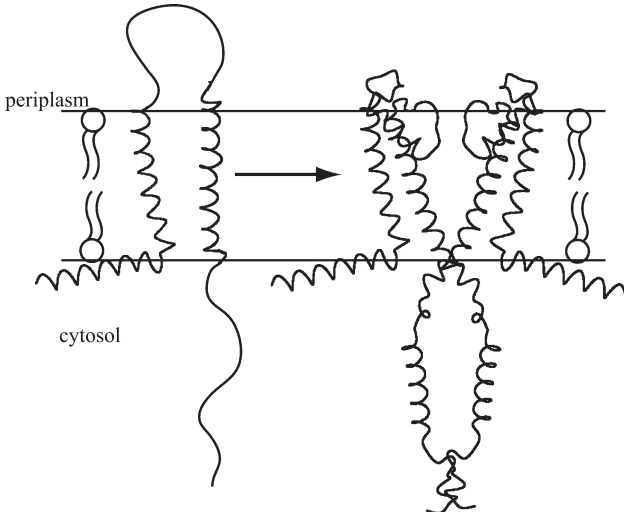

Fig. 2. Schematic drawing of the proposed KcsA assembly in the inner membrane. The KcsA monomeric periplasmic loop is detected in the periplasm, while according to the tetrameric crystallized state, this loop is folded back into the plane of the membrane. The conformation and positioning of the monomer in the membrane is speculative. For clarity, only two subunits of the assembled KcsA are shown.

brane vesicles, which is in accordance with the suggestion that oligomerization occurs in the membrane [115]. The in vitro membrane insertion of monomeric KcsA has been extensively studied in the microsomal membrane [116]. It could be shown that the N- and C-termini are at the *cis*-site of the membrane, as expected from the crystallographic data. In addition, the detection of the loop connecting the two membrane helices in the microsomal lumen demonstrated the in vitro transmembrane insertion of monomeric KcsA.

As expected for membrane protein integration, a functional SRP-pathway was found to be needed for efficient tetramer formation at the membrane [121]. Interestingly, in the absence of Ffh, the protein component of SRP, membrane association of newly synthesized KcsA was unaffected, but no tetramers are formed. Possibly, Ffh is required for correct KcsA monomer insertion. In this respect, it is plausible that SRP fulfils a chaperone-like function. As membrane-association was not affected in the absence of Ffh, but tetramerization was blocked, SRP might prevent the nascent chain from misfolding or aggregation at the membrane and thereby direct the nascent protein in a correct conformation to the membrane. Since in another study the in vivo depletion of Ffh did not change the ribosome concentration at the membrane [122], one could speculate that the important role of Ffh in membrane protein biogenesis might not only be a direct targeting function, but probably a role in directing correct insertion that is necessary for assembly into functional membrane proteins.

Unlike the expected involvement of Sec components in membrane protein insertion in general, KcsA membrane association and tetramer formation was largely unchanged in inner membrane vesicles with reduced SecY or SecE levels or depleted for YidC as compared to wild-type membranes. Even more, most efficient membrane assembly was observed in the presence of pure lipid vesicles (Ref. [121] and experimentally shown in Fig. 3). This demonstrates that the minimal requirements for KcsA membrane assembly are a functional SRP-pathway and a lipid bilayer, and suggests that the Sec-pathway does not play a major role in KcsA insertion and assembly. This shows that information for tetramerization is present in the protein itself and that no other protein components in the membrane are essential for oligomerization. The contribution of Sec-components and YidC to this process in vivo has not been determined and is still unclear.

Only few membrane proteins are reported to insert Sec-independently of which M13 procoat has been extensively investigated (see above). Also the polytopic melbiose permease, MelB, and recently the sensor kinase protein KdpD were found to insert into the membrane independent of Sec proteins, and in the case of KdpD also independent of YidC [123,124]. These Sec-independent proteins have a common topology with their N- and C-termini in the cytosol and all contain very small periplasmic loops. It is therefore believed that like for M13 described above, MelB and KdpD insert

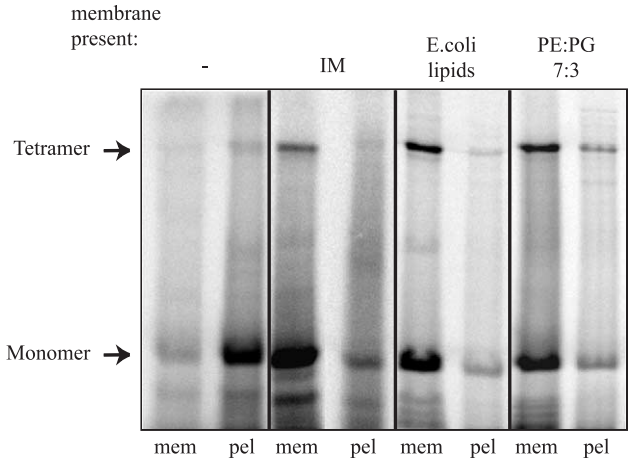

Fig. 3. In vitro synthesis and membrane assembly of KcsA. Translation in a membrane-free environment of the *kcsA* gene only results in monomers that can be pelleted through a sucrose cushion. Addition of inverted inner membrane vesicles (IM) during the translation resulted in the formation of KcsA tetramers that are associated to the membrane vesicles, as determined by sucrose gradient analysis. The presence of a membrane composed of isolated *E. coli* lipids or even synthetic lipids resembling the phospholipid composition of the *E. coli* membrane efficiently supported the tetramerization of KcsA. mem: fraction from a sucrose gradient containing the membrane vesicles; pel: pellet content after sucrose gradient analysis containing aggregated protein.

their transmembrane helices in a paired helix loop structure. The hydrophobic energy from insertion of the transmembrane parts consequently compensates for translocation of the small hydrophilic loop across the membrane. Could the KcsA monomer also insert its membrane helices in pairs, according to the M13 insertion model? Although the periplasmic loop of KcsA is short, it is still at least twice as long as the hydrophilic regions of the other studied polytopic proteins, which might hinder its translocation in a looped structure. On the other hand, the KcsA loop is considerably more hydrophobic, which will cost less energy to translocate across the lipid bilayer. In addition, tetramerization efficiency was much higher in the cotranslational assay compared to the posttranslational situation, indicating that correct monomer insertion is most efficient cotranslationally. We propose that correct membrane insertion of the two helices of KcsA occurs cotranslationally by consecutive insertion of the two helices driven by hydrophobic forces and ongoing protein synthesis at the ribosome. As a result of its relatively high hydrophobicity (according to the Kyte and Doolittle scale [125], -0.4 versus -2.2 for the M13 procoat loop), the periplasmic loop might be embedded in the membrane before it is translocated by the emergence of the second transmembrane helix.

4.5. Lipid influences on KcsA

To further explore the requirements of the lipid bilayer that support KcsA tetramer formation, membranes with different phospholipid compositions were tested. It was

shown that the large variety of lipid acyl chains as present in *E. coli* lipids is not important for KcsA tetramerization. The presence of low amounts of the negatively charged PG increased the efficiency of membrane association and tetramerization as compared to a lipid bilayer composed of only zwitterionic lipids. Also, high amounts of PE increased KcsA membrane assembly compared to a pure PC bilayer. The lipid bilayer composed of 30–40% PG in PE supported most efficiently the KcsA assembly [126]. Differences in membrane association with lipid bilayers of different composition could be explained by effects on the efficiency of targeting by the SRP-pathway, as described above. Also, a stimulatory effect on membrane association could be caused by electrostatic interactions between the newly synthesized KcsA and PG. Facilitated insertion of the KcsA monomer between the smaller headgroups of PE compared to PC could explain the increased membrane association in the presence of PE as well, similar to the facilitated interfacial insertion of Lep described above. The different effects that lipids have on tetramerization efficiency are most likely explained by influences of the lipids on the correct conformation needed for tetramerization of the KcsA monomer. Both correct docking by the SRP-route and an effect of lipids on KcsA will result in a correct conformational state of the monomer resulting in subsequent tetramerization.

The possibility of a direct influence of lipids on the KcsA tetramer was supported by the observation that the high thermal stability of tetramer in detergent is even more increased in an intact lipid bilayer [126]. The temperature needed for disassembly of the in vitro formed tetramer was similar to the temperature needed to disassemble purified KcsA after reconstitution in a lipid bilayer, indicating that the biosynthetically formed tetrameric state is similar to the purified and reconstituted tetramer which has been shown to be fully functional [108]. Tetrameric KcsA was most stable in a lipid bilayer containing negatively charged lipids. Further strong indications for a specific interaction of lipids with KcsA came from mass spectrometry measurements. The KcsA monomer could be detected and lipid adducts were clearly visible in the analysis of samples with KcsA reconstituted in lipid vesicles [127]. Strikingly, a preferential binding of PG, and to a lesser extent PE, was found over PC. Furthermore, an additional structure at the interface between two monomers was found with improved crystallographic analysis of the tetramer [128]. A lipid molecule could be modelled into this electron density (Fig. 4) and biochemical analysis resulted in the probable identification of PG specifically bound to the purified tetramer. Interestingly, these authors describe a method for unfolding of the tetramer into monomers which can be refolded again. In this refolding assay, the presence of lipids is essential. No requirement for a specific class of lipids was found; however, a large amount of the negatively charged detergent SDS had to be included in the assay which complicates the interpretation of these data on lipid specificity in refolding.

To summarize, stimulating or preferential influences of negatively charged lipids (PG), and to a lesser extent PE, have been described for in vitro membrane assembly of newly synthesized KcsA, thermo stability of the KcsA tetramer, specific binding to the monomer measured by mass spectrometry and extraction from the purified protein tetramer. In addition, negatively charged lipids are essential for the activity of KcsA [129]. These different approaches together strongly indicate a direct preferential interaction of

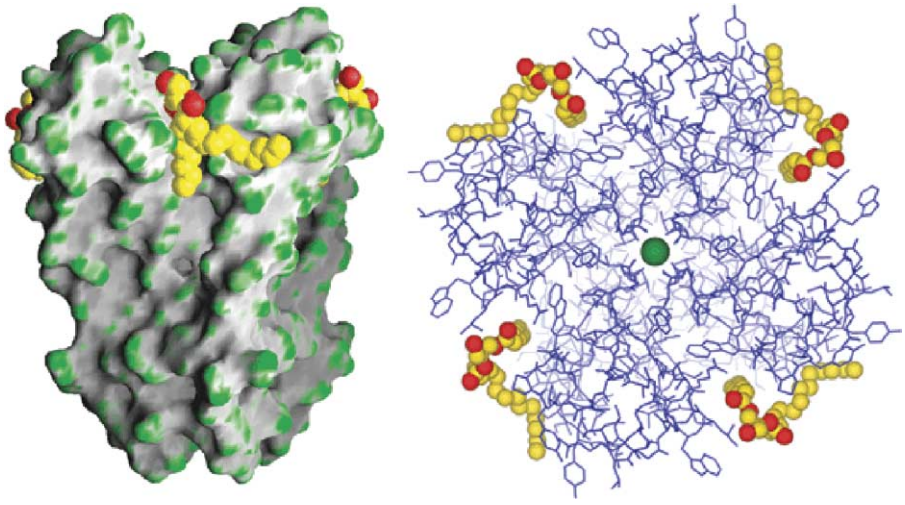

Fig. 4. Lipid binding to KcsA. A tightly bound lipid molecule could be modelled into the interface of two KcsA monomers in a refined structural analysis of KcsA. The carbon atoms of the lipid molecule (built as 1,2,-diacylglycerol) are yellow and the oxygen atoms red. Left shows surface binding of the lipid to KcsA from a side view. The KcsA surface is colored according to curvature (green, convex; grey, concave). On the right, the top view of the blue colored channel from the extracellular site is presented. The green sphere represents a potassium ion. (Reprinted with permission from Ref. [128]; Copyright (2003), American Chemical Society).

KcsA with the lipids in the membrane, which is essential for its biogenesis and subsequent functioning.

Further support for the essential role of specific lipids in protein assembly comes from the observation that lipids have been found in crystal structures of some membrane proteins and protein complexes and that specific lipids are required for functioning of different proteins (see for review, e.g. Ref. [130]). Moreover, direct evidences have been presented that lipids are required for the correct folding of the membrane protein lactose permease (LacY). Using an assay to directly measure correct folding of this multiple membrane spanning protein, a specific requirement for PE for folding of LacY into the functional state could be determined in the step after membrane insertion [131]. Misfolded LacY in cells lacking PE could be rescued by reintroduction of PE to the membrane [131,132]. Recently it was determined, both in vivo and in an in vitro reconstitution system, that part of LacY adopts an inverted topology in the absence of PE, and the wild-type topology can be recovered by newly synthesized PE [133,134]. This suggests that lipids can assist in folding and that, in response to the lipid composition, membrane proteins can rearrange after initial assembly.

5. Concluding remarks

The studies reviewed here have demonstrated that lipids are important in many aspects during the process of protein translocation, insertion and functional assembly of membrane proteins. Both major classes of the phospholipids of the *E. coli* inner membrane, the negatively charged lipids and lipids with small headgroups, have been shown to be essential in optimal functioning of these processes. The described roles of these phospholipids are:

- directing targeting of secretory proteins mediated by their signal sequence;
- directing targeting of membrane proteins via the SRP pathway;
- directing and facilitating the interfacial insertion of peripherally attached protein domains;
- influencing or even determining the correct topology of transmembrane segments;
- influencing optimal functioning of Sec-components, including the translocon;
- influencing (efficient) formation of protein complexes in the membrane;
- directing correct folding into the functional conformation.

An increasing amount of studies now reveal essential interactions of even specific classes of lipids with proteins for correct functioning, of which some examples in *E. coli* are described here but are also found in other membrane systems such as in mitochondria [135]. Therefore, it is important upon studying membrane biogenesis or membrane protein functions, not only to focus on the specific protein of interest, but also to always bear in mind its native environment in the biological membrane.

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