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Topical Review

The Role of Anionic Lipids in Protein Insertion and Translocation in Bacterial Membranes

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Introduction

Insertion of proteins into and translocation across biological membranes are essential for cellular processes such as membrane biogenesis and secretion. Both insertion and translocation involve membrane passage of hydrophilic polypeptide segments and transfer of hydrophobic segments from the aqueous environment of the cytosol to the hydrophobic core of the membrane. Over the last decade these processes have been extensively studied in several organisms and many of the components involved have been identified. Several of the proteins and lipids involved in these processes are similar in prokaryotes and eukaryotes. Therefore, it is thought that the underlying molecular mechanisms may also be universal. The purpose of this review is (i) to provide an overview of the current knowledge about the role of anionic lipids in protein translocation and membrane protein insertion and (ii) to provide a model in which common features of the influence of lipids on insertion of membrane proteins and translocation of secretory proteins are integrated.

This review will focus on the gram-negative bacterium *Escherichia coli.* This organism does not contain internal membranes and all proteins are synthesized in the cytosol. The cytosol is surrounded by an envelope consisting of an inner and an outer membrane that are separated by the so-called periplasmic space. Translocation and insertion are well studied in this organism and because of the advanced genetics, the phospholipid composition of the inner membrane can be manipulated very efficiently by controlling the key enzymes in the lipid biosynthetic pathways. In this way the role of lipids in translocation and insertion can be studied in living cells.

Phospholipids in the Inner Membrane of *E. coli*

The most abundant lipid in the inner membrane of *E. coli* is phosphatidylethanolamine (PE) which accounts for 75–80% of the total phospholipids (Raetz, 1978). PE is zwitterionic and does not carry a net charge at physiological pH. Phosphatidylglycerol (PG) and cardiolipin (CL) are negatively charged at physiological pH and account for 20% and 1–5% of the total phospholipids in the inner membrane respectively, the exact values depend on the growth conditions (Raetz, 1978). The lipids of the inner membrane are organized in a liquid-crystalline bilayer (Burnell et al., 1980), which is also the dominant organization in hydrated total lipid extracts of the inner membrane. Phospholipid biosynthesis in *E. coli* takes place at the inner leaflet of the inner membrane (Raetz, 1986; Raetz & Dowhan, 1990), but the distribution of the different lipid classes across the two halves of the bilayer is not known. For another prokaryote, it was shown that PG was present in almost equal amounts in the two leaflets (de Bony et al., 1989). Lipid biosynthesis in *E. coli* is well studied and the genes coding for the enzymes involved in headgroup diversification are characterized and knockout mutants are available. Disrupting *pgs*A

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Table. Lipid composition in *E. coli* cells

Strain (genotype)	Composition (in mol %)				
	PE	PG	CL	PA	Rest
SD12 (wt)	74	21	5	0	0
HDL11 (*pgsA*)	91	2	1	6	0
AD93 (*pssA*)	0	48	44	4	4
SD11 (*cls*)	82	18	0	0	0

The lipid composition of the inner membranes of SD12 (de Vrije et al., 1988), HDL11 (Kusters et al., 1991), AD93 (Rietveld et al., 1993) and of total cells of strain SD11 (de Vrije, 1989) are presented. HDL11 cells were grown in the absence of IPTG to prevent synthesis of anionic phospholipids. AD93 cells were grown in the presence of 50 mM $MgCl_2$.

blocks synthesis of PG and since CL is made by condensation of two PG molecules, the overall anionic lipid contents drops. In wild-type cells, PG is required to modify the major lipoprotein of *E. coli* and it was observed that below 2% PG, cells were dying. Strains without this lipoprotein, are able to survive at low PG levels (Kusters et al., 1991). Due to the accumulation of the acidic lipid precursor phosphatidic acid, a background level of 5–10% anionic lipids remains. Knocking out the *cls* gene blocks formation of cardiolipin without detectable effects for the cell (Nishijima et al., 1988). When the *pss*A gene is disrupted, no PE is synthesized and the inner membrane contains only anionic phospholipids (DeChavigny, Heacock & Dowhan 1991). However, the cells now require divalent cations and grow slowly (Rietveld et al., 1993; DeChavigny et al., 1991). By means of these lipid biosynthetic mutants the anionic lipid content of the inner membrane can be varied from below 10% to 100%. The composition of the major phospholipids in the inner membranes of wild-type and lipid biosynthetic mutant *E. coli* strains are depicted in the Table.

Components Involved in Protein Translocation

By means of biochemical and genetic techniques, a set of proteins was shown to be involved in translocation of proteins that have to function outside the cytosol. For overviews on this so called Sec-machinery the reader is referred to (Arkowitz & Bassilana, 1994; Driessen, 1994). Here we will only briefly introduce the machinery. Proteins destined for translocation are synthesized as precursors carrying N-terminal extensions called signal sequences. Precursors are often maintained in a translocation competent state by the tetrameric SecB protein (Kumamoto & Beckwith, 1985; Kusters et al., 1989; Randall, Topping & Hardy, 1990; Kumamoto, 1991) which also plays a role in targeting (de Cock & Tommassen, 1992). These SecB-precursor complexes have a high affinity for SecA which is found in the cytosol and in multiple conformations in the inner membrane where it couples ATP hydrolysis to translocation (Oliver & Beckwith, 1982; Hartl et al., 1990; Economou & Wickner, 1994). Together with SecA, the Sec-Y, -E and -G proteins constitute the basic machinery for translocation (Tokuda, 1994). Efficient in vivo translocation also requires the presence of two membrane proteins (SecD and SecF) whose exact roles are not known but may be involved in maintaining the proton motive force during translocation (Arkowitz & Wickner, 1994). After translocation signal sequences are removed by the action of signal peptidases. Some precursors require the presence of other cytosolic components such as the bacterial SRP (Luirink et al., 1992) or GroEL/ES (de Cock & Tommassen, 1992).

Beside these proteinaceous components also other, membrane related, factors play a role in efficient translocation. One of these is the proton motive force (pmf) consisting of a proton gradient (acidic in the periplasm) and an electrical component ($\Delta\Psi$, positive in the periplasm). It was shown that both components are equivalent forces in translocation (Oliver & Beckwith, 1982). Apart from the anionic lipids that play a special role in translocation and that form the topic of this review, also several other aspects of the lipid bilayer should be mentioned here. It was stated above that lipids from *E. coli* are organized in a dynamical liquid-crystalline bilayer. But also substantial and regulated amounts of nonbilayer lipids are present. These lipids are essential for survival of cells and required for dynamic processes such as protein translocation (Rietveld, Koorengevel, de Kruijff, 1995). The liquid-crystalline state of the lipids in which the acyl chains are disordered is also important for the functioning of the *E. coli* inner membrane. Lowering the temperature or incorporation of acyl chains with higher melting temperatures, results in a gel phase with more ordered acyl chains. This has many physiological consequences, one of which is inhibition of protein translocation (Kimura & Izui, 1976; Ito, Sato & Yura, 1977; Pages et al., 1978; DeRienzo & Inouye, 1979).

It was recently established that many of the proteinaceous components involved in translocation in prokaryotes and eukaryotes are conserved (Ng & Walter, 1994). Additionally, it was found that both lipids and proteins are involved in translocation across the membrane of the endoplasmic reticulum of eukaryotes (Martoglio et al., 1995). After having introduced the components involved in protein translocation, the next section will focus on the role of negatively charged phospholipids in translocation.

Involvement of Anionic Phospholipids in Precursor Translocation

Direct evidence for the involvement of anionic phospholipids in protein translocation was obtained when *E. coli* strains with a disrupted *pgsA* gene were used. It was shown that translocation of the outer membrane precursor proteins prePhoE and proOmpA was severely hampered in vivo and in vitro (de Vrije et al., 1988). The in vitro approach made use of vesicles isolated from the lipid biosynthetic mutant strains. In later studies it was shown that reintroduction of anionic phospholipids by means of a lipid transfer protein restored translocation (Kusters, Dowhan & de Kruijff, 1991). Apparently, it is only the negative charge that was important since also a variety of chemically different anionic lipids could restore translocation (Kusters et al., 1991). The use of *E. coli* strain HDL11 in which the expression of *pgsA* was under control of the *lac* operon enabled fine tuning of the amount of anionic phospholipids. It was shown that translocation efficiency was directly proportional to the amount of PG present in the inner membrane (Kusters et al., 1991). Later on the anionic lipid dependence of translocation was also found for other proteins.

The influence of anionic phospholipids on protein translocation efficiency can be either indirect or direct. Since lipids form the basic building blocks of biomembranes, altering the lipid composition of membranes could interfere with some basic function or property of the membrane. Evidence will be provided here for the direct involvement of anionic lipids in translocation, mediated by interactions with the Sec machinery or precursor proteins. We first summarize the evidence for SecA-lipid and precursor-lipid interactions as obtained from model systems. Next, the significance of these findings for the translocation process will be indicated.

SecA is a water soluble protein with an ability to associate with lipid monolayers and bilayers. It was proposed on the basis of vesicle aggregation studies and deletion mutagenesis that SecA contains two distinct lipid binding sites (Breukink, Keller & de Kruijff, 1993; Breukink et al., 1995). Breukink et al. (1992) observed very efficient penetration of SecA into lipid monolayers made from anionic lipids but not into monolayers of zwitterionic lipids. Binding of SecA to vesicles, as determined by fluorescence quenching studies, was also stimulated by the presence of anionic lipids (Ulbrandt, London & Oliver, 1992). From these studies, it was concluded that binding of SecA to membranes depended on the presence of anionic phospholipids. Circular dichroism and proteolysis experiments indicated that binding of SecA to negatively charged membranes is accompanied by changes in the conformation of the protein (Ulbrandt et al., 1992). It was proposed on the basis of experiments employing monolayers of anionic phospholipids that SecA undergoes a nucleotide-dependent membrane insertion-deinsertion cycle (Breukink et al., 1993). Binding of nonhydrolyzable ATP analogues caused deep penetration of SecA, while the ADP-bound form is more surface associated. A similar nucleotide dependent cycle was detected in bilayer systems employing Electron Spin Resonance techniques (Keller et al., 1995). In these experiments it was also found that SecA can penetrate deeply into the acyl chain region of the bilayer.

Interactions of anionic lipids with precursor proteins, especially the signal sequences, were also investigated. Peptides corresponding to signal sequences (signal peptides) were chemically synthesized and used to study interactions with model membranes. These studies showed spontaneous partitioning of signal peptides in lipid monolayers and bilayers with a preference for anionic lipids. This is consistent with the primary structure of signal sequences (von Heijne, 1985): a positively charged N-terminus is followed by a central hydrophobic core of 7–15 residues which could penetrate into the hydrophobic core of the membrane. A more polar C-terminal region of 3–7 residues precedes the cleavage site. It was demonstrated that the adoption of α-helical structures in apolar environments as trifluoroethanol (TFE) was a trademark of functional signal peptides (Briggs et al., 1986; McKnight, Briggs & Gierasch, 1989). Also a strong correlation was observed between presence of anionic lipids in the model membranes and induction of α-helical structure in the signal peptide (Keller, Killian & de Kruijff, 1992). The importance of charge interactions was demonstrated by using signal peptides in which the positive charges at the N-terminus were replaced by negative charges. These negatively charged signal sequences were less efficient in translocation. They showed reduced penetration into monolayers and also adopted less helical structure in the presence of anionic lipids, although the ability to form helical structures in TFE was comparable to wild-type peptides (Demel, Goormaghtigh & de Kruijff, 1990; Keller et al., 1992). From NMR studies on the structure of signal peptides in membrane-mimicking environments with anionic detergents, it was concluded that a functional signal peptide adopts a dynamical helix-break-helix conformation (Rizo et al., 1993; Chupin et al., 1995). Many signal peptides contain a helix breaking residue and it is thought that this structural motif is of importance for the efficiency of initiation of translocation.

After establishing the possibility of anionic lipid-SecA and anionic lipid-precursor interactions in model systems we should concern ourselves with the question whether these interactions play a role in the functional process. SecA is present in a five- to tenfold molar excess compared to the integral membrane components of the Sec-machinery and about 10–40% of this amount is bound to the membrane (Cabelli et al., 1991). Similar to

the situation described for the model systems, binding of SecA to inner membranes is dependent on the anionic lipid content (Kusters, Huijbregts & de Kruijff, 1992). Using membrane vesicles from lipid biosynthetic mutants, it was shown that anionic phospholipids together with SecY and SecE provide a high affinity binding site for SecA (Hendrick & Wickner, 1991). On the basis of several studies employing inner membrane vesicles, a model for the activity of SecA was proposed with many similarities to the nucleotide driven insertion-deinsertion cycle that was proposed on the monolayer results. It is thought that upon ATP binding SecA inserts deeply into the membrane and adopts a transient membrane-spanning conformation, concomitantly moving a part of the precursor across the membrane (Economou & Wickner, 1994). Upon ATP hydrolysis SecA de-inserts and the precursor is released from SecA. Then, the cycle can start again to move the next piece of the precursor across. It was shown by Lill, Dowhan and Wickner that the ATPase activity of SecA was stimulated by anionic phospholipids (Lill et al., 1990).

It is therefore concluded that interactions with anionic lipids play a role in the translocation activity of SecA. Important remaining questions concern especially the surroundings of SecA during the insertion-deinsertion cycle. It is not known whether SecA inserts between the lipids or in the proteinaceous environment formed by other Sec proteins, or in between.

Also interactions between precursors and anionic lipids in the functional process were studied. Translocation of the M13 procoat precursor requires the presence of anionic lipids, while it does not require the activity of SecA (Kusters et al., 1994). This will be further discussed in the next section. Moreover, translocation of various chimeric proOmpF-Lpp precursors with artificial signal sequences was investigated, employing membrane vesicles of HDL11 with variable anionic lipid content. It was observed that translocation efficiencies of precursors with long, hydrophobic signal sequences depended neither on the presence of positive charges at the N-terminus nor on anionic lipids in the membrane (Phoenix et al., 1993*a,b*). Translocation of precursors with shorter hydrophobic cores was stimulated by the presence of positive charges at the N-terminus and negatively charged lipids in the membrane (Phoenix et al., 1993*a,b*). Strikingly, translocation of all these precursors with positive charges at the N-terminus required SecA (Hikita & Mizushima, 1992). From this it was concluded that the residual amount of anionic lipids in HDL11 vesicles (in these experiments 9%) was sufficient for SecA function. Furthermore, it was suggested that the stimulation of translocation efficiency by anionic lipids was due to electrostatic interactions between precursor and lipids. This idea was corroborated by the finding that shielding the negatively charged lipids of wild-type vesicles with positively charged compounds as doxorubicin and polylysine also decreased translocation efficiencies of the positively charged precursors (Phoenix et al., 1993*a*).

These results show that anionic lipids can stimulate translocation both by their effects on the action of SecA and by interaction with precursors. The results with the chimeric precursors suggested that relatively small amounts of anionic lipids were already sufficient for proper SecA functioning while larger amounts were required for optimal interactions with precursors. But also other components involved in translocation can be influenced by the anionic lipid contents of the membrane. *SecG* null mutants do not have an aberrant phenotype at 37°C, but it was found that their growth was arrested at 30°C (Nishiyama et al., 1994). This arrest could be alleviated by extra synthesis of anionic lipids (Kontinen & Tokuda, 1995). It is unclear whether this points to a direct interaction between SecG and lipids under physiological conditions.

Involvement of Anionic Lipids in Membrane Protein Assembly

Membrane proteins come in many flavors. Some are only peripherally associated with the membrane while others reside integrally in the membrane. Integral membrane proteins can span the bilayer either with hydrophobic α-helices or with amphiphilic β-sheets. It seems at the moment that the latter type is restricted to the outer membranes of gram-negative bacteria and mitochondria. Membrane proteins with membrane spanning α-helices are more common and the remainder of this section will deal with their characteristics and integration. These membrane proteins span the membrane by means of alternating signal anchor (SA) and stop transfer (ST) sequences (von Heijne & Gavel, 1988). They both contain a stretch of hydrophobic residues (typically 18–25) but SAs are preceded by positive charges and STs are followed by positive charges. From this it is clear that especially SA segments share similarities with signal sequences. In fact the only major differences seem to be the presence of a cleavage recognition site and a smaller hydrophobic length in signal sequences (von Heijne, 1990). The orientation of membrane proteins obeys the positive inside rule which states, on the basis of statistical analysis of membrane proteins, that hydrophilic loops rich in positive charges are predominantly located in the cytoplasm whereas loops largely devoid of positive charges are most often found in the periplasm (von Heijne, 1986). The structural and topological similarities of some membrane proteins and precursors are depicted in Fig. 1.

Membrane integration of proteins can follow two pathways depending on the lengths of the hydrophilic segments which have to pass the membrane. It was

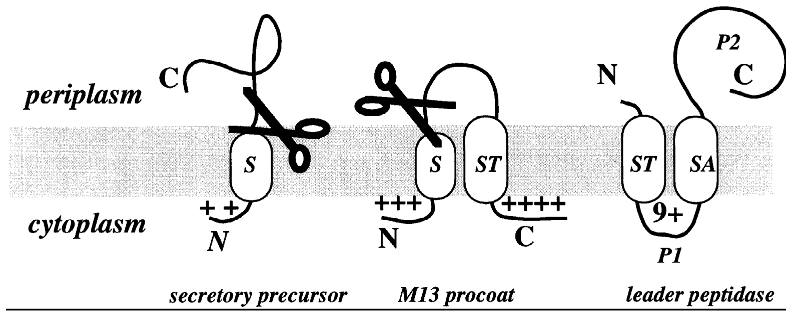

Fig. 1. Schematic representation of the similarities between precursors and membrane proteins. Uncleaved forms of a secretory precursor protein (left) and M13 procoat (middle) are drawn. The cleavage site directly behind the signal sequence (S) is indicated by scissors. Presence of a stop transfer (ST) and a signal anchor (SA) are indicated in a presentation of the transmembrane orientation of leader peptidase (right). The membrane is indicated by a grey bar.

shown in vivo that segments longer than approximately 60 amino acids depend on the function of the Sec-machinery including SecA while shorter loops to not require this (von Heijne, 1989; Lee et al., 1992: Andersson & von Heijne, 1993). Leader peptidase (Lep) from *E. coli* is a protein with a large C-terminal periplasmic segment which is preceded by two transmembrane regions and a cytoplasmic loop (*see* Fig. 1). Integration of Lep requires the action of the Sec-machinery both in vivo and in vitro (Moore, Dalbey & Wickner, 1988; Lee et al., 1992). The anionic lipid dependency of the integration was tested by shielding anionic phospholipids on wild-type vesicles by the positively charged agent polymyxin and by using vesicles from HDL11 with low anionic lipid contents. From these experiments it was concluded that efficient integration of this membrane protein required anionic phospholipids (W. van Klompenburg et al., 1997).

Also the anionic lipid requirement of membrane integration of the phage M13 precursor protein procoat was tested. This protein starts with a cleavable signal sequence, which is followed by a short periplasmic loop, a membrane spanning segment and a short C-terminal cytoplasmic segment (Fig.1). Because of the small size of the periplasmic loop, insertion of procoat was independent of the Sec machinery and could even take place in model membranes without Sec proteins (Silver, Watts & Wickner, 1981; Watts, Silver & Wickner, 1981; Watts, Wickner & Zimmermann, 1983). Membrane insertion of this protein depended on the presence of acidic lipids just as a procoat derivative with an extended periplasmic domain which did require SecA (Kusters et al., 1994). These experiments show that anionic lipid dependency of integration can be due to direct interactions of lipids with the newly synthesized protein. But which part of procoat would be prone to interact with anionic lipids? As described in general terms in the positive inside rule, the hydrophobic segments of the procoat protein are flanked by positively charged segments which remain in the cytosol. Replacing these by negative charges resulted in decreased integration efficiencies (Gallusser & Kuhn, 1990). Moreover, while wild-type procoat bound very efficiently to anionic lipid vesicles, the negatively charged mutants did hardly show any binding (Gallusser & Kuhn, 1990). This strongly suggests that the positive charges are involved in anionic lipid dependent penetration into the membrane.

The influence of positive charges on membrane protein topology was most convincingly shown by studies on Lep. For topology of wild Lep *see* Fig. 1. By genetic means all but one of the positive charges in P1 were removed and four positive charges were added to the periplasmically located N-terminus. After expression, the orientation of this construct was tested and shown to be completely inverted with now both N- and C terminus in the cytoplasm and P1 in the periplasm (von Heijne, 1989; Nilsson & von Heijne, 1990). This means that the positive charges are strong determinants of protein orientation.

An obvious possibility is that the anionic lipids interact with positive charges to establish membrane protein orientation. To get insight into this possibility very recent experiments combined the availability of the lipid biosynthetic mutant strain HDL11 and constructs derived from Lep with various charge distributions (van Klompenburg et al., 1997). In cells with wild-type lipid composition, constructs with one positive charge in the P1 loop and two or more positive charges at the N-terminus are exclusively found with the N-terminus in the cytosol. Lowering the anionic lipid contents of 25% to 10% facilitated passage of up to four positive charges at the N-terminus of Lep (van Klompenburg et al., 1997). This is the most direct proof thus far that interactions between newly synthesized membrane proteins and lipids are important in establishing orientation of membrane proteins.

Also the proton motive force (pmf) or the membrane dipole may contribute to the cytosolic location of positively charged loops. The dipole potential, which is positive in the membrane interior, favors the passive diffusion of hydrophobic anions across the bilayer over diffusion of hydrophobic cations (Flewelling & Hubbel, 1986). It is believed to arise from oriented dipoles at the membrane-water interface which can be caused by (i) the polar parts of the lipids and (ii) by oriented water molecules. Because the membrane dipole potential is a direct result of the structure of lipids, its influence on protein insertion will be hard to dissect. Of the total proton motive force (positive and acidic in the periplasm of *E.*

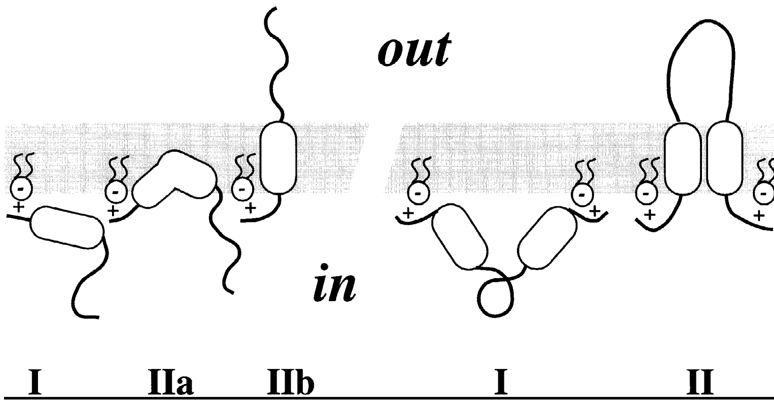

Fig. 2. Model for the interactions between anionic lipids and precursor and membrane proteins in initial stages of translocation and insertion. For detailed description *see* text.

coli), the electrogenic component $\Delta\Psi$ was proposed to retard the membrane passage of positive charges. Membrane passage of one or two lysines at the N-terminus of Lep was possible in the absence, but not in the presence of a pmf (Andersson & von Heijne, 1994). It was not possible to translocate more positive charges across the membrane, regardless of the absence or presence of the pmf. These data do not yield quantitative insights into the relative roles of pmf and anionic lipids, but it is clear that they both play a role.

Model for the Interaction of Anionic Lipids and Newly Synthesized Membrane and Precursor Proteins

Studies on translocation of precursor proteins and on the insertion of membrane proteins revealed important similarities in these processes such as their dependency on anionic lipids. The presence of a positive charge-hydrophobic segment cluster in both precursors and membrane proteins was shown to be important for translocation and insertion, and for the interaction with anionic lipids. How do we envisage the role of anionic lipids? There are several possibilities: (i) Anionic lipids can lower the pH at the membrane surface compared to the bulk, thereby protonate acidic amino acid residues and facilitate their membrane passage (Krishtalik & Cramer, 1995). (ii) Their presence gives rise to a negative surface charge in which positively charged ions can accumulate. (iii) They could directly bind to positively charged amino acids.

Which one of these three possibilities is most important, is not known. It is doubtful whether the 25% anionic lipids present in the *E. coli* inner membrane could lower the surface pH sufficient to allow protonation of aspartates and glutamates. Important in this respect is the pH of the cytoplasm of *E. coli.* It is conceivable that in some eukaryotic organelles in which the bulk pH is already low, the effect of the anionic lipids on surface pH is in fact sufficient to protonate acidic residues. This could for instance be important for the action of toxins or entry of viruses. Consequences of the low surface pH will not be taken into account in our model for translocation and insertion in *E. coli.* On the basis of the presented data it is not possible to clearly distinguish between the influence of a negative surface charge and of direct binding of lipids to proteins.

To summarize the current view on the involvement of lipids in both insertion and translocation, a model is presented (Fig. 2). In this model, we did not aim to include the knowledge on the involvement of proteins or the membrane potential in precursor translocation. In the first stage (I) the anionic lipids attract the positive charges at the N-terminus of signal sequences or in the hydrophilic loops of membrane proteins. Subsequently, the hydrophobic segments of membrane proteins and signal sequences will partition into the membrane (II). This can in principle happen with either N-terminal or C-terminal hydrophilic segments passing the membrane. Positive charges which initially interact with one side of the membrane will remain there. For signal sequences the membrane integration of the signal sequence may happen via a looped conformation (IIa) which is promoted by the helix-breaking residue in the middle of the signal sequence. After stretching of the signal sequence, the N-terminus of the mature part moves across the membrane (IIb).

It is likely that step IIb of signal sequence insertion in our model can only take place in the presence of the Sec proteins since the mature part of the precursor is in general hydrophilic and unlikely to reside in the hydrophobic core of the membrane. Consistent with this suggestion, it was observed that a concerted action of the pmf causes the stretching of the signal sequence and the activation of the Sec machinery (Nouwen, de Kruijff & Tommassen, 1996*a,b*). Because of the similarities between the translocation processes across different cellular and intracellular membranes and the fact that anionic

lipids are present there, we think that the model proposed here may be valid for those systems as well.

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