Protein-Lipid Interactions in Assembly and Function of Leader Peptidase

Het belang van eiwit-lipide interacties voor membraan-assemblage en functie van leader peptidase

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Abbreviations

ATP	adenosine 5' tri phosphate
BSA	bovine serum albumine
CL	cardiolipin
DOPC	1,2-dioleolyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-dioleolyl-*sn*-glycero-3-phosphoethanolamine
DOPG	1,2-dioleolyl-*sn*-glycero-3-phosphoglycerol
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetic acid
ER	endoplasmic reticulum
GAR-PO	goat anti rabbit horseradish peroxidase
IM	inner membrane
IPTG	iso propyl thio galactopyranoside
Lep	leader peptidase
LPS	lipo poly saccharide
LUVETS	large unilamellar vesicles made by extrusion techniques
OM	outer membrane
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
pmf	protonmotive force
PMSF	phenyl methyl sulfonyl fluoride
SBTI	soy bean trypsin inhibitor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane

Chapter 1

Introduction

Biomembranes

Cells are the entities of life and they at least consist of one aqueous compartment separated from the environment by a membrane. The emergence of membranes marks the transition of prebiotic evolution to cellular life (Koch, 1984) and it is thought that by confining the information bearing molecules like nucleic acids and their products to essentially one space, membranes facilitate the action of natural selection (Koch, 1995).

The essential feature of all membranes is the selective permeability to solutes. Membranes thereby enable the compartments they enclose to be vastly different from their environment in a chemical and structural sense. Because of this, membranes are at the basis of structural and functional differentiation between, and also within cells. Intracellular compartmentalisation, as observed in eukaryotic organisms, reduces the distance across which solutes and enzymes have to diffuse and allows for functional specialisation. Moreover, enzymes involved in subsequent steps of multistep processes can be attached to or integrated in membranes to form multi enzyme complexes. It is thought that the products released by one enzyme are in this way "channelled" to the next enzyme, which would facilitate complex metabolic conversions.

Membranes are non-covalent macromolecular assemblies of lipids and proteins of which the exact composition is highly variable and depending on cell or organelle type. In general membrane lipids are amphiphilic molecules which upon dispersion in water can spontaneously form relatively impermeable liquid crystalline bilayers. These bilayers form the structural backbone of biomembranes. Proteins form the functional framework of biomembranes and take care of for instance communication and solute transport. These controlled and directional membrane processes imply a functional asymmetry between the inner and outer face of membranes. This is reflected by differences in lipid (Bretscher, 1972; Verkleij *et al.*, 1973) and protein composition of the two faces of the membrane. Moreover, natural membranes are highly complex dynamical structures in which lipid and proteins may diffuse laterally to some degree (Singer and Nicolson, 1972). At the other hand, it is known that diffusion of some membrane proteins is limited or not random and even specialised domains may exist (Jacobson *et al.*, 1995).

Bacteria are relatively simple unicellular organisms which only contain one or two membrane systems. The need for internal compartments is largely circumvented by being small (Koch, 1990). This thesis and the major part of this introduction focuses on the inner membrane of the gram negative bacterium *Escherichia coli* which is a member of the family *enterobacteriaceae*. It is such an attractive model in membrane research for two reasons. Key

processes as membrane biogenesis, energetics, signal transduction and transport which in eukaryotic cells are divided over several membranes, all take place here. On the other hand both the lipid and protein composition of the *Escherichia coli* inner membrane can be genetically manipulated.

The envelope of *E. coli*

The cytoplasm of *E. coli* is surrounded by an envelope (Lugtenberg and van Alphen, 1983) which is comprised of the inner and outer membrane, which are separated by the periplasmic space (Fig. 1).

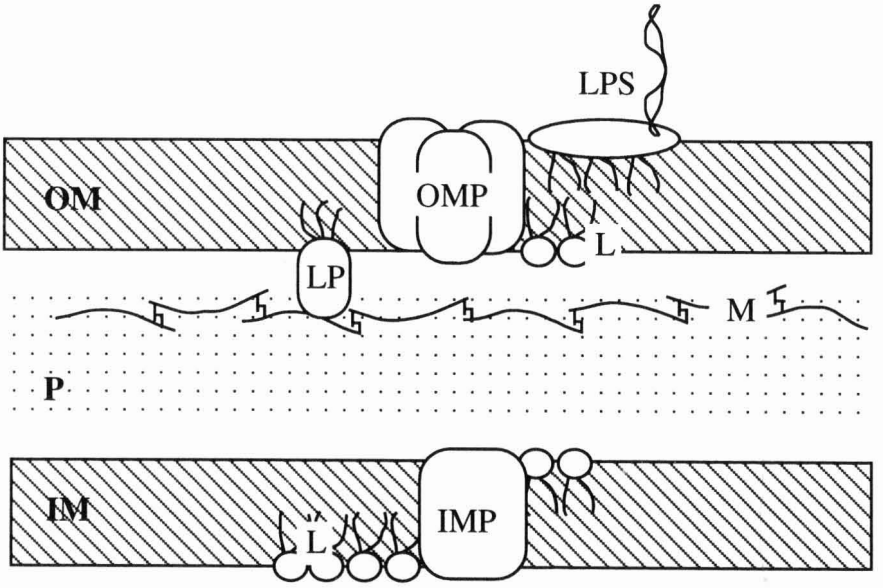


Figure 1. Schematic representation of the cell envelope of *E. coli*. The cytoplasm of *E. coli* is surrounded by the inner membrane (IM), the periplasm (P) and the outer membrane (OM). The IM contains phospholipids (L) and proteins (IMP). The gel state of the periplasm is indicated by the dotted area. The murein sacculus (M) gives the cell its shape and is linked to the OM via Braun's lipoprotein (LP). In the outer membrane, proteins (OMP), phospholipids (L) and lipopolysaccharide (LPS) are found. The presence of periplasmic space spanning structures like for instance flagella is not indicated.

The outer membrane (OM) protects gram negative bacteria against a putative hostile environment with harmful compounds as detergents (e.g. bile salts), antibiotics and phospholipases (Nikaido and Vaara, 1985). The lipid constituent of the OM is highly asymmetric (Nikaido and Vaara, 1987); at the inner leaflet exclusively phospholipids and lipoprotein and in the

outer leaflet mainly lipopolysaccharide (Fig. 1, LPS) are found. LPS consists of a hydrophobic part, lipid A and a hydrophilic part composed of an oligosaccharide core and in most bacterial strains a moiety called the O antigen (Schnaitmann and Klena, 1993). LPS is negatively charged and forms complexes with cations (Lugtenberg and van Alphen, 1983). The importance of these complexes is demonstrated by the destabilising effect of compounds like EDTA which scavenge divalent cations. Destabilising or removal of LPS, renders the whole cell more susceptible to agents added from the outside. The outer membrane (Fig. 1, OM) contains a large amount of relatively few different integral membrane proteins, mainly diffusion pores allowing the passage of hydrophilic molecules with molecular weights lower than 600 Da (Payne and Gilvarg, 1968; Decad and Nikaido, 1976).

The compartment between the OM and inner membrane (IM) is called the periplasm (Fig. 1). From electron microscopic studies (Leduc *et al.*, 1985) and from estimates of the lateral diffusion rate (Brass *et al.*, 1986), it was inferred that this compartment is filled with a gel. This gel consists of proteins (5% of the total cellular protein content) and large amounts of hydrated heteropolymers. Two types of polymers are present. Membrane derived oligosaccharides (MDOs) are highly charged glucose polymers substituted with phosphoglycerol, ethanolamine and succinyl ester groups (Kennedy *et al.*, 1976). They are claimed to keep the periplasm isotonic to the cytoplasm (Kennedy, 1982). Therefore the hydrostatic pressure in both cytoplasm and periplasm is higher than in the environment. Without the presence of a highly cross-linked second polymer, the peptidoglycan (also called murein, Fig. 1, M) (Weidel and Pelzer, 1964), the membranes would yield to the turgor pressure. The peptidoglycan consists of sugar chains (on average 21 disaccharide units) which are linked by peptides to form the so called murein sacculus which gives the cell its rigidity and shape (Braun *et al.*, 1973). The murein sacculus is linked to the outer membrane mainly through the *lpp* gene product (Braun, 1975), Braun's lipoprotein (Fig. 1, Lpp). Lpp is covalently linked via the carboxy-terminal lysine to the murein (Braun and Bosch, 1972) while the amino-terminal cystein is modified with a palmitate and a diacylglycerol moiety which anchors the protein to the OM (Hantke and Braun, 1973).

The inner membrane (IM) consists of phospholipids which are organised in a dynamical, liquid crystalline bilayer, and of about 6 - 9 % of the total amount of cellular proteins (Cronan *et al.*, 1987). Many structural components of the cell envelope, like LPS, MDO and murein are synthesised by proteins associated with the IM (Osborn *et al.*, 1972; van Golde *et al.*, 1973; Waxman and Strominger, 1983). The IM also plays a key role in energy transduction. The oxidation of organic substrates is coupled to the generation of a proton motive force (pmf) (Ingledew and

Poole, 1984), which in turn can drive flagellar motion (Manson *et al.*, 1977), ATP synthesis (Futai and Kanazawa, 1983) or the transmembrane transport of solutes (see below). The IM is the actual diffusion barrier (Cronan *et al.*, 1987) of the envelope and only allows passage of solutes via specific transport systems. Some of these transport systems could simply mediate diffusion of substrates across the membrane, as for example the glycerol facilitator encoded by the *glpF* gene (Heller *et al.*, 1980). Other transport systems create a concentration difference for their substrates between both sides of the membrane at the expanse of energy. Examples of these are the transport of lactose (Zilberstein *et al.*, 1979) histidine (Hobson *et al.*, 1985), and mannitol (Jacobson *et al.*, 1983; Leonard and Saier, 1983). In these cases energy is provided by the proton motive force, ATP consumption or chemical modification of the substrate respectively. Also proteins which are synthesised on cytosolic ribosomes but have to function outside the cytosol, for instance as components of the cell envelope, are transported across the IM at the expanse of the proton motive force and ATP consumption (see also section on protein translocation and for reviews: (Wickner *et al.*, 1991; Driessen, 1994).

Phospholipids

Structure and function of phospholipids

Biomembranes contain an overwhelming diversity of lipids which on basis of their chemical structures can be divided in different classes. The inner membrane of *E. coli* contains only glycerol based phospholipids (Fig. 2). On basis of their headgroups the lipids of the inner membrane are divided in three major classes. The zwitterionic phosphatidylethanolamine (PE) accounts for 75 % of the total phospholipid of *E. coli*. The remaining 25% is formed by phosphatidylglycerol (PG) and cardiolipin (CL), which are both negatively charged at physiological pH (Raetz, 1978). The most common fatty acids in *E. coli* are palmitic acid (16:0), palmitoleic acid (16:1) and *cis*-vaccenic acid (18:1; Δ11-12), while also cyclopropane fatty acids (17:0cyc and 19:0cyc) are found. Polyunsaturated fatty acids are not synthesized.

This lipid diversity in *E. coli* is not compatible with the all too simple notion that lipids only provide the building blocks of bilayer membranes. At least three different reasons can be given for the observed diversity: 1) Some phospholipids have specific functions involved in different cellular processes (Jackson and Kennedy, 1983; Miller and Kennedy, 1987; Xia and Dowhan, 1995). 2) For optimal membrane functioning it is believed that the acyl chains of lipids should be predominantly in a liquid crystalline state. The selective synthesis and incorporation of

different fatty acids enable *E. coli* to maintain the fluidity of the bilayer over a range of temperatures. Membranes from cells grown at low temperatures contain more *cis*-vaccenic acid and less palmitic acid and therefore have a decreased melting temperature (Marr and Ingraham, 1962; Cronan and Gelmann, 1975). 3) Biomembranes have to contain lipids with different shapes (Lindblom *et al.*, 1986; Goldfine *et al.*, 1987; Rietveld *et al.*, 1993).

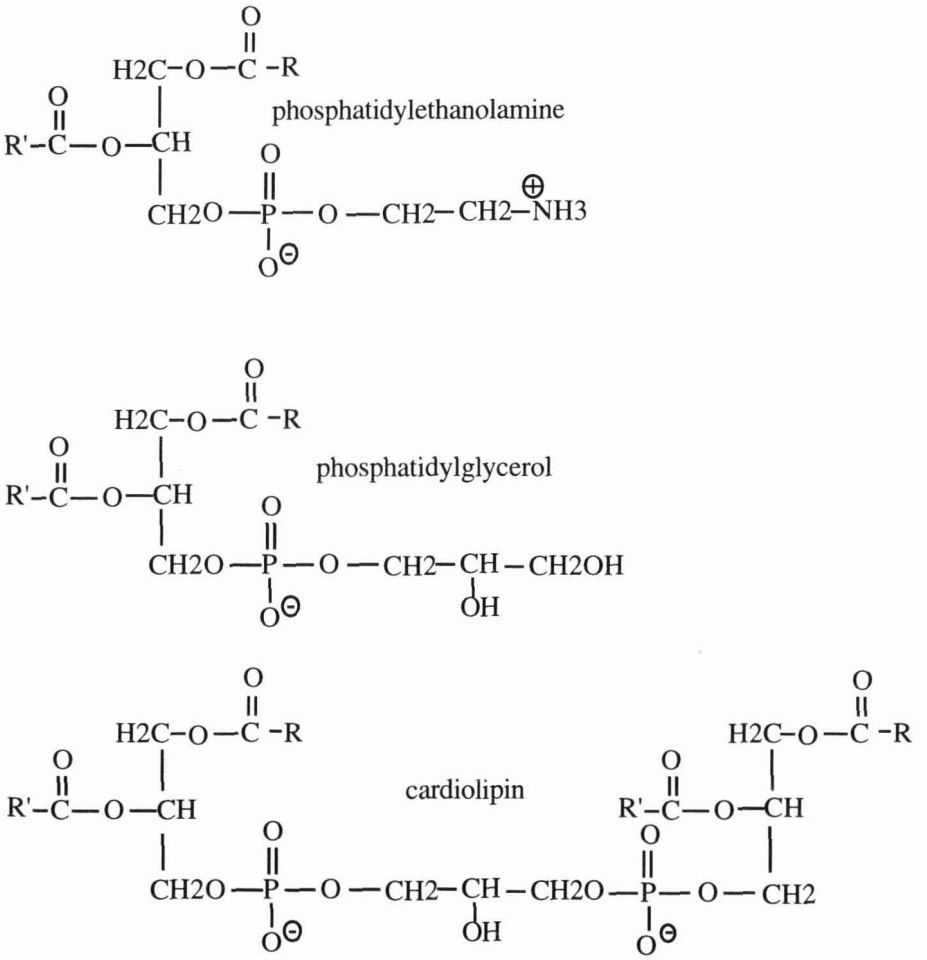


Figure 2. Chemical structure of the major phospholipids of the inner membrane of *E. coli*. *R* and R' indicate the acyl chain at the *sn* 1 and *sn* 2 position, respectively.

Due to characteristics of both headgroup and acyl chains, lipids can adopt different liquid crystalline structures, an ability commonly referred to as lipid polymorphism (Cullis and de Kruijff,

1979). When the cross-sectional areas in the headgroup region and acyl chain region areas are similar, the lipid will be cylindrical and pack easily into bilayer structures. When however, the cross-sectional area in the headgroup is smaller than in the acyl chain region, as is the case with PE of *E. coli*, the lipids will be cone shaped and give rise to so called inverted hexagonal (H_{II}) structures. The latter are called non-bilayer preferring lipids. The structural preferences of lipids are strongly influenced by temperature, pH, ionic strength, divalent cations and membrane proteins (Killian and de Kruijff, 1986). Biomembranes contain both bilayer preferring and non-bilayer preferring lipids and it was found that the membrane composition is tightly regulated in such a way that the lipids are in a bilayer configuration, but with a tendency to form non-bilayer structures (Tate *et al.*, 1991).

Synthesis of phospholipids

Biosynthesis of phospholipids in *E. coli* starts in the cytosol where the fatty acyl chains are made. All later steps take place at the cytosolic surface of the IM. The first acyl chain is transferred from the acyl carrier protein (ACP) to the first position of the glycerol part of *sn*-glycerol-3-phosphate by the *plsB* gene product (Lightner *et al.*, 1980). Phosphatidic acid (PA) is formed by transfer of the second acyl chain (Fig. 3). PA normally turns over very rapidly into CDP-diglyceride. Diversification of the polar headgroups starts by the exchange of CDP for either L-serine or *sn*-glycerol-3-phosphate to result in phosphatidylserine (PS) and phosphatidylglycerol phosphate (PG-P), by the action of the *pss* and *pgsA* gene products, respectively (Fig. 3) (Raetz, 1986). PS is normally decarboxylated very fast to the zwitterionic end product PE. PG-P is dephosphorylated to PG. Cardiolipin synthase (*cls*) catalyses the condensation of two PG molecules to form cardiolipin.

A substantial part of these lipids does not stay at the site of synthesis, but has functions in different locations. PE is the major phospholipid of the OM (Lugtenberg and Peters, 1976) and PG is used for the modification of Braun's lipoprotein (Sankaran and Wu, 1994). Both PE and PG are used in synthesis of MDOs (Jackson and Kennedy, 1983; Miller and Kennedy, 1987). Moreover, most organisms have an asymmetric distribution of phospholipids over the two leaflets of their membranes. Although for *E. coli* the lipid distribution is not yet determined, for some prokaryotic organisms a slightly asymmetric distribution of PG was found (de Bony *et al.*, 1989). About the mechanisms involved in lipid transport and maintaining lipid asymmetry, very little is known at present.

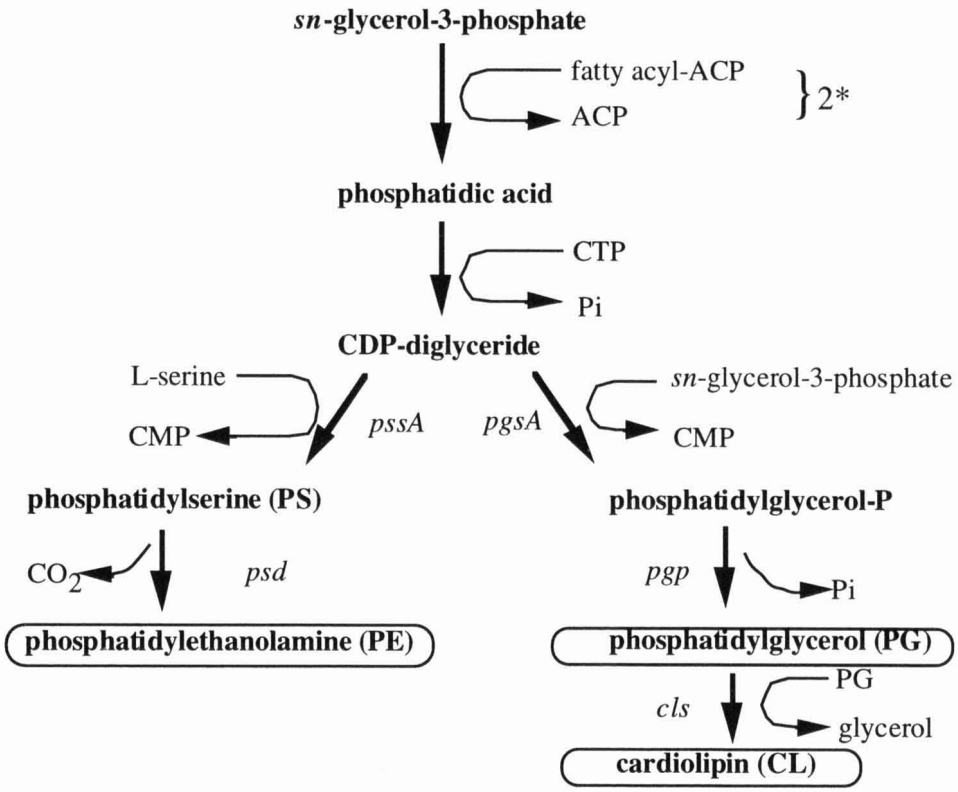


Figure 3. Terminal part of the phospholipid synthesis pathway. Names written in **bold** indicate intermediate- or end-products, boxed names indicate end products which occur in wild type *E. coli* cells, *italics* indicate the structural genes involved in headgroup diversification.

Phospholipid headgroup composition is in *E. coli* probably not regulated at the level of enzyme synthesis since it is not affected by overexpression of the synthetic enzymes (van den Boom and Cronan, 1989; Raetz and Dowhan, 1990). The only way of substantially changing the headgroup composition is by making chromosomal deletions in the lipid biosynthetic genes. By knocking out the *pssA* gene (DeChavigny *et al.*, 1991) cells could be grown which were completely devoid of PE. The loss of the only non-bilayer preferring phospholipid of *E. coli* is fatal to the cells. However, addition of divalent cations such as magnesium or calcium to the growth medium

restored cell growth by induction of non-bilayer behaviour of cardiolipin (Rietveld *et al.*, 1993; Killian *et al.*, 1994). Null alleles of the *pgsA* gene resulted in very low concentrations of the anionic phospholipids PG and CL (Miyazaki *et al.*, 1985)but when the anionic phospholipid concentrations reaches 1-2 %, growth ceases (Heacock and Dowhan, 1987; Heacock and Dowhan, 1989) unless the synthesis of Braun's lipoprotein was abolished. Modification of the lipoprotein consumes substantial amounts of PG. Furthermore the amount of *pgsA* synthesis and thereby the anionic phospholipid composition could be regulated after reintroducing a copy of *pgsA* under transcriptional control of the *lac* operon (Kusters *et al.*, 1991). The resulting headgroup compositions in some lipid biosynthetic mutant strains are depicted in table I.

Table I. Lipid composition of wild type and mutant *E. coli* cells. The lipid composition of the inner membranes of SD12 (de Vrije, 1989), 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 $MgCl_2$.

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

Membrane proteins

Both the inner and outer membrane of *E. coli* contain peripheral and integral membrane proteins. Peripheral proteins will be discussed in a following section dealing with lipid-protein interactions The three-dimensional structures of some integral outer membrane proteins were solved and it was found that these span the bilayer by means of so-called β barrel scomposed of many amphiphilic anti parallel β strands (Cowan *et al.*, 1992). This class of membrane proteins seems to be restricted to the outer membrane of gram negative bacteria and the outer mitochondrial membrane. Integral inner membrane proteins and the vast majority of eukaryotic membrane proteins, on the other hand, are thought to span the bilayer by means of α helices of approximately 20 hydrophobic amino acid residues. Experimental evidence for this suggestion is

provided by X-ray analysis of several membrane proteins (Deisenhofer *et al.*, 1985; Allen *et al.*, 1986; Tsukihara *et al.*, 1996). Attention will be focused on this class of proteins.

Integral membrane proteins are classified according to the number of membrane spanning helices and their orientation in the bilayer (Fig. 4). Bitopic membrane proteins contain only one membrane spanning segment and they share structural similarities with proteins which have to pass the prokaryotic inner membrane. These secretory proteins (also called precursors) are synthesised with a hydrophobic segment at their N-terminus. These segments are called signal sequences and are removed by signal peptidases after their function in the translocation process. The major signal peptidase of *E. coli* is called leader peptidase. The similarities between precursors and membrane proteins are visualised in figure 4. Moreover, precursors can be converted into membrane proteins by preventing cleavage of the signal sequence (Dalbey and Wickner, 1985). The transmembrane spanning region of a bitopic membrane protein acts as either a signal-anchor (SA) or a stop-transfer (ST) sequence (Fig. 4 upper panel). A SA sequence is located in front of a translocated domain and initiates its membrane passage, while a ST sequence marks the end of the translocated domain.

Bitopic membrane proteins are divided into four classes. Type I proteins are synthesised with signal sequences. A C-terminal ST sequence anchors these proteins in the bilayer. Type II proteins are anchored to the membrane via a SA sequence which acts as a signal for translocation of the C-terminal domain. Alternatively, when the (usually) small N-terminus translocates, a type III membrane protein is obtained and the hydrophobic segment is called a ST domain. Type IV proteins are anchored to the membrane via a C-terminal hydrophobic segment. All multiple spanning (polytopic) membrane proteins exist of alternating SA and ST domains. Depending on the number (odd or even) of transmembrane helices and the location of the termini, there are in principle four possible transmembrane folds (topologies): N_{in}-C_{in}, N_{out}-C_{out}, N_{in}-C_{out}, and N_{out}-C_{in} as depicted in the lower panel of figure 4

Figure 4 also indicates the strong correlation between the orientation of membrane helices and the distribution of positive charges in all classes of membrane proteins. The non-translocated hydrophilic segments are enriched in positively charged amino acid residues compared to the translocated segments. This phenomenon was called the positive-inside or cis-positive rule and observed in several cellular and organellar membranes (von Heijne, 1986; Gavel *et al.*, 1991; Gavel and von Heijne, 1992; Sipos and von Heijne, 1993).

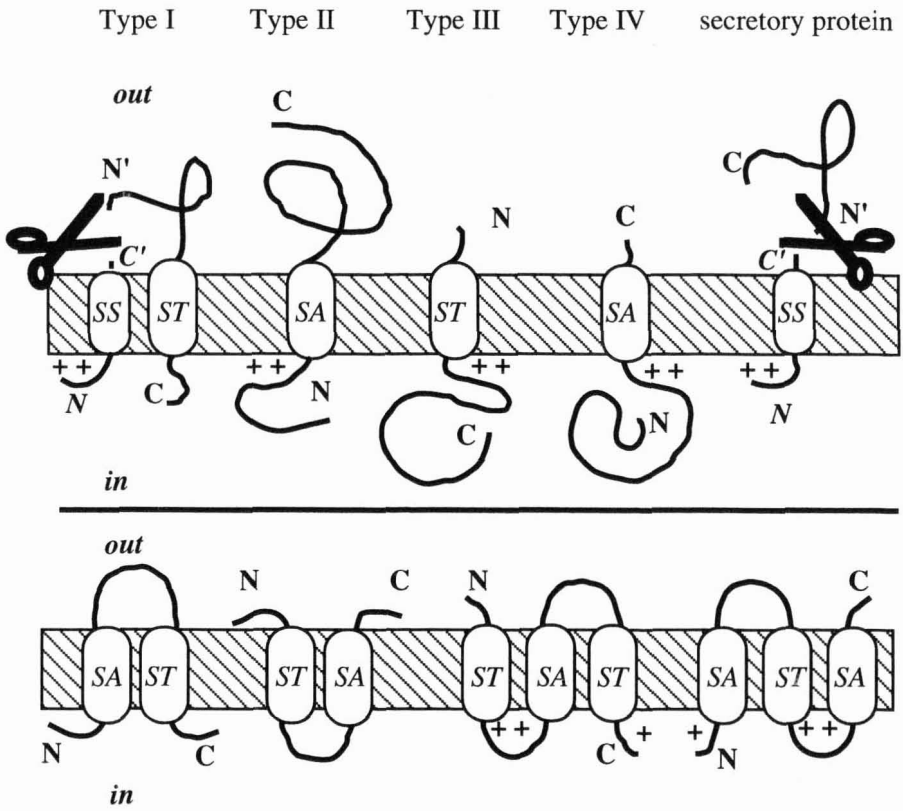


Figure 4. Structure of membrane proteins. The positions of signal sequences (*SS*), signal anchors (*SA*) and stop transfer sequences (*ST*) are indicated. Upper panel: bitopic and secretory proteins. The positions of the N- and C-termini relative to the membrane are indicated. Scissors indicate cleavage by leader peptidase. Lower panel: The four different topologies which can be assumed by polytopic membrane proteins. The two possibilities for proteins with even numbers of transmembrane helices (left) and odd numbers (right) are indicated. Most often membrane proteins contain more membrane spanning helices.

The importance of positive charges was demonstrated by experiments in which the charge balance around the transmembrane helices was inverted. By adding positive charges to the N-terminus of leader peptidase, and removing the positive charges in the single cytoplasmic loop, the orientation of this polytopic membrane protein could be inverted from N_{out}-C_{out} to N_{in}-C_{in} (von Heijne, 1989; Nilsson and von Heijne, 1990; Andersson *et al.*, 1992) Cytochrome *P*450, a type III protein could be converted to a type II protein by adding positive charges to the N-terminus (Monier *et al.*, 1988; Szczesna-Skorupa and Kemper, 1988; Szczesna-Skorupa and Kemper, 1989). In a similar way

type II proteins (ASGP receptor subunit H1 and paramyxovirus HN) were partially converted into type III proteins (Parks *et al.*, 1989; Beltzer *et al.*, 1991; Parks and Lamb, 1991). The incomplete conversion to type III proteins may be caused by the length and folding of the hydrophilic domains. In general the N-terminal loops of natural type III proteins are very short (Rohrer and Kuhn, 1990).

In summary; statistical analyses suggested a role for positively charged residues as topological determinants which was confirmed by mutagenesis studies. It is not known why the positively charged loops prefer cytoplasmic localisations, but one of the interesting possibilities is binding of these loops to negatively charged lipids.

Protein translocation and membrane protein insertion

Both secretory proteins and periplasmic loops of integral membrane proteins have to pass the bacterial inner membrane. Therefore translocation and integration mechanisms of secretory proteins and membrane proteins of *E. coli* will be briefly summarised here. Secretory proteins are synthesised in the cytosol as precursors with signal sequences as already mentioned in section 1.4. Signal sequences consist of three portions, a positively charged N-terminus, a hydrophobic core of 7-15 amino acid residues with often a helix breaking residue in the middle, and a C-terminal recognition site for enzymes which remove the signal sequence after translocation (von Heijne, 1985). A stretch of approximately 30 amino acid residues following the signal sequence is depleted in positively charged residues (Andersson and von Heijne, 1991).

Efficient translocation of precursors requires metabolic energy in the form of a protonmotive force and ATP. Anionic phospholipids (de Vrije *et al.*, 1988) and non-bilayer phospholipids (Rietveld *et al.*, 1995) are also required for translocation. By genetic and biochemical means, a set of proteins was also found to be involved in the translocation of secretory proteins (Arkowitz and Bassilana, 1994). SecB interacts with a subset of precursor proteins and keeps them in a translocation competent state (Kumamoto and Beckwith, 1985; Kusters *et al.*, 1989; Kumamoto, 1991). Some precursor proteins may use other components, like the GroEL/ES chaperone (Bochkareva *et al.*, 1988; Kusukawa *et al.*, 1989) or the bacterial SRP (Luirink *et al.*, 1992), to stay en route to the membrane. The SecB-precursor complex can associate with the SecA protein (Hartl *et al.*, 1990) which is found both in the cytosol and associated with the inner membrane (Oliver and Beckwith, 1982). ATP hydrolysis by membrane bound SecA and the proton motive force drive the translocation of the secretory proteins. The

mechanistic details of this process are not clear, but it was shown that binding and hydrolysis of ATP drives an insertion-deinsertion cycle of SecA in the membrane (Breukink *et al.*, 1992; Economou and Wickner, 1994; Kim *et al.*, 1994) which in turn may drive inversion of the topology of SecG (Nishiyama *et al.*, 1996). ATP hydrolysis is stimulated by the SecB-precursor complex, anionic phospholipids and the SecYEG complex (Lill *et al.*, 1990). The SecY, SecE and SecG proteins are polytopic membrane proteins and constitute together with SecA the minimal machinery for efficient protein translocation (Tokuda, 1994; Douville *et al.*, 1995).

After translocation the signal sequences from precursors, but rarely from membrane proteins, are removed by signal peptidases (processing). Although specialised signal peptidases do exist, like the lipoprotein signal peptidase lspA (Dev and Ray, 1984) or the prepillin peptidase (Meyer *et al.*, 1984), most precursor proteins are processed by leader peptidase (Lep) (Dalbey and Wickner, 1985; Wickner *et al.*, 1987), also called signal peptidase I. After cleavage the signal sequence is probably degraded very efficiently as was shown for prelipoprotein signal sequences (Ichihara *et al.*, 1984; Novak and Dev, 1988).

Most membrane proteins described in literature have a unique topology and it is generally believed that this is achieved during their integration in the membrane. This integration involves membrane passage of hydrophilic loops and transfer of the hydrophobic helices from the cytosol to the hydrophobic core of the membrane. Hydrophilic loops of membrane proteins can probably pass the membrane in two essentially different ways. The translocation of longer loops makes at least partially use of the action of the Sec machinery as described above for precursor proteins (Lee *et al.*, 1992; Andersson and von Heijne, 1993). Much less is known about membrane insertion of proteins with shorter periplasmic loops. Membrane integration of the type I protein procoat from the bacteriophage M13 could occur in a pure lipid bilayer in which only leader peptidase was included to remove the signal sequence (Silver *et al.*, 1981; Watts *et al.*, 1981; Geller and Wickner, 1985). Integration of this M13 protein required the presence of both hydrophobic segments, and positive charges in the cytosolic loops (Galluser and Kuhn, 1990). From these experiments and others, involving constructs derived from leader peptidase, it was concluded that loops with less than 50-60 amino acid residues can probably pass the membrane in a spontaneous fashion not requiring the aid of other integral membrane proteins (Andersson and von Heijne, 1993). The transfer of the hydrophobic segments from an aqueous environment to the hydrophobic core of the bilayer is probably an energetically favourable event and could offset some of the energy costs of membrane passage of hydrophilic segments (von Heijne and Blomberg, 1979; von Heijne,

1980). However, still very little is known about the mechanisms involved in the insertion process and most studies employed small peptides and artificial membrane systems. Thusfar only few studies aimed at elucidating the mechanisms involved in the integration of polytopic membrane proteins in living cells. This was, at least partly, due to the lack of sensitive assays for integration of most membrane proteins.

Lipid-protein interactions

Protein-lipid interactions are important for both integral and peripheral membrane proteins and therefore some of the possible interaction mechanisms are briefly mentioned here. For integral membrane proteins such as discussed above, hydrophobic interactions between transmembrane segments and lipid acyl chains can occur. The lipids directly surrounding an integral membrane protein form the so called annulus and the properties of the lipids in this ring can be different from that of the bulk lipids. It is shown that proteins may have specific structural preferences for certain lipids in their annulus (Esmann *et al.*, 1985). Peripheral membrane proteins can contact the membrane in many different ways as is depicted in figure 5, and some of the biological examples are given below.

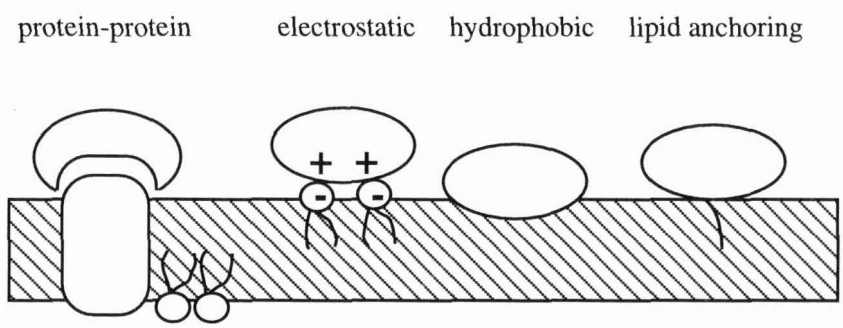


Figure 5. Possible interaction modes for binding of peripheral membrane proteins to membranes. For examples: see text.

The integral membrane component of the H^+-ATPase is responsible for the membrane association of the F_1 portion (Hoppe and Sebald, 1984), which therefore does not have to bind to lipids itself. Alternatively, covalent attachment of one or more acyl chains to a protein as is the case with

murein lipoprotein (Braun, 1975) can also anchor a protein to the membrane. Binding can also be mediated by electrostatic interactions between positively charged protein segments like in myelin basic protein (Cheifetz *et al.*, 1985) and the headgroups of negatively charged phospholipids which are present in all natural occurring membranes. Pyruvate oxidase of *E. coli* binds to the membrane via hydrophobic interactions (Chang and Cronan, 1995) without traversing the membrane. Peripheral membrane proteins can also bind to membranes by combining some of the interactions described above. The MARCKS (myristoylated alanine rich C kinase substrate) peptide carries both an acyl chain and interacts via positively charged amino acids (McLaughlin and Aderem, 1995). The bee venom protein mellitin was proposed to lie as an α-helix on top of the phospholipid headgroups with hydrophobic residues pointing downwards into the core of the bilayer and positively charged hydrophilic residues pointing towards the surface (Terwilliger *et al.*, 1982). Often positively charged residues on the protein and negatively charged phospholipids play a role in the interactions (Van der Waart *et al.*, 1983; Sixl *et al.*, 1984; Yang and Glaser, 1995).

Insight into these protein-lipid interactions is very often derived from model systems. These at least comprise the protein of interest and a lipid aggregate representing a bilayer or one half of the bilayer. Rehydration of dry lipid films can be performed to produce large multilayered vesicles, which can be converted into single walled vesicles of defined sizes by extrusion techniques. These vesicles are very useful to study various aspects of protein-lipid interactions. When for instance large vesicles are used, centrifugation protocols can be employed to determine the binding of proteins (see for example: (van 't Hof and de Kruijff, 1994). Also monolayer experiments are very often performed to determine whether proteins have an affinity for lipids. In these type of experiments the changes in surface pressure of a monolayer of lipids, representing one half of the bilayer, can be measured on line. When a protein is able to penetrate the lipid monolayer, this will be measured as an increase in surface pressure (Demel, 1994).

Leader peptidase

In this thesis the integration and function of leader peptidase (Lep) of *E. coli* is studied. The primary amino acid sequence of Lep is depicted in figure 6. Lep is an extensively studied membrane protein which has homologues in each living organism (Dalbey and von Heijne, 1992) and some background information on assembly and function of Lep will be provided here.

MANMFALILVIATLVTGILWCVDKFFFAPKRRERQAAAQAAA 42
GDSLDKATLKKVAPKPGWLETGASVFPVLAIVLIVRSFIYEPFQ 86
IPSGSMMPTLLIGDFILVEKFAYGIKDPIYQKTLIETGHPKRGDIV 132
VFKYPEDPKLDYIKRAVGLPGDKVTYDPVSKELTIQPGCSSGQ 175
ACENALPVTYSNVEPSDFVQTFSRRNGGEATSGFFEVPKNET 217
KENGIRLSERKETLGDVTHRILTVPIAQDQVGMYYQQPGQQLA 260
TWIVPPGQYFMMGDNRDNSADSRYWGFVPEANLVGRATAIW 301
MSFDKQEGEWPTGLRLSRIGGIH

Figure 6. Primary amino acid sequence of Lep. Membrane spanning helices are underlined. This is a corrected version, the originally published sequence reads an arginine at position 42. Using improved sequence methodology it was shown that R42 must be replaced by A and G (Andersson, 1993).

Lep spans the inner membrane with two transmembrane segments and with an overall N_{out}-C_{out} topology (Fig. 7). The short cytoplasmic loop (Fig. 7, P1) carries nine positively charged lysyl and arginyl residues (Laws and Dalbey, 1989), while such residues are absent from the N-terminus and rare in the first 30 residues from the large periplasmic (Fig. 7, P2) domain. Translocation of the P2 domain makes use of the Sec machinery *in vivo* (Lee and Kuhn, 1992). The orientation of Lep in the membrane could be manipulated, in agreement with the positive inside rule, by starting with a construct from which all but one of the positive charges in the P1 domain were removed (Nilsson and von Heijne, 1990). By adding as little as two positive charges to the N-terminus, the overall orientation goes from N_{out}-C_{out} to N_{in}-C_{in}. Therefore Lep is an attractive tool to study factors involved in determining membrane protein orientation.

The catalytic mechanism of Lep was also extensively studied. Lep cleaves 5-7 residues downstream of the hydrophobic core of signal sequences. The consensus sequence contains a turn inducing residue (glycine or proline) at position -6 and small residues, preferably alanine, at positions -3 and -1 with respect to the cleavage site (von Heijne, 1983; von Heijne, 1985; Fikes *et al.*, 1990) For the processing of a water soluble precursor *in vitro* a k_{cat} of almost $9s^{-1}$ was found (Chatterjee *et al.*, 1995). Sequence comparisons, site directed mutagenesis and deletion studies showed that only a serine and a lysine residue were absolutely required for catalysis which pointed to a new catalytic mechanism (Bilgin *et al.*, 1990; Sung and Dalbey, 1992; Black, 1993). Both catalytic residues reside in the periplasmic P2 domain. Since the N-terminus of a signal sequence probably stays at the cytosolic site of the membrane (Kuhn, 1987) and since the hydrophobic core

of the signal sequence comprises only 7-15 amino acids, it seems that the catalytic site of Lep has to move very close to the membrane. One of the possible ways to achieve this is by interacting with the lipids.

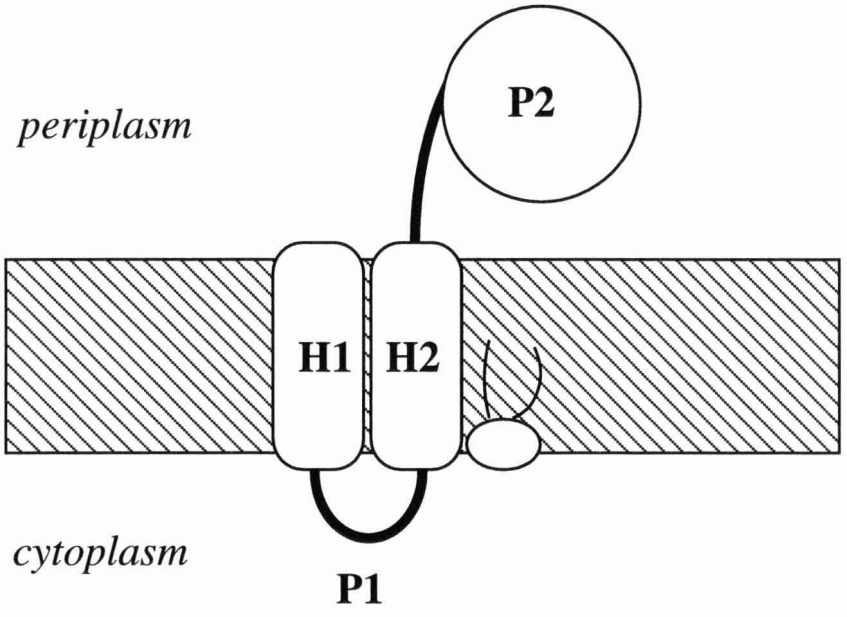


Figure 7. Orientation of leader peptidase in the inner membrane of *Escherichia coli*. The two transmembrane helices (H1 and H2), the cytoplasmic loop (P1) and the periplasmic domain (P2) are indicated.

1.8 Outline of this thesis

In the studies described in this thesis, Lep is used as a model to study the influence of lipids on the functioning and integration of membrane proteins.

In the first half of this thesis the influence of lipids on activity of Lep is studied. In chapter 2 a new method to isolate this protein and the influence of lipids on its activity are presented. In chapter 3 it is shown that in absence of its membrane anchors, the catalytic domain of Lep is able to insert into the lipid phase of membranes. The insertion is characterised and the implications for the mode of action of Lep are discussed.

In the second half of this thesis Lep is used as a model to study the role of lipids during the integration of membrane proteins. In chapter 4 it is shown that the anionic lipid content of the membrane is an important determinant for the orientation of membrane proteins. In chapter 5 a new *in vitro* system for the membrane integration of Lep is described in which the influence of anionic phospholipids and other important factors contributing to assembly can be studied in detail. In chapter 6 the results are summarized and discussed. Section 6A is concerned mainly with the results from chapter 2 and 3 and in section 6B the data from chapter 4 and 5 are related to literature data about the involvement of anionic lipids in membrane protein assembly and protein translocation.

Chapter 2

A quantitative assay to determine the amount of leader peptidase in *E. coli* and the orientation of membrane vesicles

based on publication (III)

Summary

The number of leader peptidase molecules per *E.coli* cell was determined using western blot techniques. Different strains were found to contain approximately one thousand Lep molecules per cell during exponential growth. Based on the activity of Lep *in vitro* it could be estimated that this amount is sufficient to process all translocated precursors. Lep did not appear to be under growth phase dependent control, but was constitutively expressed. The quantitative western blot technique was also used to establish the orientation and intactness of isolated inner membrane vesicles.

Introduction

For normal growth *E.coli* has to target a large number of proteins to the periplasm and outer membrane. These proteins have to cross the inner membrane by a process which is usually mediated by an N-terminal 20-30 amino acid residues long extension, the signal sequence (von Heijne, 1985). After translocation which is catalyzed by the so-called Sec machinery (Arkowitz and Basiliana, 1994), the signal sequence is removed by a signal peptidase and the passenger protein is released into the periplasm. Leader peptidase (Lep, also named Signal PeptidaseI) processes most of the periplasmic and outer membrane proteins (Dalbey and Wickner, (1985) with the exception of the lipoproteins which are processed by SPaseII (Dev and Ray, 1984).

The assembly and function of Lep has been extensively studied. Lep is comprised of two transmembrane helices separated by a cytoplasmic loop (P1). The second transmembrane helix acts as an uncleaved signal sequence for translocation of the large periplasmic P2 domain containing the active site (Dalbey, 1991) Sequence comparisons (van Dijl *et al.*1992; Black, 1992) and site directed mutagenesis (Black, 1992) point to a new proteolytic mechanism with similarities to class A β-lactamases. This idea is supported by inhibition studies with β–lactam (Kuo *et al.*, 1994). Processing activity has been assayed *in vitro* and k_{cat} and K_m values for different substrates were determined (Kuo *et al.*, 1994; Chatterjee *et al.*, 1995). To link these values to Lep activity *in vivo*, the amount of Lep per cell should also be known. Therefore a quantitative procedure was used to estimate Lep expression during growth and in different genetic backgrounds. In combination with protease accessibility experiments the method was also used to establish the orientation of frequently used membrane vesicles.

Results

Quantification of Lep

To determine the amount of leader peptidase in *E.coli,* a western blot procedure was employed. The range, sensitivity and accuracy of this method was first tested using purified Lep. Samples containing between 5 and 70 ng purified protein were subjected to SDS PAGE and transferred to nitrocellulose which was incubated with antibodies and subjected to chemiluminescent detection. The resulting autophotograph is shown in figure 1A, upper panel. The major band corresponds to purified Lep as determined by total protein staining of the blot (results not shown). In addition, two minor bands (together reproducibly 10% of total intensity) corresponding to previously described bands in purified Lep preparations (Tschantz and Dalbey, 1994) were present.

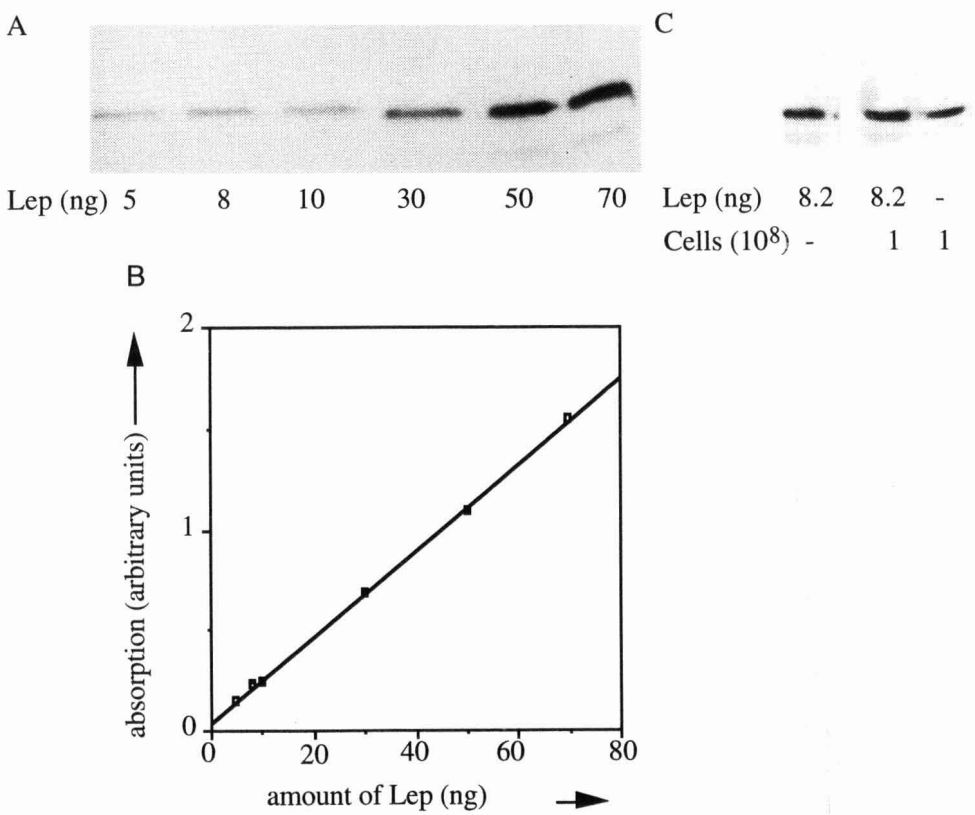


Figure 1. Sensitivity and reliability of quantification of Lep by Western blotting A) six samples of purified Lep(His)6 were subjected to SDS-PAGE and blotted to nitrocellulose. The blots were immuno-decorated and developed with ECL-reagents to illuminate X-ray film. B) Linear relation between absorbance and amounts of Lep, results correspond to figure 1A.. C) Quantification of Lep in a background of whole cells. Samples were prepared as described in the text.

The intensities of the bands on film were quantified by laser scanning densitometry. A linear relationship (correlation coefficient = 0.999) between the amount of Lep and absorption was observed throughout this range (Fig. 1B). In order to determine whether components present in *E.coli* cells interfered with the results from this method a control needed to be performed. In the control 8.2 ng of purified Lep (Fig. 1C, lane 1) was added to $1*10^8$ MC1061 cells. The intensity of the band corresponding to Lep from $1*10^8$ cells (Fig. 1C, lane 3) was subtracted from that of the complex sample (Fig. 1C, lane 2). The resulting intensity corresponded to 8.0 ± 0.1 ng Lep (average of an experiment in duplicate). From this we conclude that there is no interference of cell constituents with this procedure. To further validate the method, a comparison was made to the much less sensitive method of Coomassie Brilliant Blue (CBB) staining of gels. Appropriate aliquots of purified Lep and whole cells from MC1061 overproducing Lep were subjected to SDS-PAGE and gels were either CBB stained or blotted and treated as before. The Lep concentrations estimated from both CBB stained gels and illuminated films were found to be within 10% of each other (data not shown). The western blot method was therefore taken to yield reliable quantitative insights into the amount of Lep in *E.coli* cells. Cells of four different wild type strains were grown to exponential phase (OD_{660} = 0.5-0.9) and the amount of Lep per ml sample was quantified. Simultaneously the number of viable cells was determined. All tested strains were found to contain around 1000 Lep molecules per cell (Table I).

Table 1. Amount of Lep in various *E.coli* strains.

Strain	**Lep (10^3molecules/cell)**
MC1061	1.1 ± 0.1
MC4100	0.82± 0.1
MRE600	0.91± 0.1
W3110	1.4 ± 0.1
HDL11 (+IPTG)	1.9 ± 0.1
HDL11 (-IPTG)	2.1 ± 0.4

To see whether the level of Lep is dependent on growth phase, we followed the number of Lep molecules in a culture of MC1061 cells from lag phase to early stationary phase.

Determinations later in stationary phase were not made because of the reduction in the amount of viable cells. From exponential through to stationary phase the number of Lep molecules per cell was constant at around 1000 molecules per cell (Fig. 2). In freshly diluted cultures slightly elevated numbers of Lep molecules were observed. This is possibly caused by Lep from non-viable cells present in the inoculum. From this experiment it is concluded that *E.coli* does not adapt Lep expression to growth phase.

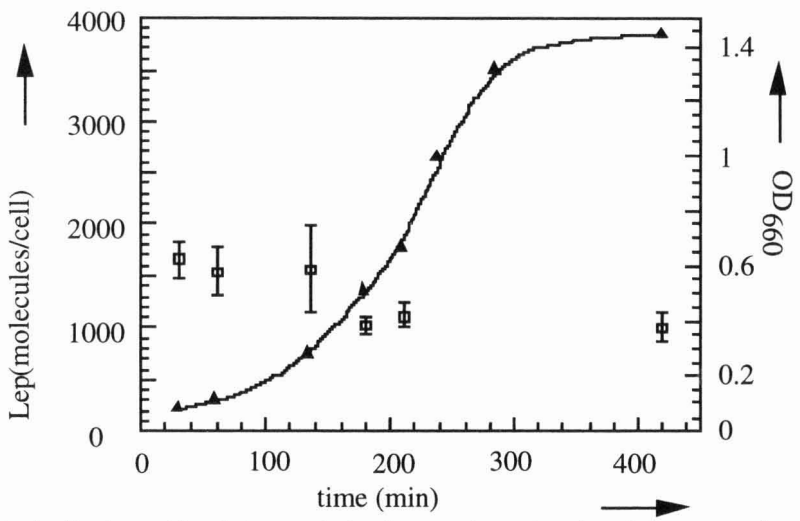


Figure 2. Levels of leader peptidase during growth. Samples were taken at the indicated time points and analyzed as described in the text. Squares indicate the amount of Lep molecules per cell and trinagles represent the optical density at 660 nm of the cell culture.

Previously, it was reported that precursor translocation is impaired in absence of anionic phospholipid synthesis (de Vrije *et al.*, 1988) Therefore Lep expression was tested in *E.coli* strain HDL11 in which anionic phospholipid biosynthesis depends on the addition of IPTG to the medium. Each HDL11 cell was found to contain approximately 2000 Lep molecules independent of addition of IPTG (table I). The reason that this value is higher than in wild type strains may be due to a difference in genetic background. In HDL11 the gene encoding the major lipoprotein is deleted (Kusters *et al.*, 1991) and this causes major changes in the cell envelope. Because of the insensitivity of Lep expression to anionic phospholipid synthesis, processing activity against a purified natural substrate, prePhoE, was tested in the absence and presence of phospholipids. Addition of phospholipids to an octyl glucoside-Lep(His)6 mixture resulted in a large stimulation

of processing (Fig. 3). The negatively charged dioleolylphosphatidylglycerol (DOPG) was slightly more effective than the zwitterionic dioleolylohosphatidylcholine (DOPC) or dioleolylphosphatidylethanolamine (DOPE, not shown).

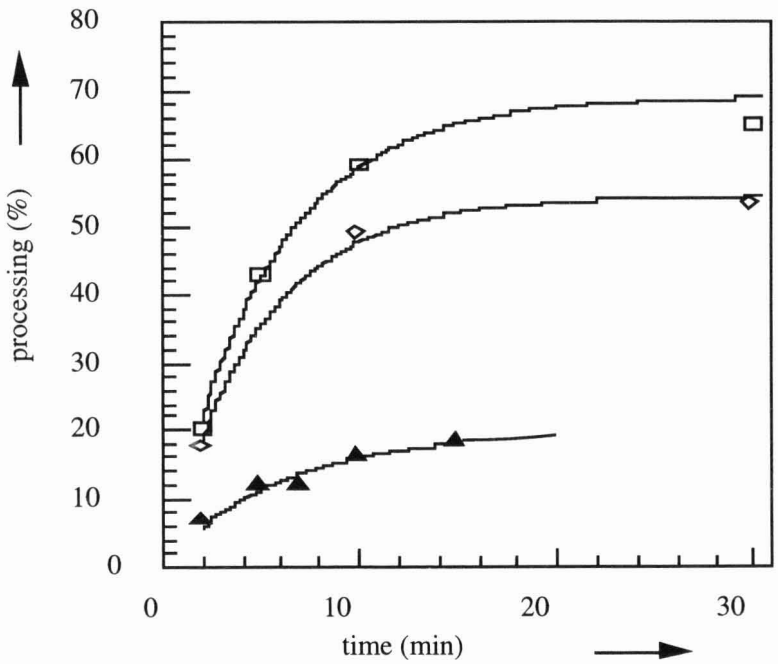


Figure 3. Processing of prePhoE by Lep *in vitro*. Lep activity was determined in 34 mM octyl glucoside after addition of 0.27 mM octyl glucoside (black triangles), DOPC (diamonds) or DOPG (squares).

Lep as a tool in topology determination of vesicles

Investigations into solute transport across membranes and protein translocation often involves the use of inner membrane vesicles. Employing the quantitative Western blot technique and using the topology of Lep as a marker, the orientation of these vesicles can be tested by protease treatment. French pressure cell passage of *E.coli* cells yields inverted membrane vesicles (de Vrije *et al.*, 1987) in which the immunoreactive P2 domain of Lep is in the lumen and P1 is exposed on the exterior of the vesicles (Fig. 4A, left panel). Protease treatment should therefore not cause a loss of immunoreactive material. When loss does occur, it is indicative of contamination with either right-side out vesicles or leaky vesicles. Osmotic shock of spheroplasts yields sealed right-side out

vesicles (Kaback, 1971) in which P2 of Lep should be on the exterior face of the vesicles (Fig. 4A, right panel). Protease treatment of these vesicles should result in a complete loss of immunoreactive material. Presence of protease-resistant Lep is therefore indicative of contamination with inverted vesicles.

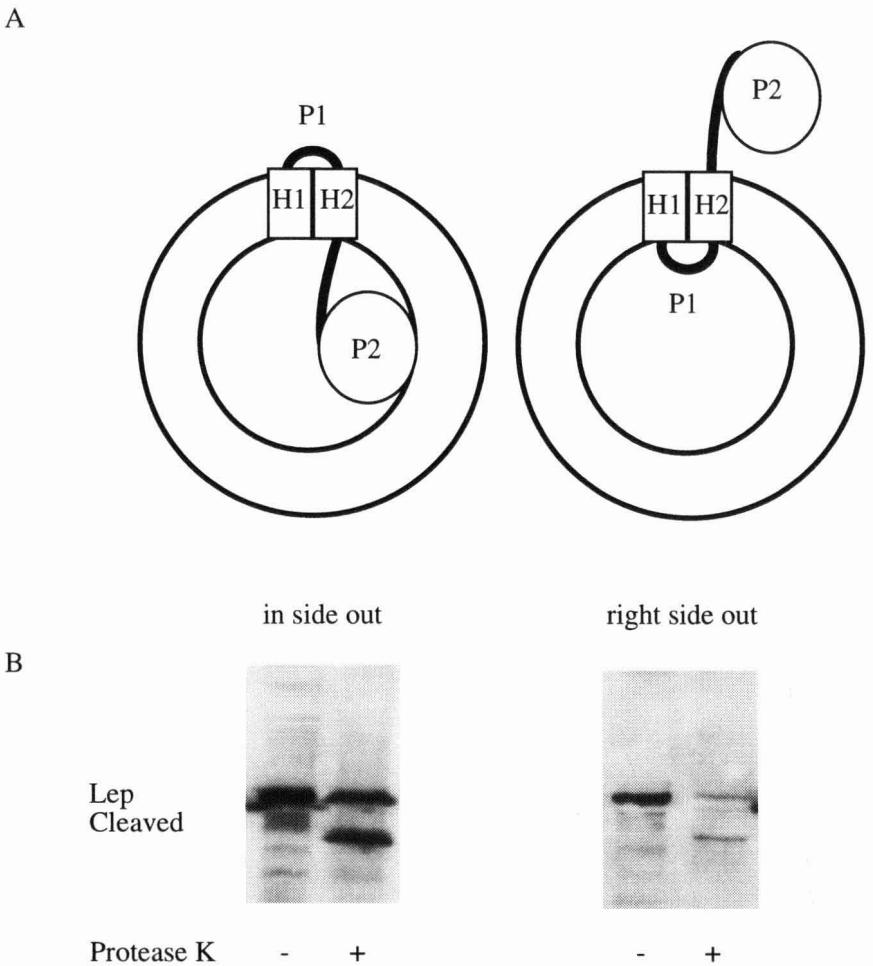


Figure 4. Leader peptidase as a tool in determining vesicle topology. A) Schematic drawing of the orientation of Lep in inside out (left panel) and rightside out (right panel) vesicles. B) Protease K treatment of vesicles. The autophotograph shows presence of intact Lep (Lep) and Lep digested in the P1 loop (cleaved).

Membrane vesicles of MC1061 with both topologies were subjected to protease treatment and after protease inhibition, samples were separated by SDS-PAGE, transferred to nitrocellulose and developed as before. Results are shown in figure 4B. After protease treatment of inverted inner membrane vesicles (Fig. 4B, left panel) Lep was quantitatively retrieved (100 ± 2 %) either as full length protected form or shifted to a lower molecular weight. The latter corresponding to molecules with a digested P1 loop. Both forms contribute around 50% to the total value. Incubation in the presence of up to 0.5 mg/ml protease K did not result in further digestion and only after solubilization of the membranes with Triton X 100 the two bands could be digested (results not shown). As predicted in right-side out vesicles the majority of the Lep molecules were digested (Fig. 4B, right panel) by protease K, however 10-20% Lep remained which could only be digested by protease after Titon X 100 treatment (result not shown). This is most probably due to contamination with inverted membrane vesicles during isolation.

Discussion

In this report a method is presented for the quantification of leader peptidase of *E.coli* based on Western blotting and enhanced chemiluminescence detection. This method was used to gain insight into the number of Lep molecules in *E. coli* and to establish the topology of vesicles that were isolated according to established procedures. Intactness and correct orientation of inverted membrane vesicles was demonstrated by protection of Lep against complete proteolytic breakdown by protease K. From protease protection of Lep in right side out vesicles it appears that these were contaminated with 10-20% inverted membrane vesicles. It is concluded that this method is useful in determining orientation and intactness of vesicles routinely used in studying solute transport and protein translocation.

In this study it was observed that various wild type strains contained around 1000 Lep molecules per cell. For the processing of a water soluble chimeric precursor protein *in vitro* a k_{cat} value of 8.73 s^{-1} was obtained (Chatterjee *et al.*, 1995). Applying this number to an *in vivo* situation it would mean that an *E.coli* cell can process half a million precursor molecules every minute. An average cell in balanced growth with a mass doubling time of 40 minutes contains 0.156 pg of protein (Neidhardt, 1987) of which approximately 75% is in the cytoplasm (Silhavy *et al.*, 1979)and a further 6-9% in the inner membrane (Cronan *et al.*, 1987). Assuming an average substrate molecular weight of 35 kDa and subtracting the 0.7 million murein lipoprotein molecules (Inouye, 1974) which are not a substrate for Lep, we estimate 10 000 proteins per cell

which have to be processed by Lep per minute. It therefore seems that there are many more Lep molecules than necessary to process this amount of precursor proteins. One possible explanation is that Lep is in functional excess. Alternatively this large amount might be necessary because the active site of Lep is not saturated with precursor proteins, but has to find its substrate by diffusion. The cellular amounts of most of the membrane components involved in translocation are known. For SecD, SecE and SecY, 500 copies per cell (Matsuyama *et al.*, 1992) were estimated, indicating that Lep is in excess. Furthermore Lep does not copurify with any of the Sec proteins (Brundage *et al.*, 1990; Tokuda *et al.*, 1991; Akimara *et al.*, 1991; Matsuyama *et al.*, 1992). This suggests that it does not form a stable complex with the rest of the translocation machinery. Therefore it is most likely that processing occurs as a result of a transient rather than a stable interaction of Lep with the translocator.

Experimental procedures

Estimation of the number of Lep molecules per cell

Overnight cultures of wild type strains MC1061 (Sasakawa and Yoshikiwa, 1987), MRE600 (Cammack and Wade, 1965), MC4100 (Casadaban, 1976), W3110 (Bachmann, 1972) and lipid biosynthetic mutant strain HDL11 (Kusters *et al.*, 1991) were diluted 1:50 in fresh L-broth which for HDL11 was supplemented with chloramphenicol (25 μg/ml), kanamycin (50 μg/ml) and tetracycline (10 μg/ml) and when appropriate 100 μM IPTG (Sigma, Poole, UK), and grown at 37 ^{o}C. At different time points samples of 1 ml were taken and centrifuged 4 minutes at 8000 g.

Half of each sample was resuspended in sample buffer, subjected to SDS-PAGE and transferred to nitrocellulose. The blot was treated with Lep antiserum and GAR-PO (BioRad, Hercules, USA) and developed employing enhanced chemiluminescence (ECL) reagents (Amersham, Buckinghamshire, UK) and X-ray film (Fuji, Japan). Band intensities were quantified by laser scanning densitometry on an Ultroscan XL (Pharmacia LKB, Bromma, Sweden). After comparison to known amounts of purified Lep, which were run on the same gel, the amount of Lep in the samples could be calculated. The remainder of each of the samples were resuspended in physiological salt solution to make dilution series for viability counting on LB agar plates.

Purification of Lep(His)6

pLep(His)6 was constructed by introducing five extra histidine residues at the C-terminus of Lep by site-directed mutagenesis. The resulting protein was overexpressed as described before for the

wild type protein (Tschantz and Dalbey, 1994). Cells were harvested (15 minutes, 4,000 x g) and resuspended in 10 mM Tris-HCl pH 8.0, and lysed by addition of 5mM EDTA and 10mg/ml lysozyme, freeze-thawing and sonication. After removing cell debris by low speed centrifugation (10 minutes, 1000 x g), membrane fractions were collected by centrifugation (20 minutes, 23,000 x g) and resuspended in 10 mM Tris-HCl pH 8.0, 34 mM octyl glucoside (Sigma, Poole, UK), 15 mM imidazole (Sigma), 100mM NaCl (buffer A) and centrifuged again (20 minutes 23,000 x g). After applying the cleared solution to a chelating sepharose column loaded with Cu^{2+} (Pharmacia, Uppsala, Sweden) and washing with buffer A, pure Lep (>99% densitometrically, 2-4 mg/l culture) was eluted in 10mM Tris-HCl pH8.0, 50mM imidazole, 34mM octyl glucoside (fig. 5, lane 6) Protein concentrations were determined according to Bradford (Bradford, 1976) and by Coomassie Brilliant Blue staining of gels using BSA as a standard. Purified Lep to raise antibodies in rabbits was a gift from Dr. W. Wickner, Dartmouth Medical School.

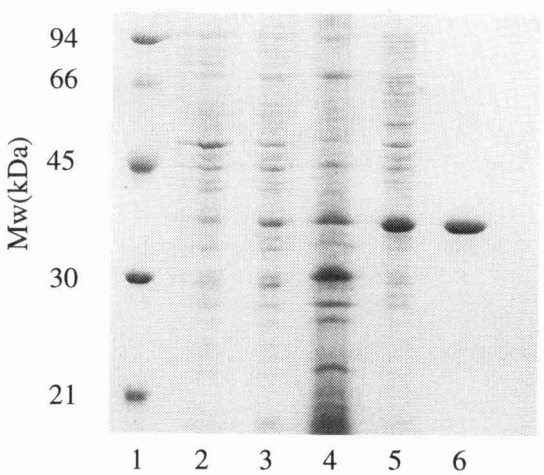


Figure 5. Purification of His tagged Lep analysed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. Lane 1, molecular weight marker; lane 2, non induced cells; lane 3, cells induced with 0.2% arabinose; lane 4, crude membrane pellet resuspended in 1% octyl glucoside; lane5, cleared membrane solution; lane 6, pure His tagged Lep.

Processing assay

Purified His tagged Lep (78pmol) was diluted in assay buffer (10 mM Tris/HCl pH8.0, 34mM octyl glucoside and mixed with phospholipids (0.27mM final concentration) in assaybuffer. Lipids were purchased from Avanti Polar Lipids (Alabama, USA). The processing reaction was started by

addition of 10 pmol ^{35}S labelled prePhoE which was purified as described before (Nouwen *et al.*, 1994). The processing reaction was stopped by rapid freezing in a dry ice-ethanol bath. Samples were analysed by SDS-PAGE followed by autoradiography. Films were densitometrically scanned and the amounts of precursor and mature PhoE were calculated after correction for the loss of one methionine in the signal sequence after processing.

Topology determination of vesicles

Inverted inner membrane vesicles and right side out inner membrane vesicles were isolated from *E.coli* strain MC1061 following published procedures (de Vrije *et al.*, 1987; Kaback, 1971). The vesicles were incubated in 10 mM Tris/HCl pH 8.0, 10 mM $MgSO_4$ in the absence and presence of 0.2 mg/ml proteinase K (Boehringer Mannheim,Germany) at room temperature. 20% Sucrose was included in the incubation buffer for inverted vesicles to maintain an isotonic environment. After 25 minutes at room temperature, 2 mM PMSF (Sigma) was added and the samples were left on ice for 5 minutes. Sample buffer was added and the samples were separated by SDS-PAGE and blotted to nitrocellulose. After immuno-detection Lep was quantified by densitometry.

Chapter 3

The catalytic domain of leader peptidase inserts in a phosphatidylethanolamine dependent way in the outer leaflet of the inner membrane of *E. coli*

based on publication (V)

Summary

Leader peptidase is an integral membrane protein of *E. coli* that catalyses the removal of most signal peptides from translocated precursor proteins. Here, we show that when the transmembrane anchors are removed *in vivo*, the remaining catalytic domain can bind to inner and outer membranes of *E. coli*. Furthermore, the purified catalytic domain binds to inner membrane vesicles and vesicles composed of purified inner membrane lipids with comparable efficiencies. It is shown that the interaction is caused by penetration of a part of the catalytic domain between the lipids. Penetration is mediated by phosphatidylethanolamine, the most abundant lipid in *E. coli*, and does not seem to depend on electrostatic interactions. A mildly hydrophobic segment around the catalytically important residue serine 90 is required for the interaction with membranes.

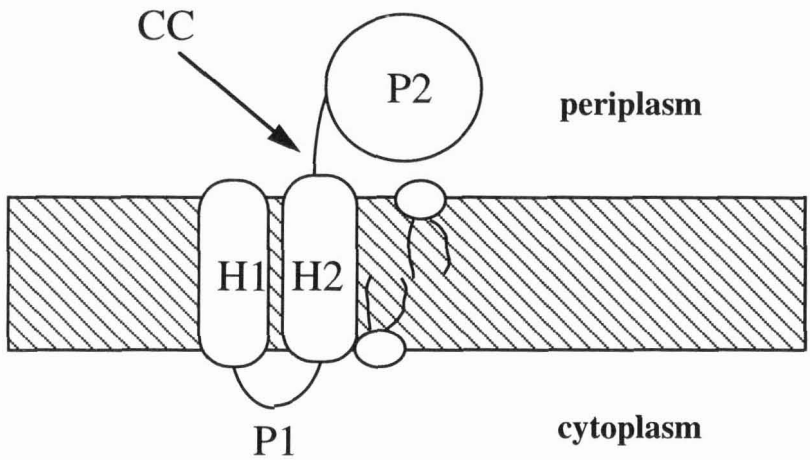
Introduction

Protein translocation is an essential process in both prokaryotic and eukaryotic cells. Approximately 20% of all proteins synthesized by the gram negative bacterium *E. coli* function outside the cytosol (Cronan *et al.*, 1987) and therefore must at least pass the cytosolic membrane. Passage of the cytoplasmic membrane is most often catalysed by the combined action of a proteinaceous secretion (sec) machinery (Arkowitz and Basiliana, 1994), anionic phospholipids (De Vrije *et al.*, 1988) and the membrane potential (Bakker and Randall, 1984).

For recognition by the export machinery proteins are usually synthesized as precursors with amino terminal extensions called leader- or signal- sequences. Signal sequences contain a hydrophobic core region which is preceeded by an amino terminal positively charged domain (von Heijne, 1985). After translocation signal sequences are removed by the action of leader (signal) peptidases which cleave 5 to 7 residues downstream of the hydrophobic core of the signal sequence (von Heijne, 1985). Most signal sequences are removed by Lep (also called leader peptidase or signal peptidase I) which is encoded by the *LepB* gene (Date and Wickner, 1981). It recognizes a turn inducing residue (glycine or proline) at position -6 and small residues, preferably alanine, at positions -3 and -1 with respect to the cleavage site (von Heijne, 1983; Fikes *et al.*, 1990).

Lep is comprised of an amino terminal part containing two transmembrane helices (H1 and H2) separated by a highly positively charged cytosolic loop (P1) and a carboxy terminal large

periplasmic domain (P2) (Fig. 1). Site directed mutagenesis studies suggested that the proteolytic mechanism involves a serine at position 90 and a lysine at 145 and that all residues required for cleavage are located in P2 (Black, 1993; Tschantz *et al.*, 1993).



----TGASVFPPVLAVLIV*SPSAQAA*RSFIYEPFQIP---

H2 CC P2

Figure 1. Orientation of wild type Leader peptidase in the membrane and indication of the position of the engineered cleavage cassette (CC) behind the second transmembrane helix. The amino acid sequence of the region around H2 of a construct bearing a cleavage cassette is depicted. H2 (from residue 68 to 76) is underlined and the cleavage cassette between residues 76 and 77 is depicted in italics.

The substrates for Lep are membrane bound and may still be in the vicinity of the translocation machinery at the moment of processing. It is therefore conceivable that Lep finds its substrate by having affinity for components of the sec machinery. In this study this idea was tested using constructs corresponding to the periplasmic domain. The association of this domain to membranes with or without the translocation machinery was compared. It is found that the presence of the sec machinery is not required for efficient binding. Interestingly the periplasmic domain of Lep is able to penetrate into pure lipid bilayers and shows a profound specificity for the lipid phosphatidylethanolamine. The results are discussed in relation to the mode of action of Lep.

Results

The periplasmic domain of Lep associates with membranes after removal of the membrane anchors

The catalytic domain of Lep is localised in the periplasm (Fig.1). To study possible interactions of this domain with membranes *in vivo*, a mutant was required in which the catalytic P2 domain could be separated from the membrane spanning segments H1 and H2. Downstream of H2 a recognition sequence for cleavage by Lep was engineered (Fig.1) and the resulting construct was named H2-CC. This construct was efficiently expressed and cleaved *in vivo* probably by the native population of Lep molecules in the membrane (Nilsson and von Heijne, 1991). When spheroplasts were prepared the cleaved form was accessible to proteases (Nilsson and von Heijne, 1991, results not shown). Periplasmic fractions of pulse labelled cells expressing H2-CC were isolated by osmotic shock and screened by immunoprecipitations. While most of the periplasmic marker protein β-lactamase was recovered in the soluble periplasmic fraction (75±6%, n=3), the cleaved domain of Lep was found predominantly in the pellet (84±7%, n=3) (results not shown). The catalytic domain is therefore probably associated with membranes. To investigate more precisely the localisation of the periplasmic domain, membrane fractions were prepared of cells expressing H2-CC. Inner and outer membranes were separated on a sucrose gradient and the different fractions analysed by SDS PAGE. Antibodies against Lep were used to stain blots made from the different fractions (Fig.2 insert).

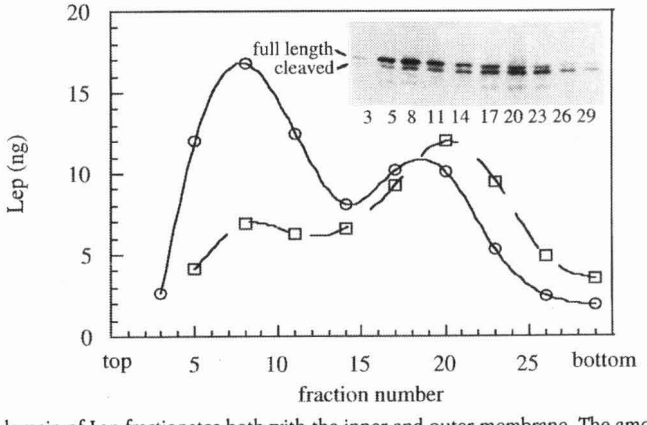


Figure 2. The P2 domain of Lep fractionates both with the inner and outer membrane. The amount of Lep and the cleaved P2 domain was determined in fractions which were collected from a sucrose gradient on which *E. coli* membranes were separated. The amount of full lenth Lep (circles) and cleaved form (squares) were plotted against the fraction number. The inner and outer membranes were found around fractions 8 and 20 respectively. The insert shows the result of the corresponding western blot treated with Lep antibody.

Two bands reacted with the antibody, one of 36 kDa coinciding with endogenous Lep was as expected found mainly in the inner membranes around fraction 8 and to a lesser extent in the outer membrane (Fig. 2, circles) as reported before (Zwizinski *et al.*, 1981), while a band corresponding to the cleaved off P2 domain was also found in the outer membranes around fraction 20 (Fig. 2 squares). It is therefore concluded that the periplasmic domain of Lep has affinity for membranes.

Binding of the periplasmic domain of Lep to membranes does not require a specific proteinaceous component

The ability of the periplasmic domain of Lep to bind to membranes was confirmed by vesicle binding experiments. For this purpose we made use of a purified, enzymatically active construct (Δ2–75) lacking the 2 membrane spanning segments (Kuo *et al.*, 1993). This construct is expressed in the cytoplasm and can be purified in large amounts. By means of ultracentrifugation experiments the binding of Δ2–75 to right side out inner membrane vesicles and outer membranes was determined. In the absence of membranes Δ2–75 was quantitatively recovered in the supernatant after ultracentrifugation (Fig.3 upper panel, lanes 1-3). Right side out inner membrane vesicles (Fig. 3, upper panel lanes 4-6) contain many different proteins as judged by Coomassie Brilliant Blue staining of gels, while outer membranes (Fig. 3, lower panel lanes 1-3) showed a characteristic pattern with only two dominant bands. Both types of vesicles were pelleted efficiently. When Δ2–75 was incubated with vesicles prior to centrifugation part of the molecules sedimented with the vesicles (Fig.3 upper panel lanes 7-9 and Fig. 3 lower panel lanes 4-6). So Δ2–75 is apparently capable of binding to both inner and outer membranes while the native population of Lep is found mostly in the inner membrane of *E. coli*. This suggests that the periplasmic domain does not require specific inner membrane components for binding. To investigate the possibility that the P2 domain recognises the lipid component of membranes the binding of 5 μg Δ2–75 to inner membranes and to single walled lipid vesicles made from purified inner membrane lipids were compared. The same amount of lipid phosphorous was used for both types of vesicles. The amount of bound protein was determined by laser scanning densitometry. Right side out inner membrane vesicles bound 1.7 ± 0.3 μg of Δ2–75 while the lipid vesicles bound 1.6 ± 0.3 μg, this strongly suggests that the membrane binding of Δ2–75 is mediated by lipids.

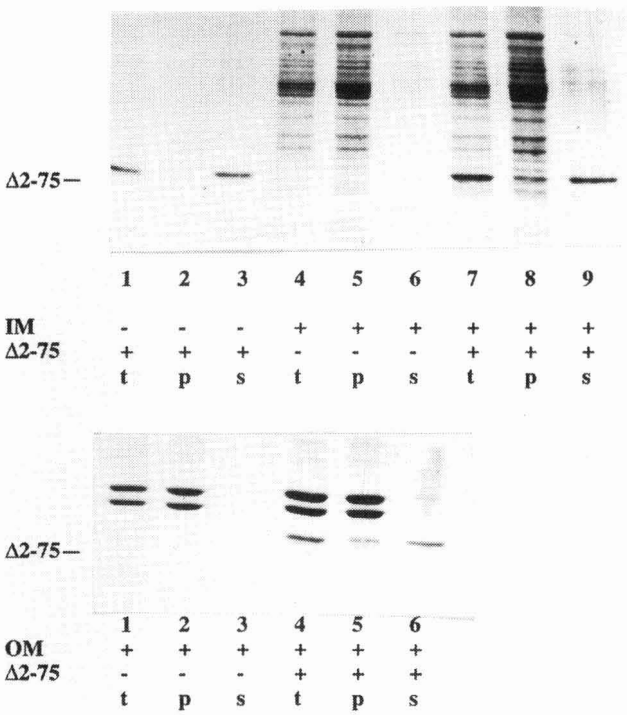


Figure 3. Purified Δ2–75 associates both with inner and outer membrane vesicles. Samples with (upper panel, lanes 1-3 and 7-9; lower panel, lanes 4-6) or without Δ2–75 (upper panel, lanes 4-6; lower panel, lanes 1-3) were incubated in the absence (upper panel, lanes 1-3) or presence of membranes before ultracentrifugation. After centrifugation samples were split in pellet (p) and supernatant (s) fractions and compared to the total (t) amount before centrifugation. The upper panel shows incubations with inner membranes and the lower panel with outer membranes. Gels were stained with Coomassie Brilliant Blue.

Since membrane binding of the periplasmic domain of Lep does not seem to be caused by proteinaceous interactions we looked for a general feature such as hydrophobicity that enables proteins to interact with membranes. Figure 4 shows a hydrophobicity plot of Lep. Besides the two transmembrane segments H1 and H2 a third hydrophobic segment H3 stands out. This region contains also the catalytic important serine 90 residue. A mutant lacking H3 (Δ2–98, Fig.4) was purified and the binding of the two purified constructs to lipid vesicles were compared. Different amounts of the constructs were added to the lipid vesicles and subjected to ultracentrifugation. The amount of protein associated with vesicles was quantified and plotted against the amount of added protein (Fig. 5).

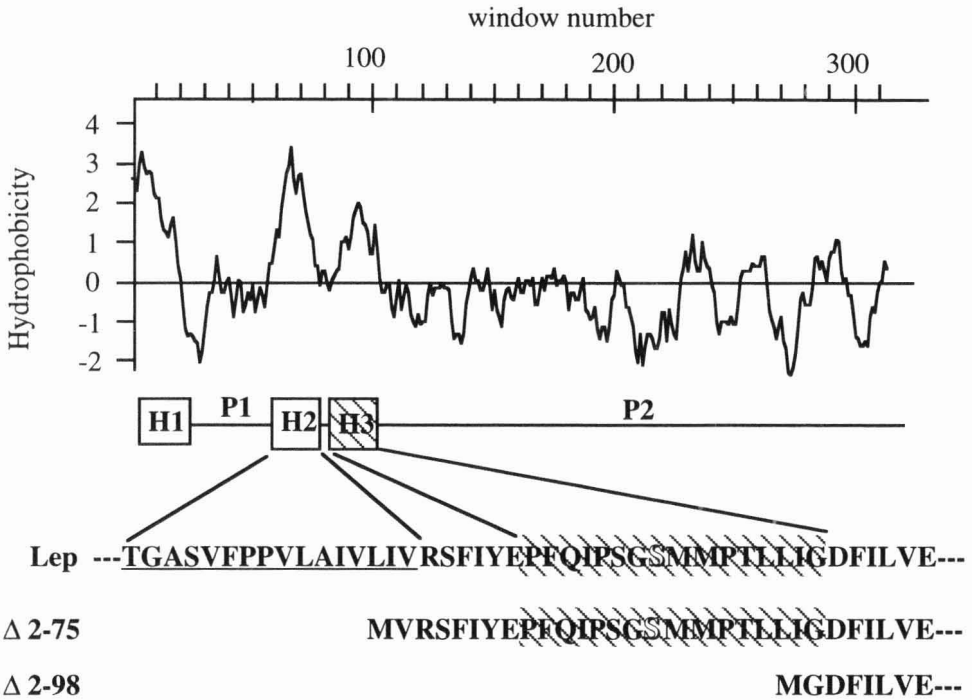


Figure 4. Hydrophobicity, domain structure and partial sequences of Lep and two truncated forms of Lep. The hydrophobicity of Lep was determined according Kyte and Doolittle using a window of 11 amino acids. The domain structure shows the relative positions of the hydrophobic parts. In the amino acid sequence the second transmembrane segment (H2) and H3 are indicated. The catalytic important serine 90 is depicted in white.

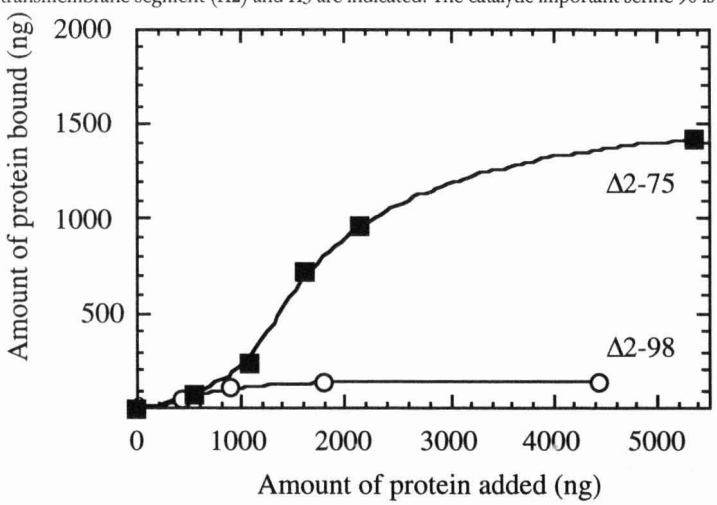


Figure 5. comparison of lipid vesicle binding by Δ2–75 and Δ2–98. Binding of Δ2–75 (closed squares) and Δ2–98 (open circles) were determined as described in materials and methods.

The sigmoidal binding curve of Δ2–75 might indicate that cooperativity is involved in binding of Δ2–75 to the vesicles. In any case it is clear that Δ2–75 binds much more strongly to the lipid vesicles than Δ2–98 indicating that the interaction of the periplasmic domain of Lep could be mediated via the H3 region.

Interaction of Δ2–75 with membranes is caused by penetration between the lipids

The association of Δ2–75 with lipid vesicles can be caused by binding to the surface as well as by insertion of a part of the protein between the lipids. To gain insight into the nature and specificity of the interaction of the periplasmic domain of Lep with lipids we made use of monolayer experiments. In these experiments lipids are spread on top of a buffer solution in a teflon trough. The lipids orient themselves with the head groups towards the buffer solution and their apolar acyl chains away from the aqueous phase. They thereby resemble one half of the bilayer. Proteins can be added to the aqueous phase and when they are able to insert between the lipid molecules this will increase the surface pressure which can be measured online by means of a microbalance. Lipids isolated from purified inner membranes of *E. coli* were spread to an initial surface pressure of 22 mN/m (Fig.6A). When Δ2–98 was injected under these layers a small increase in surface pressure was observed which stabilised in 30-40 minutes. Δ2–75 gave rise to a much larger pressure increase indicating a more efficient interaction with lipids. Since proteins that only interact with the lipid head group without penetration between the lipids do not give rise to a pressure increase (Demel *et al.*, 1973), the results show actual insertion between the phospholipids in the monolayer.

The limiting surface pressure is the initial surface pressure at which a protein is just able to insert into a monolayer, and therefore an important characteristic of insertion. In biological membranes the packing densities of the lipids were found to correspond to surface pressures between 31 and 35 mN/m (Demel *et al.*, 1975). To investigate whether the constructs are able to penetrate into membranes with physiological lipid packing densities, monolayers with different initial surface pressures were made. From figure 6B it is clear that Δ2–75 can insert in monolayers with much higher initial pressures than Δ2–98. Δ2–75 is able to insert into monolayers with initial pressures as high as 35mN/m and therefore this construct is expected to be capable of penetrating between the lipids in biological membranes.

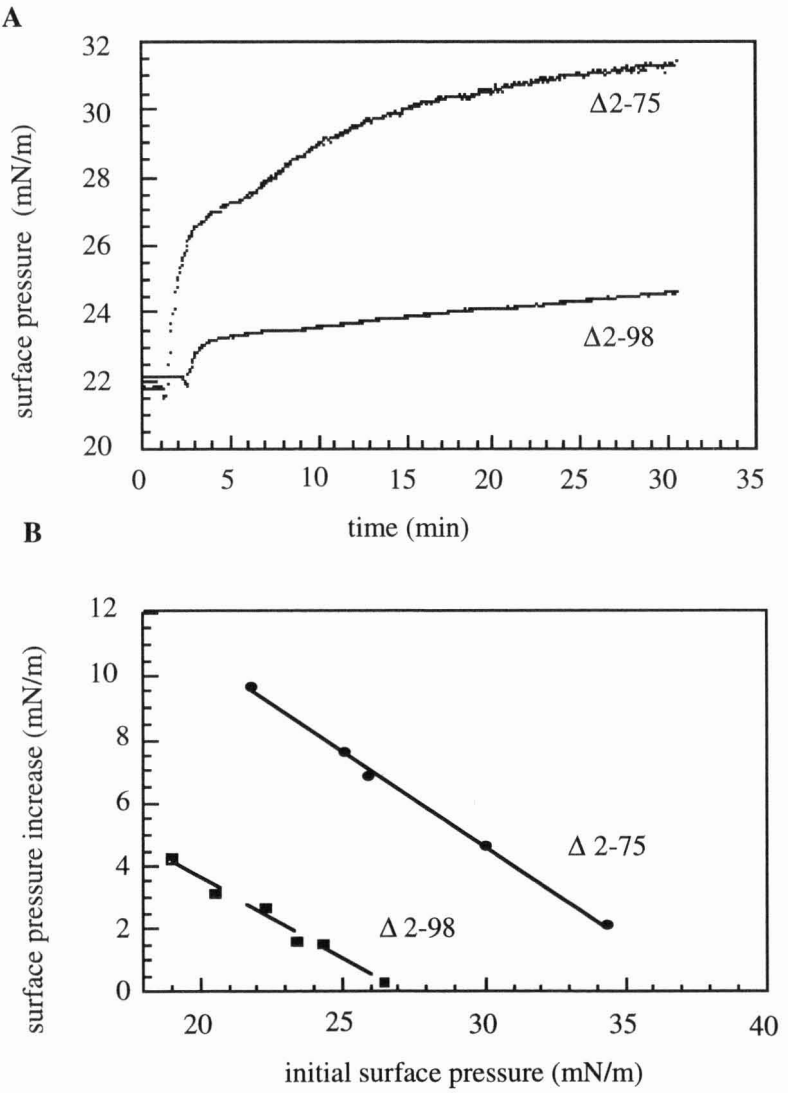


Figure 6. Interaction of Δ2–75 and Δ2–98 with monolayers. (**A**) insertion profile of Δ2–75 and Δ2–98 into monolayers of *E. coli* inner membrane lipids which were spread until 22mN/m initial pressure. After injection of protein the changes in surface pressure were followed. (**B**) Ability of Δ2–75 and Δ2–98 to penetrate into monolayers as function of the initial pressure of the monolayer.

Phosphatidylethanolamine is required for efficient insertion of Δ2–75 in monolayers

Electrostatic interactions often contribute to the binding of proteins to lipids. In those cases positively charged amino acid residues bind to negatively charged phospholipid head groups. Increasing the ionic strength results in shielding of the charges and therefore reduces the interaction. To test this possibility monolayers with surface pressures of 25mN/m were spread on top of buffer solutions with sodium chloride concentrations ranging from 50 mM to 1200 mM. The surface pressure increase caused by insertion of Δ2–75 was measured. The increase at 50 mM was set at 100 and the surface pressure increases at higher salt concentrations were related to this value. Figure 7 shows that the insertion of Δ2–75 is increased instead of being reduced at higher salt concentrations demonstrating that electrostatic interactions do not contribute significantly to the binding of Δ2–75 to the monolayer. One of the possible reasons for the slight increase in interaction may be the shielding of repulsive charges.

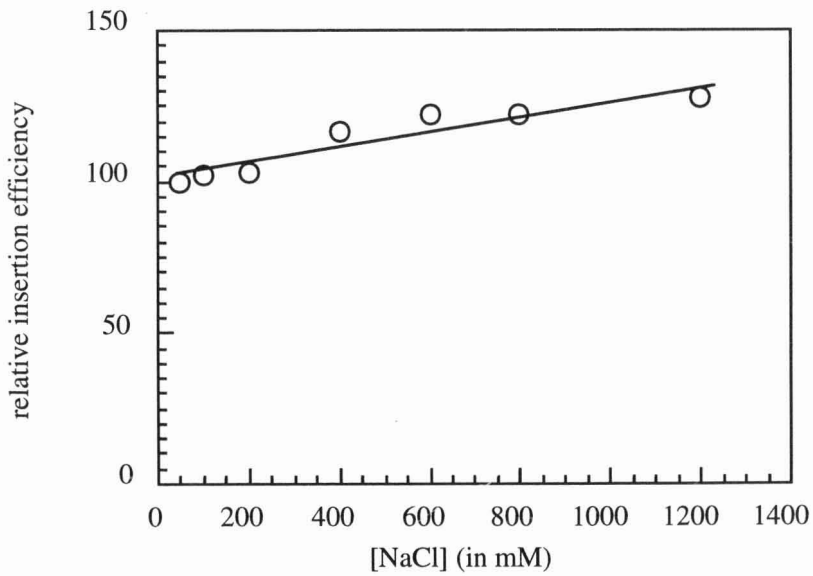


Figure 7 Electrostatic interactions are not important for insertion of Δ2–75 in a monolayer derived from *E. coli* lipids. Δ2–75 was injected underneath monolayers in buffers of various ionic strength. The surface pressure increase at different salt concentrations were compared to that at 50 mM NaCl. See text for details.

Charge interactions are apparently not important for the insertion of the P2 domain into monolayers. To get more insight into the characteristics of interaction, insertion into monolayers of pure lipids were compared.

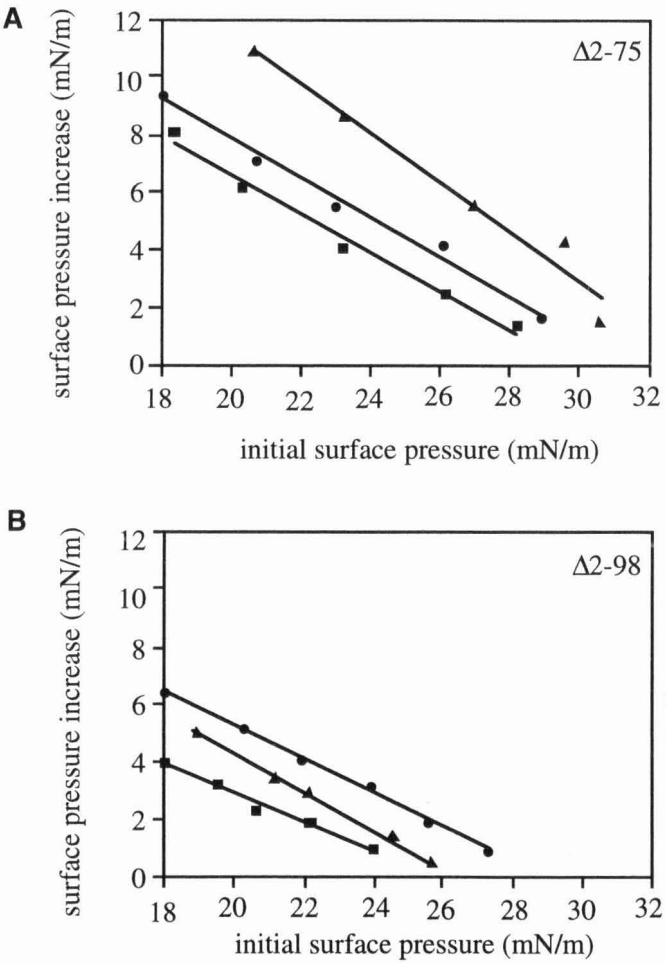


Figure 8. Specific interaction of Δ2–75 and Δ2–98 with different lipid head group classes. (**A**) Surface pressure increase caused by insertion of Δ2–75 into monolayers of DOPE (triangles), DOPC (squares) and DOPG (circles) as function of the initial surface pressure. (**B**) Surface pressure increase caused by insertion of Δ2–98 into monolayers of DOPE (triangles), DOPC (squares) and DOPG (circles) as function of the initial surface pressure.

Phosphatidyl ethanolamine (PE) has a small zwitterionic headgroup and it accounts for approximately 75 % of the phospholipids in the *E.coli* inner membrane (Raetz, 1978). The second most abundant lipid is the negatively charged phosphatidyl glycerol (PG) which accounts for about 20% (Raetz, 1978). Insertion of Δ2–75 in dioleolyl phosphatidyl ethanolamine (DOPE) and dioleolyl phosphatidyl glycerol (DOPG) monolayers were measured as function of the initial pressure. From figure 8A it is clear that Δ2–75 inserts best in DOPE monolayers. To see whether this is only due to the zwitterionic character of DOPE also dioleolyphosphatidyl choline (DOPC) was tested. The phosphatidyl choline head group is also zwitterionic but much bulkier than the phosphatidyl ethanolamine head group. Insertion in DOPC is considerably less efficient and almost comparable to insertion in DOPG. Therefore also other characteristics of the head group seems to be important.

Next the interaction of Δ2–98 with the same range of lipids was tested and while interaction with all lipids was less than in the case of Δ2–75, it was striking that especially the interaction with DOPE was very much reduced. These results together suggest that the membrane affinity of the periplasmic domain of Lep is mediated by the hydrophobic H3 segment and lipids with the zwitterionic phosphatidyl ethanolamine head group.

Discussion

This study reports on the interaction of the catalytic domain of leader peptidase, one of the key enzymes in preprotein translocation, with membranes. Evidence for membrane binding activity was obtained using two approaches. Fractionation studies with the construct H2-CC from which the membrane spanning regions are separated from the periplasmic domain *in vivo* showed that the periplasmic domain is associated both with inner and outer membranes indicating no affinity for specific components of the inner membrane. This finding was confirmed by studies using purified Δ2–75 resembling the periplasmic domain of Lep. Δ2–75 binds to lipid vesicles and right side out inner membrane vesicles equally well, again suggesting that the catalytic domain does not bind to proteinaceous components of the preprotein translocation (sec) machinery but has a high affinity for the membrane lipids.

The periplasmic domain not only has catalytic (Kuo *et al.*,1993; Tschantz *et al.*, 1995) but also membrane binding activity. It therefore seems that the function of H1 and H2 is limited to assembly, in addition by firmly anchoring the protein to the cytoplasmic membrane, the P2 domain is prevented to move to the outer membrane. The lack of sequence conservation in H1, P1

and the amino terminal half of the H2 segment between leader peptidases from different sources (Dalbey and von Heijne, 1992) is consistent with this limited role in function. Alternatively the membrane anchors could in principle also be involved in recognition of components of the sec machinery although indications for such a recognition were not reported. Furthermore Lep does not copurify with any of the known sec proteins and Lep is able to process precursors which do not make use of a functional sec machinery.

The binding to lipids was also demonstrated by efficient insertion of Δ2–75 into monolayers derived from inner membrane lipids. Insertion does not seem to depend on electrostatic interactions (Fig.7) Remarkably, while anionic phospholipids are important for the insertion of a lot of proteins such as the signal sequence of prePhoE (Batenburg *et al.*, 1988), and the translocation ATPase SecA (Breukink *et al.*, 1992), Δ2–75 displayed best penetration in lipid films made from the zwitterionic lipid phosphatidylethanolamine. The limiting surface pressure for insertion into this type of lipids was very close to that of complete extracts of the *E.coli* inner membrane which contain about 75% PE. This not only corroborates the relative unimportance of electrostatic interactions, it also points to the important role PE can play because of its specific structural properties. Insertion into monolayers of DOPC which is also zwitterionic was very much reduced. This difference in insertion of proteins into PE compared to PC was also found in case of the precursor to ferredoxin (van't Hof *et al.*, 1993), SecA (Breukink *et al.*, 1992) and prePhoE (van Raalte *et al.*, 1996). One of the possible reasons for efficient interactions with PE is the ability of PE to form intermolecular hydrogen bridges. It is also very likely that the difference in insertion efficiency between PC and PE is caused by the differences in size of the head groups. The PE head group is smaller and could offer more space for proteins to insert. The size of the head group relative to the diameter of the acyl chains is indeed so small that these lipids are unable to form stable bilayers by themselves. This class of non-bilayer forming lipids is of great importance in biological membranes and their relative amounts are strictly regulated (Rietveld *et al.*, 1993). Apparently their presence in membranes can give rise to specific packing conditions which are essential for membrane functions as, for example, protein translocation (Rietveld *et al.*, 1995).

Phosphatidyl ethanolamine was also shown to be important for specific interactions with other proteins. It promotes the folding of a periplasmic loop of lactose permease (Bogdanov *et al.*, 1996), regulates the activity of glycero phosphate acyl transferase (Snider and Kennedy, 1977) and was recently found in the crystals of cytochrome C oxidase (Tsukihara *et al.*, 1996).

In the present study it is shown that the catalytic domain of Lep binds to lipids while it was demonstrated before that activity of the full length Lep was enhanced by addition of phospholipids to an *in vitro* assay (van Klompenburg *et al.*, 1995). In that system all phospholipids tested stimulated processing but anionic phospholipids were slightly more effective than zwitterionic. This could indicate that anionic phospholipids act on the conformation of either the signal peptide (Chupin *et al.*, 1995) or the membrane anchors of Lep. It is also possible that anionic phospholipids indeed have a special role in the catalysis of Lep. In this case it can be envisaged that PE mediated insertion orients the catalytic site in such a way that anionic phospholipids can get close to the active site and fulfill their function.

Considering the nature and specificity of the interaction between the periplasmic domain of Lep and lipids it is most likely that membrane association is caused by interaction of a hydrophobic segment within the periplasmic domain with the lipids. Deletion of H3 (residue 83 to 98) which is the most hydrophobic part within the periplasmic domain indeed diminished both insertion into the lipid monolayers and association with the lipid vesicles.

The insertion into the lipid phase as described in this study has important implications for the mode of action of Lep. There is compelling evidence that during translocation the amino terminus of the signal peptide stays at the cytosolic side of the membrane (Kuhn, 1987; Shaw *et al.*, 1988), while the hydrophobic core is probably too short to span the hydrophobic part of the bilayer. This means that in order to reach its substrate, the catalytic site of Lep must be at least very close to the membrane. Insertion of the periplasmic domain into the lipid phase and possible involvement of H3, which carries the catalytic serine 90 residue, implies that the active site of Lep may actually be (partially) buried in the membrane.

Interestingly it was observed that lengthening the hydrophobic core of signal sequences resulted in reduced processing of preproteins without much effect on translocation (Chou and Kendall, 1990). In light of the foregoing this can be interpreted as moving the cleavage site of the preprotein out of the membrane and therefore away from the catalytic site of leader peptidase.

Materials and methods

Isolation and purification of proteins and lipids.

Two truncated forms of Lep were isolated essentially as described before(Kuo *et al.*, 1993; Tschantz *et al*, 1995) but detergent was left out. The proteins were stored in 20 mM Tris/HCl, pH 7.4. Total lipid extracts from the inner membrane were prepared by extraction (Bligh and Dyer,

1959) and further purified by column chromatography on a sillica column (sillicagel 30-60 μm, Baker B.V.). After equilibration in chloroform lipids were eluted in a choloroform : methanol 1:1 mixture and converted to their sodium salts (Smaal *et al.*, 1985)

Isolation of the periplasmic fraction.

An overnight culture of MC1061 bearing a plasmid encoding H2-CC was diluted into fresh M9 medium supplemented with ampicillin (50 μg/ml), 0.2% fructose, thiamin and all amino acids except for methionine. In early-exponential phase expression was induced with 0.2% arabinose and cells were labelled with 50 μCi ^{35}S methionine. After 2 minutes cells were harvested by centrifugation in an Eppendorf centrifuge, washed and resuspended in 30mM Tris/HCl, pH7.5. An equal volume of the same buffer with 40% sucrose and EDTA (final concentration: 0.1 mM) was added and the mixture was incubated at room temperature for 10 minutes. Cells were pelleted and the pellet was quickly resuspended in ice cold 0.5 mM $MgCl_2$ and incubated on ice for 10 minutes. After centrifugation for 8 minutes the supernatant was separated from the pellet. Both fractions were immunoprecipitated with Lep and β–lactamase antibodies and analysed by SDS-PAGE followed by autoradiography.

Vesicle isolation and preparation.

Inverted and right side out inner membrane vesicles (IIMVs and RSOs) and outer membranes (OMVs) of *E. coli* strain MC1061 were isolated according to published procedures. Identity of the fractions were confirmed by lipopolysaccharide (LPS) staining and immunodetection of OmpA, Lep and SecY. Large unilamellar vesicles (LUVETs) were prepared by means of extrusion through a polycarbonate filter (Nucleopore: 0.2 μm pore size) of a rehydrated (10mM Tris/HCl, 50 mM NaCl, pH 7.5) total lipid extract from the inner membrane of MC1061.

Vesicle binding assay.

LUVETs corresponding to 200 nmol lipid were incubated with the indicated amounts of proteins in 300μl 10mM Tris/HCl, 50 mM NaCl, pH 7.5 for 1 hour at room temperature. Inner membrane vesicles and OMVs were incubated in 300μl 50 mM triethanolamine/HAc, 250 mM sucrose, 1 mM DTT, pH7.5 to maintain the same environment as during isolation. Vesicles were pelleted by centrifugation for 30 minutes at 236 * 10^3 g at room temperature in a TL 100.3 rotor using a TL 100 ultracentrifuge (Beckmann Instruments Inc. Palo Alto, California, U.S.A.). Pelleting efficiencies of

the vesicles were calculated after phosphorus determination (Rouser *et al.*, 1970) on supernatant and pellet. The amount of bound protein was determined after SDS-PAGE and Coomassie Brilliant Blue staining. The intensities of the bands were quantified by densitometry (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, U.S.A.) and compared to calibration curves of the same proteins which were run on the same gels. The amount of bound protein was corrected for the pelleting efficiencies which were always above 80%.

Monolayer experiments.

The Wilhelmy plate method was used to measure protein induced changes in surface pressure of a monomolecular layer of phospholipids at constant surface area. Surface pressures were measured at 26 ± 1^0C using a Cahn 2000 microbalance while continuously stirring the subphase with a magnetic bar. Unless stated otherwise, a subphase of 5 ml 10mM Tris/HCl, 50 mM NaCl, pH 7.5 was placed in a teflon trough. The monomolecular lipid layers were spread from a chloroform/methanol 3:1 (by vol.) solution at the air/buffer interphase to give initial surface pressures between 18 and 35mN/m. Lower initial surface pressures were not used since both proteins gave rise to surface pressures of 18 mN/m in the absence of a lipid monolayer. In all experiments saturating amounts of protein (for both proteins 4μg/ml) were added in the subphase from a hole in the edge of the trough. The surface pressure was measured until a constant level was reached.

Chapter 4

Anionic phospholipids are determinants of membrane protein topology

based on publication (IV)

Summary

The orientation of many membrane proteins is determined by the asymmetric distribution of positively charged amino acid residues in cytoplasmic and translocated loops. The positive inside rule states that loops with large amounts of these residues tend to have cytoplasmic locations. Orientations of constructs derived from the inner membrane protein leader peptidase from *Escherichia coli* were found to depend on the anionic phospholipid content of the membrane. Lowering the contents of anionic phospholipids facilitated membrane passage of positively charged loops. On the other hand elevated contents of acidic phospholipids in the membrane rendered translocation more sensitive to positively charged residues. The results demonstrate that anionic lipids are determinants of membrane protein topology and suggest that interaction between negatively charged phospholipids and positively charged amino acid residues contribute to the orientation of membrane proteins.

Introduction

The vast majority of all membrane proteins is exclusively found in one orientation. How is this achieved? Features of both the preexisting membrane and the newly synthesized proteins are likely to be involved. The orientation of many proteins is dictated by arginyl and lysyl residues in short loops (<60 residues) connecting the hydrophobic transmembrane helices (von Heijne and Gavel, 1988; Gavel *et al.*, 1991; Gavel and von Heijne, 1992; Sipos and von Heijne, 1993). Positively charged residues are enriched in loops that do not translocate across the membrane, whereas translocated loops are largely devoid of them (the 'positive inside' rule). In addition, positively charged residues are able to block the translocation of larger domains if they are placed immediately downstream of a signal peptide or transmembrane helix (Yamane and Mizushima, 1988). How the positively charged residues exert their influence on membrane protein topology is largely unknown. Evidence for a role of the proton motive force (pmf) was published (Andersson and von Heijne, 1994) and involvement of lipid composition hypothesized (Andersson *et al.*, 1992; Krishtallik and Cramer, 1995) In the present study we report on the involvement of negatively charged phospholipids in preventing transmembrane passage of positive charges. Anionic phospholipids are present in all membranes (Zambrano *et al.*, 1975; Shibuya *et al.*, 1985; Douce and Joyard, 1990; Hovius *et al.*, 1990) and fulfill diverse functions, often based on electrostatic interactions with positively charged protein domains(Van der Waart *et al.*, 1983; Sixl *et al.*, 1984; Yang and Glaser, 1995) We reasoned that charge interactions between arginyl and lysyl residues

and anionic phospholipids might determine the orientation of membrane proteins. To test this hypothesis lipid biosynthetic *E. coli* mutants, in which the anionic phospholipid content could be manipulated between 10% and 100%, were employed. In these strains the transmembrane orientations of constructs derived from leader peptidase (Lep, also called Signal Peptidase I) were determined. Lep is a well characterized inner membrane protein from *Escherichia coli* which removes signal peptides from translocated precursor proteins (Wolfe *et al.*, 1985). It is used as a model to study membrane protein topogenesis and the orientation of Lep can be altered by the addition or removal of positively charged residues in appropriate locations as was predicted by the positive inside rule (von Heijne, 1989). Analysis of the orientations of these constructs in the lipid mutant strains demonstrates that negatively charged phospholipids can inhibit translocation of positively charged domains, thereby contributing to the control of membrane protein topology.

Results

In order to test whether anionic phospholipids in the inner membrane of *E. coli* influence the orientation of membrane proteins, constructs derived from the membrane protein Lep were expressed in strain HDL11. In this strain expression from the *pgsA* gene which encodes the key enzyme for the synthesis of anionic phospholipids is under control of the *lac* promoter. (Kusters *et al.*, 1991) When cells are grown in the presence of IPTG the inner membrane contains wild type levels of the anionic phospholipids phosphatidylglycerol (PG, 16%) and cardiolipin (CL, 3%) (Kusters *et al.*, 1991). In the absence of IPTG the levels of PG and CL drop to 2 and 1% respectively. However, this is partially compensated by accumulation of the precursor lipid phosphatidic acid such that a level of 9% acidic phospholipids remains (Kusters *et al.*, 1991).

Lep was chosen as a model in our studies because its assembly and orientation are well established. The wild type protein has two hydrophobic transmembrane helices (H1 and H2) separated by a positively charged cytoplasmic loop (P1), and a large periplasmic domain (P2) (Fig. 1A). The orientation of Lep in the inner membrane is measured by protease accessibility assays on spheroplasts (figure 1 and von Heijne, 1989). Different constructs based on Lep with one positive charge in the P1 loop and varying amounts of positive charges at the N-terminus were used. This series is called the nK/K series where n indicates the number of lysines at the N-terminus. HDL11 cells were grown in the presence of IPTG and construct 0K/K was expressed. After conversion to spheroplasts the topology was determined by trypsin treatment. Apparently 0K/K inserts in the same N_{out}-C_{out} manner as the wild type protein (Fig. 1A) because trypsin removes the immunogenic P2 domain (Fig. 1B, lanes 1 and 2). As

expected from the positive inside rule, addition of four positively charged residues to the N-terminus (construct 4K/K) causes the protein to insert with an 'inverted' orientation (Fig. 1A), as shown by trypsin cleavage in the P1 loop of 4K/K (Fig. 1B, lanes 3 and 4). When constructs insert in the inverted orientation, usually also a small portion of undigested molecules is observed (Nilsson and von Heijne, 1990).

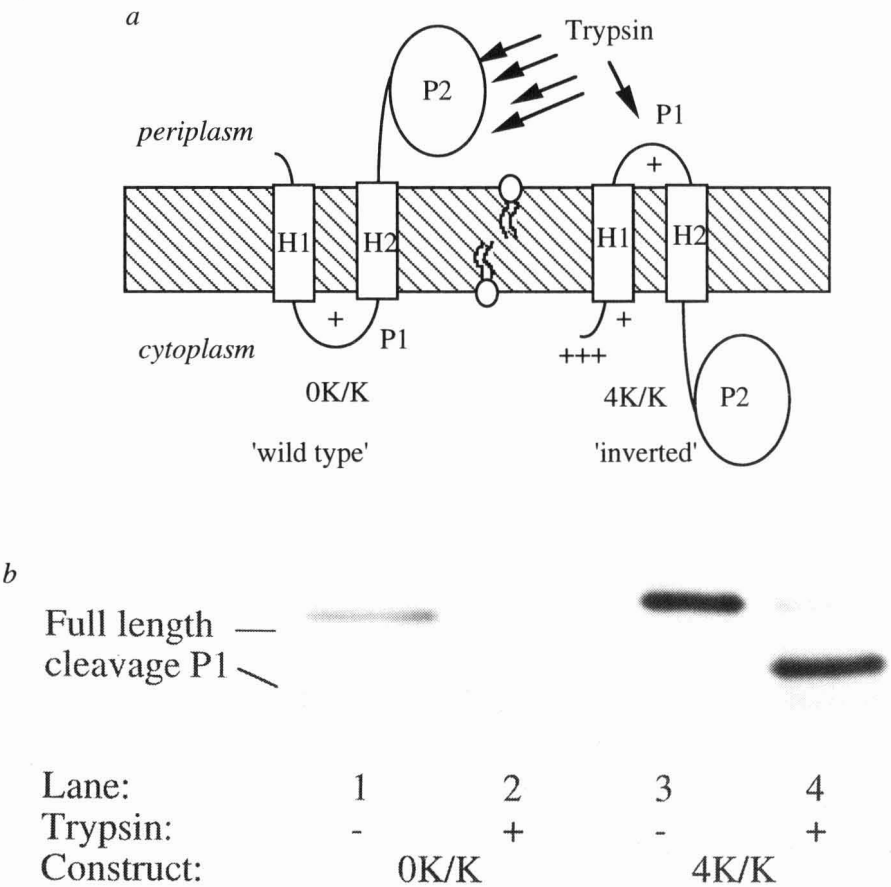


Figure 1. Orientation of two constructs derived from Lep in the inner membrane of wild type *E.coli*. cells.A: Schematic representation of the topology of constructs 0K/K (left) and 4K/K (right). Apart from the varying number of lysines inserted between residues 4 and 5 , these constructs differ from wild type Lep by a replacement of the highly positively charged segment 30-52 (in the P1 loop) by a nine residue spacer and the substitution of lysine 56 by asparagine. The resulting nK/K series (one lysine in the P1 loop, varying numbers of lysines in the N-terminal tail) was described before (Nilsson and Von Heijne, 1990) . B: Trypsin treatment of spheroplasts derived from *E.coli* HDL11 cells removes the P2 domain from construct 0K/K (lanes 1 and 2) and gives cleavage in the P1 loop of construct 4K/K (lanes 3 and 4).

Constructs with intermediate amounts of lysines at the N-terminus were also tested. The percentage of inverted molecules as function of the amount of N-terminal lysines is depicted by the open symbols in figure 2. These orientations were comparable to those found using a wild type strain (Nilsson and von Heijne, 1990). The orientation of K/K is a special case; this construct has equal amounts of charges at the N-terminus and in P1. Therefore half of the population of K/K molecules insert in the inverted and the other half in the wild-type orientation. When HDL11 cells were grown in the absence of IPTG, constructs with 2, 3 or 4 lysines at the N-terminus show a significant decrease in the fraction of inverted molecules (Fig. 2, closed symbols) paralleled by an increase in the fraction of wild-type-like oriented molecules. This implies that with decreased anionic phospholipid contents, positive charges at the N-terminus can pass the membrane more readily. Strikingly, also when cells are grown in the absence of IPTG construct K/K displays equal amounts of the two orientations.

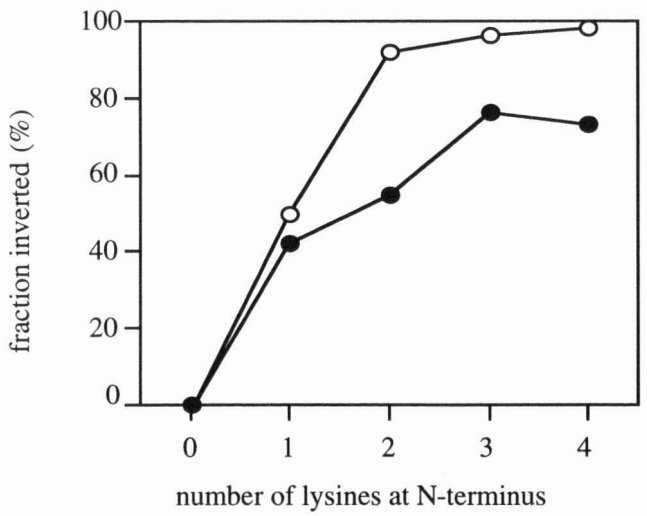


Figure 2. Influence of negatively charged lipids on the fraction of inverted molecules of the nK/K series in HDL11. Overnight cultures were diluted into mimimal medium without (closed circles) or with 50μM IPTG (open circles) to induce phosphatidylglycerol synthesis. The topologies of the constructs were determined as described under Materials and Methods. Topologies of each individual point were determined 2-7 times with less than 10% variation.

The two possible orientations of Lep derivatives are achieved by different mechanisms; while translocation of the P2-domain is catalyzed by the proteinaceous secretion (Sec) machinery (Lee *et al.*, 1992), membrane passage of the P1 loop does not require functionality of this machinery (Andersson

and von Heijne, 1993). To show that a putative decreased functioning of the Sec machinery in cells with lower anionic phospholipid contents cannot account for the effects shown in figure 2, the function of the Sec machinery was blocked by azide. The rationale of this experiment is displayed in figure 3A.

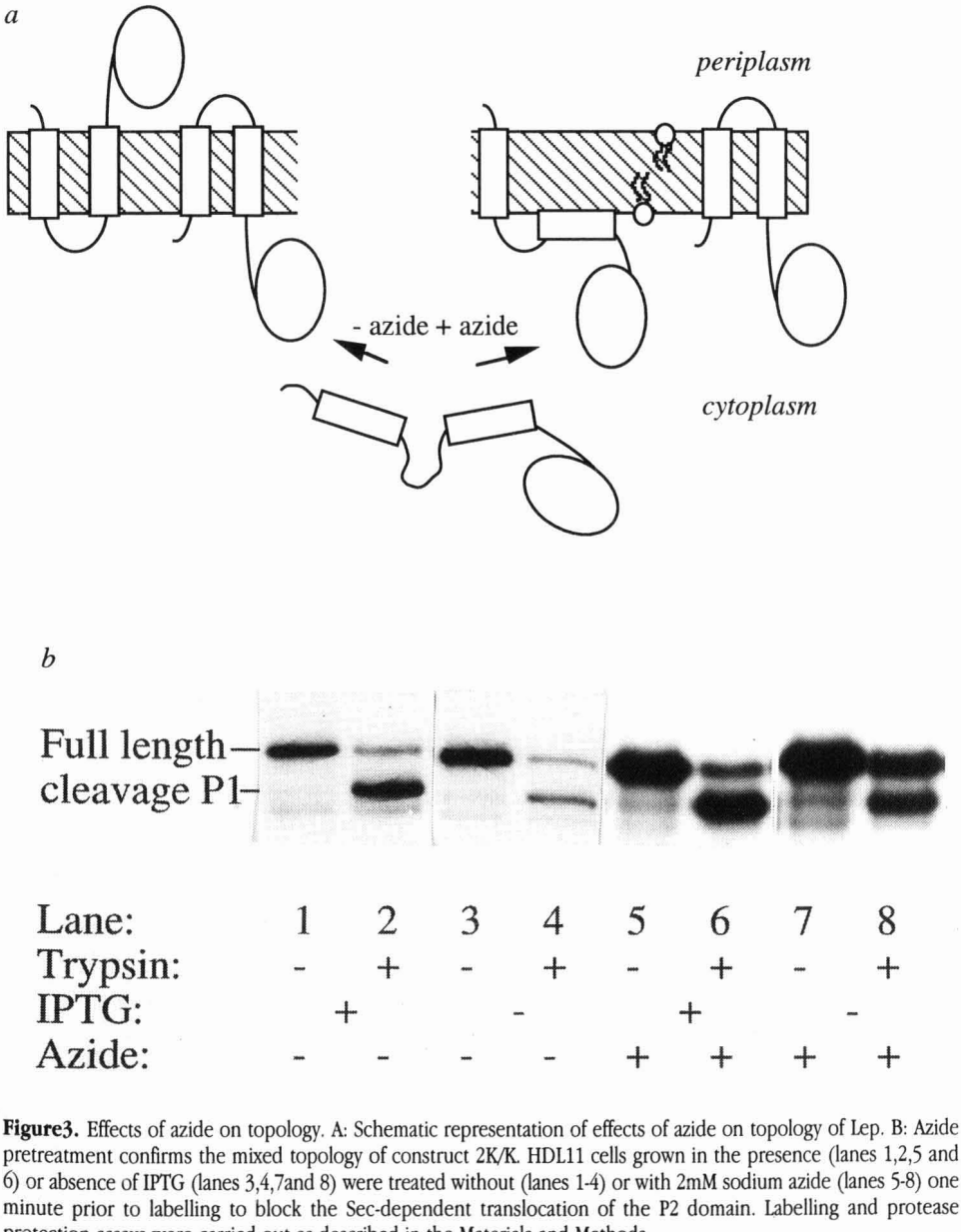


Figure3. Effects of azide on topology. A: Schematic representation of effects of azide on topology of Lep. B: Azide pretreatment confirms the mixed topology of construct 2K/K. HDL11 cells grown in the presence (lanes 1,2,5 and 6) or absence of IPTG (lanes 3,4,7and 8) were treated without (lanes 1-4) or with 2mM sodium azide (lanes 5-8) one minute prior to labelling to block the Sec-dependent translocation of the P2 domain. Labelling and protease protection assays were carried out as described in the Materials and Methods

Azide blocks the Sec-dependent translocation of the P2 domain, insertion of P1 is unaffected under these conditions. Therefore, neither P1 nor P2 of molecules which were on the pathway to wild-type like insertion pass the membrane. As a result an increase of fully protease resistant molecules is expected and the ratio between fully protected molecules and those cleaved in P1 is a measure of the ratio between molecules inserting in the wildtype and the inverted orientations.

Cells were grown in the presence of IPTG and the topology of construct 2K/Kwas analyzed. More than 90% of the 2K/K molecules can be cleaved in the P1 domain (Fig. 3B, lanes 2 and 6) both in the absence and presence of azide. In the absence of IPTG only 55% is cleaved (Fig. 3B, lanes 4 and 8) in presence and absence of azide, again demonstrating a strong effect of anionic phospholipids on topology. In the absence of anionic phospholipid synthesis, azide gives rise to a large increase of fully protease protected material. This shows that in the absence of IPTG, HDL11 cells can indeed translocate the P2 loops of about half of the 2K/K moleucles, making use of a functional Sec machinery. It also shows that blocking the Sec dependent pathway for translocation does not force the molecules into the Sec independent pathway for translocation of P1 but rather renders the molecules untranslocated.

Previously, the proton motive force (pmf) in *E.coli* was shown to be involved in membrane protein topogenesis (Andersson and von Heijne, 1994) . To exclude that the effects shown in Figures 2 and 3 are due to changes in the pmf, the pmf in HDL11 was measured and the magnitudes of $\Delta\psi$ and ΔpH were found to be independent of IPTG (van der Goot *et al.*, 1993, results not shown); ΔpH was measured according to Breeuwer (Breeuwer *et al.*, 1996, results not shown).

When placed immediately downstream of a transmembrane segment, positively charged residues can also control topology by blocking the Sec-dependent translocation of large periplasmic domains.(Andersson and von Heijne, 1991) We tested the effect of anionic phospholipids in this context by determining the localisation of the periplasmic marker alkaline phosphatase (Manoil and Beckwith, 1985) fused to Lep constructs with 0-3 arginines placed next to H2. These constructs are called //nR where n indicates the number of arginines. Enzymatic activity of alkaline phosphatase (PhoA) depends on periplasmic localisation and is therefore a good marker for this compartment. Activity can be determined on whole cells and is therefore also useful in strains from which no stable spheroplasts can be obtained. The localisations of the P2-PhoA domain in constructs //0R and //3R in a wild type strain were also determined by trypsin treatment of spheroplasts (Fig.4A). The P2-PhoA domain of construct //0R is fully accessible to trypsin (Fig 4A, lanes 1 and 2) while that of //3R is fully protected (fig, 4A, lanes 3 and 4).

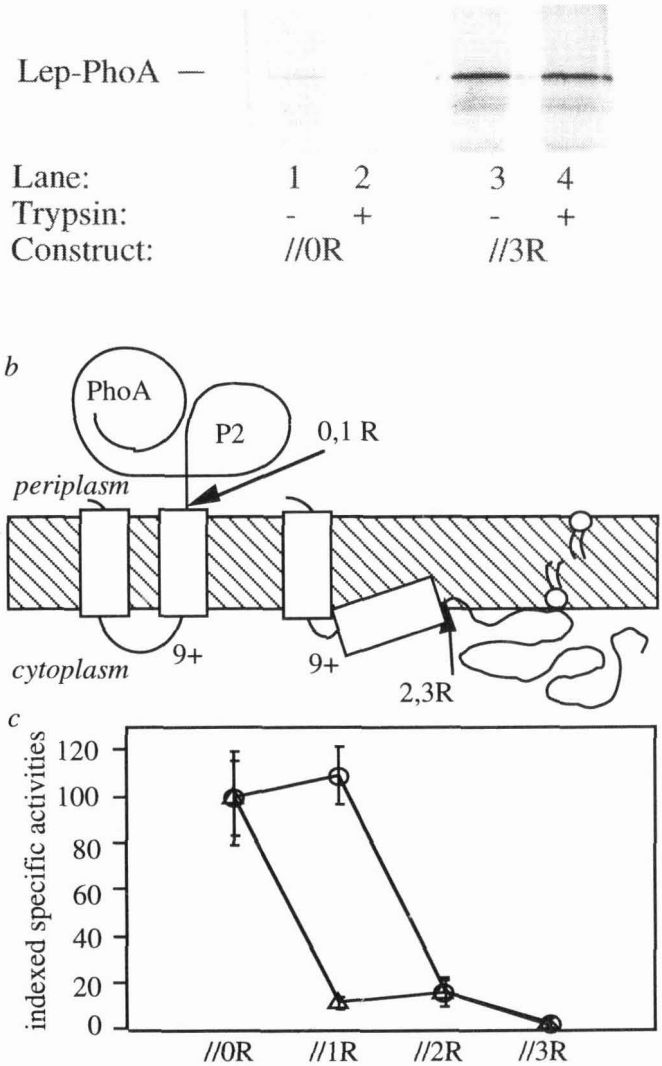


Figure 4. Translocation of a large periplasmic domain is more sensitive to positively charged residues in cells with exclusively anionic phospholipids in the inner membrane.A: Lep-PhoA fusion constructs (left) and //3R (right) were expressed in wild type cells After conversion to spheroplasts they were trypsinated as described under Materials and Methods.B: Schematic representation of the topologies of constructs //0R and //1R (left) and //2R and //3R (right) in the inner membrane of *E.coli*. cells with wild type lipid composition. C: Specific alkaline phosphatase activities of Lep-PhoA constructs in JM109 (circles) and AD93 (triangles). The indexed specific alkaline phosphatase activities were determined as in Materials and Methods and plotted against the amount of charges behind H2.

When all the constructs of the //nR series were expressed in strain JM109 (Yanisch-Perron *et al.*, 1985) with wild type lipid composition it was observed that constructs //0R and //1R display high alkaline phosphatase activity while constructs //2R and //3R are inactive (Fig. 4C, circles). These results establish the topologies depicted in figure 4B. In constructs with 2 or 3 arginines, H2 does not assume a transmembrane orientation.

To investigate the effects of increased amounts of anionic phospholipids expression we performed the same experiments in *E.coli* strain AD93 (De Chavigny *et al.*, 1991) This strain lacks an intact *pss* gene and therefore does not synthesize the only zwitterionic lipid class of *E. coli*, phosphatidylethanolamine. This results in a membrane with only negatively charged lipids. For growth and survival this strain requires divalent cations. Therefore, conventional EDTA-lysozyme treatment does not result in stable spheroplasts. The alkaline phosphatase assay is especially suited to study localisation in this strain. The fusion constructs were expressed and the localisations determined. Now, a single arginine is sufficient to block translocation (Fig. 4B, triangles). Thus, membrane integration of a hydrophobic segment is more sensitive to positively charged residues placed immediately downstream of a transmembrane segment at high densities of anionic phospholipids in the membrane.

Discussion

In this paper we establish a role for anionic phospholipids in determining the topology of a protein in a membrane. For several constructs of Lep with defined distributions of lysines around the first membrane spanning segment, transmembrane orientation in the *E. coli* inner membrane depends on the concentration of the negatively charged lipids. Lowering this concentration below wild type levels allows the positive charges to move more readily across the membrane.

The anionic lipids might exert this effect directly by interacting with the incoming protein or indirectly possibly by influencing the translocation machineries. This latter possibility should be seriously considered because Lep achieves a wild type orientation by making use of the Sec-machinery, which mediates membrane passage of the periplasmic domain. Sec dependent translocation requires anionic phospholipids for maximal efficiency. This at least partially originates from SecA which requires anionic phospholipids for full activity. By blocking the activity of SecA by azide it could be demonstrated that the fraction of the Lep molecules with an inverted orientation depends on the anionic lipids but not on function of the Sec machinery. In addition it was observed that a Lep construct with one lysine at both sides of H1 inserts in a random orientation in the membrane independent of the phospholipid composition. This clearly demonstrates that anionic lipid dependency of the functioning of the insertion

machineries is not responsible for the anionic lipid dependend topological decisions of the Lep constructs. Because also the pmf was found to be independent of the anionic lipid content of the membrane we conclude that a direct interaction between the newly synthesized protein and the membrane lipids is responsible for the observed effects.

When positive charges are present at both sides of a hydrophobic segment they can prevent it from assuming a transmembrane orientation. In Lep there are nine positive charges upstream of H2 and in cells with wild type lipid composition, two arginines downstream of H2 are required to prevent H2 from spanning the membrane (Fig.4). Increasing the anionic lipid content to 100% rendered one positive charge sufficient for this effect. Two aspects of membrane protein topology are sensitive to the positioning of positive charges. The orientation of a whole protein can be inverted and the membrane integration of a hydrophobic segment prevented. Here it is shown that in both cases anionic phospholipids play a role. The results indicate that anionic lipids prevent the translocation of positive charges, probably via electrostatic interactions.

In *E. coli* now both the pmf and anionic phospholipids are shown to exert control on membrane protein orientation. It was observed (Andersson and von Heijne, 1994) that membrane passage of one or two lysines at the N-terminus was possible in the absence of a pmf, but not in its presence. No effect of the pmf was found for constructs with more positive charges at the N-terminus. Reduction of the anionic lipid content from 25% to below 10% resulted in facilitated membrane passage of up to four positive charges. On basis of these results it is not possible to gain quantitative insight into the contributions of the pmf and the anionic lipids to membrane protein orientation. But it is clear that they can have both very pronounced effects. Interestingly, for some membrane systems to which the positive inside rule applies, like the eucaryotic endoplasmic reticulum and the thylakoids of chloroplast no or only a small $\Delta\psi$ is found. In these cases the anionic lipids may be the major determinants of membrane protein topology.

Materials and methods

Enzymes and chemicals TPCK treated trypsin, soy bean trypsin inhibitor, chicken egg white lysozyme, phenylmethylsulfonylfluoride (PMSF) and isopropylthiogalactoside (IPTG) were from Sigma; paranitrophenylphosphate (PNPP) was obtained from Serva.

Strains , growth conditions and plasmids JM109 (*endA1 recA1 gyrA96 thi hsdR17 recA1 supE44* D*(lac-proAB)*/F' *traD36 proAB laclqZ* D(M15)) (Yanisch-Perron *et al.*, 1985) and AD93 (*pss93:: kan recA srl*::Tn*10 nadB*$^+$) (De Chavigny *et al.*, 1991) were grown in LB supplemented with 50 mM

$MgCl_2$ and 10 mM KPi, pH7.3. HDL11 (*pgsA::kan* F(*lacOP-pgsA*$^+$) *lacZ' lacY*::Tn9 *lpp2 zdg*::Tn10) (Kusters *et al.*, 1991) was grown in M9 medium supplemented with chloramphenicol (20 μg/ml), kanamycin (50 μg/ml) and tetracyclin (10 μg/ml) and when appropriate with 50 μM IPTG. These strains were used to express constructs derived from Lep from the pING1 plasmid after induction by arabinose.

Assays of membrane topology for constructs of the nK/K series HDL11 cells were grown overnight in the presence of 50 μM IPTG to synthesize wild type levels of PG and CL and ampicillin (50 μg/ml) to select for the presence of plasmids. Overnight cultures were washed twice in physiological salt and diluted 1:40 in fresh medium with or without 50μM IPTG to induce anionic phospholipid synthesis. Induction, radiolabelling, protease treatment of spheroplasts and immunoprecipitatons with antisera to Lep, OmpA (an outer membrane protein as control for complete conversion of cells to spheroplasts, data not shown) and AraB (a cytoplasmic protein as a marker for intactness of the inner membrane, data not shown) were performed as described (Nilsson and von Heijne, 1990). The percentage of inverted molecules (P_i) was calculated as $P_i = 100*(7/6) * (C_{inv}/C_{tot})$. C_{inv} and C_{tot} are the background and lysis-corrected counts after quantification on a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) of, respectively, the trypsin cleaved species indicative of N_{in}-C_{in} orientation and the full length molecules before trypsin treatment. Lysis was corrected for by normalizing all counts from Lep in a lane to the counts of the cytoplasmic marker AraB'. The factor 7/6 corrects for the loss of 1 methionine upon trypsination. Variation between different experiments was $\leq$10%. Phospholipid composition of HDL11 cells was checked after lipid extraction (Bligh and Dyer, 1959) and thin layer chromatography with the eluent mixture choroform: methanol: acetic acid (65:25:10) and found to be in accordance with the results of Kusters (Kusters *et al.*, 1991).

Assays for membrane topology of the Lep-PhoA fusion proteins JM109 and AD93 cells were grown until OD_{660} = 0.8. Arabinose was added (1 %) to express the fusion constructs and after one hour cells were harvested and alkaline phosphatase activity was measured and calculated as described (Manoil and Beckwith, 1985). To calculate specific activities, cell contents were separated by SDS-PAGE and transferred to nitrocellulose after which the amount of Lep-PhoA fusion construct was quantified by making use of Lep antibodies and enhanced chemi luminescence (ECL) detection as described for Lep (van Klompenburg *et al.*, 1995). The specific activity of //0R in both strains was indexed at 100 and the other activities were related to this value. The topologies of constructs //0R and //3R in JM109 were confirmed by trypsination assays on spheroplasts as described above. The topologies of constructs //0R and //3R in AD93 were confirmed by alkaline phosphatase assays performed on inverted vesicles of

AD93 bearing //0R in the absence or presence of detergent and by trypsin treatment of vesicles bearing //0R and //3R (results not shown).

Chapter 5

In vitro membrane integration of leader peptidase depends on the Sec machinery and anionic phospholipids and can occur posttranslationally

based on publication (VI)

Summary

A cell-free system based on a lysate and membrane vesicles from *E. coli*, is used to study characteristics of the membrane integration reaction of the polytopic membrane protein leader peptidase (Lep). Integration into inverted inner membrane vesicles was detected by partial protection against externally added protease. Integration is most efficient when coupled to translation but can also occur posttranslationally and depends on the action of the proteinaceous Sec machinery and availability of anionic phospholipids. Lep is the first example of a membrane protein without cleavable signal sequence which requires anionic lipids for integration *in vitro*.

Introduction

Leader peptidase (Lep) from *E. coli* is an integral inner membrane protein which spans the membrane with two hydrophobic segments which are connected by a short cytoplasmic loop (Wolfe *et al.*, 1983). The second transmembrane segment precedes a large periplasmic domain which during assembly has to pass the inner membrane (Dalbey and Wickner, 1987). In recent years Lep is extensively used as a model to study membrane protein insertion *in vivo* and it appeared that the mechanism for translocation of periplasmic loops depends on loop length. Loops smaller than 60 amino acid residues pass the membrane bilayer in an apparent spontaneous process, while translocation of longer loops like the periplasmic domain of Lep requires the action of the so called Sec machinery (Lee *et al.*, 1992; Andersson and von Heijne, 1993).

Originally, the Sec machinery was found to be essential for the translocation of periplasmic and outer membrane proteins across the inner membrane (for review see: Schatz and Beckwith, 1990). These periplasmic and outer membrane proteins are synthesized as precursors with N-terminal extensions called signal sequences and are often maintained in a translocation competent state by the tetrameric SecB protein (Kumamoto and Beckwith, 1985; Kusters *et al.*, 1989; Kumamoto, 1991). 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 the progress of translocation (Oliver and Beckwith, 1982; Hartl *et al.*, 1990; Economou and Wickner, 1994). Together with SecA, the Sec-Y, -E and -G proteins constitute the basic machinery for translocation (Tokuda, 1994). Anionic phospholipids, which represent about 25 % of the membrane lipids of *E. coli*, are also involved in translocation (de Vrije *et al.*, 1988), they stimulate the ATPase activity of SecA (Lill *et al.*, 1990)and were found capable of interacting with signal peptides (Keller *et al.*, 1992). Efficient *in vivo*

translocation also requires the presence of a proton motive force (pmf) (Bakker and Randall, 1984) and 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 and Wickner, 1994). After translocation the signal sequences are removed by the action of signal peptidases such as Lep (Dalbey and von Heijne, 1992). Translocation of some precursors require other cytosolic components like the GroEL chaperone (Bochkareva *et al.*, 1988) or the prokaryotic SRP homologues (Luirink *et al.*, 1992) .

In vitro translocation systems, employing radiolabelled precursors and purified inner membrane vesicles, contributed much to the current knowledge about precursor protein translocation. In this study we establish an *in vitro* system for membrane integration of Lep which involves membrane passage of the periplasmic domain. The requirements for this process are investigated by using various types of vesicles and by adding agents to the assembly reactions which block the function of components possibly involved in assembly.

Materials & methods

Materials

Trinucleotides (sodium salts), T4 ligase and the restriction endonucleases *Sma*I and *Sal*I were obtained from Pharmacia and folinic acid (calcium salt), phosphocreatine (di Tris salt) polymyxin B sulphate, phenyl methyl sulfonyl fluoride (PMSF) and amino acids from Sigma (USA). SP6 RNA polymerase, creatine phosphokinase, tRNA, puromycin dihydrochloride and proteinase K were purchased from Boehringer (germany). Lep(His)$_6$ was purified as described before (van Klompenburg *et al.*, 1995) SecA and β-lactamase antibodies were a gift from Dr. H. de Cock, Utrecht University.

Strains and growth conditions

E. coli strain MRE 600 (Cammack and Wade, 1965) was used for the production of S-135 lysate and grown in Giston broth at 37^{0}C until early exponential phase. Reference vesicles were isolated from strain SD 12 (Shibuya *et al.*, 1985) which was grown at 37^{0}C in LB. PC 2977(Shiba *et al.*, 1984) was used as a source of secY^{ts} vesicles, cells were grown overnight at 30 ^{o}C in LB with 10μg/ml tetracyclin and at 42 ^{o}C, 3 hours prior to vesicle isolation. Vesicles with low contents of anionic lipids were isolated from HDL11 (Kusters *et al.*, 1991), which was grown in LB supplemented with 10μg/ml tetracyclin, 25μg/ml chloroamphenicol and 50 μg/ml kanamycin.

In vitro transcription, translation and assembly

S-135 cell extracts and inverted inner membrane vesicles were prepared from *E. coli* according to published procedures (de Vrije *et al.*, 1987). Plasmid pSPLep was created by cloning the *Sal*I-*Sma*I fragment containing *lepB* from a pING1 derived *in vivo* expression vector to the *in vitro* expression vector pSP64. DNA was purified using wizard minipreps DNA purification resin (Promega, USA) and used to direct the transcription of the *LepB* gene by SP6 RNA polymerase during a 30 minutes incubation at 40^0C following the manufacturers instructions. Translations were carried out at 37^0C as described (de Vrije *et al.*, 1987). Five minutes after initiation of translation, inverted inner membrane vesicles were added to a final concentration of 4mg/ml (protein) and the incubation was continued for 25 minutes at 37^0C. Correct assembly of Lep was demonstrated by protection of the P2 domain against the action of externally added proteinase K (200μg/ml final concentration). The protease treatment was carried out for 20 minutes at room temperature after which 2 mM PMSF was added. The samples were analysed by SDS-PAGE and exposure of the gel in a Phosphor Imager. To asses the influence of additions (antibodies or polymyxin), these were mixed with the vesicles prior to incubation in the translocation mixture.

Immunoprecipitation

Immunoprecipitations using antiserum directed against Lep were performed as described (von Heijne, 1989) using Protein A coupled sepharose CL4B (Pharmacia, LKB, Sweden).

ProOmpA translocation

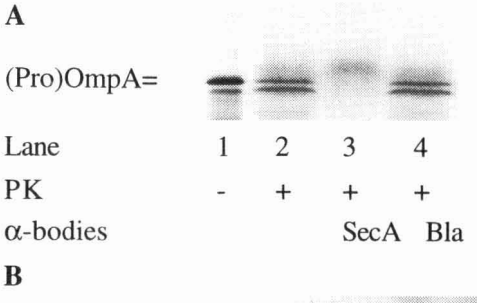
[^{35}S]-proOmpA was purified as described (Nouwen *et al.*, 1996) and stored in 8 M urea. [^{35}S]-proOmpA was diluted 50-fold into 25 μl translocation buffer (40 mM Tris-Acetate pH 8.0, 10.8 mM Magnesiumacetate, 28 mM Potassiumacetate, 2 mM DTT, 0.5 mg/ml BSA) containing inverted inner membrane vesicles (0.40 mg protein/ml), 5 μl S-135 cell extract and 4 mM ATP (Kusters, de Vrije, 1989). When indicated 1 μl polyclonal antiserum directed against SecA or β-lactamase was added. Translocation was allowed to proceed for 20 min. at 37 ^{0}C, followed by analysis as described for the assembly of Lep.

Results

To allow *in vitro* synthesis of mRNA by SP6 RNA polymerase, the *LepB* gene was cloned into an *in vitro* expression vector. Most expression plasmids carry the gene coding for the selectable marker β-

The characteristics of cotranslational membrane integration of Lep were studied using antibodies against SecA and by using vesicles deficient in SecY function. The validity of the antibody approach was tested by studying the influence of SecA antibodies on the translocation of purified proOmpA. ProOmpA is the precursor form of an outer membrane protein of *E. coli* and its translocation is dependent on a functional Sec machinery. Translocation was performed as described in materials and methods and antibodies were added to the vesicles prior to the translation-translocation reaction. Translocation was defined as the amount of protease protected proOmpA and OmpA. In the absence of antibodies (Fig. 3A, lane 2) 20% translocation was observed, while the presence of SecA antibody prevented translocation of proOmpA completely (Fig. 3A, lanes 3). As a control, translocation was studied in the presence of the β lactamase antibodies (Fig. 3A, lane 4) which did not influence translocation efficiency.

A



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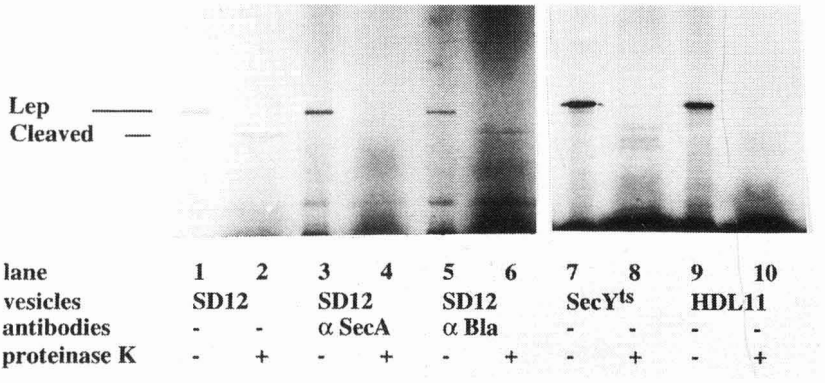

Figure 3. Requirements for membrane assembly of Lep. A: Translocation of purified proOmpA can be blocked by antibodies against SecA. The amount of translocated OmpA and proOmpA were compared to 20% of the added precursor (lane 1). Translocation was carried out in the presence of SecA antibodies (lane 3), β–lactamase antibodies (lane 4) or in the absence of antibodies (lane 2). B: Assembly of Lep in wild type vesicles (lane 1, 2), in vesicles treated with antibodies against SecA (lane 3, 4) or with antibodies against β-lactamase (lanes 5, 6), vesicles depleted of functional SecY (lanes 7, 8) or vesicles reduced in anionic phospholipid contents (lanes 9, 10). The amount of protease protected Lep (even numbered lanes) was compared to 20% of the Lep synthesized (odd numbered lanes) in presence of vesicles.

Since the SecA antibodies block Sec-dependent translocation, this procedure was used to establish whether SecA is required in the *in vitro* assembly reaction of Lep. In the absence of antibodies Lep assembles into IMV's with an efficiency of 20 ± 1 % (n=4) (Fig. 3B, lanes 1 and 2). In the presence of SecA antibody this efficiency drops below 1 % (lanes 3 and 4) while no influence of the addition of β lactamase antibodies was measurable (lane 5 and 6). The requirement for SecY was tested using vesicles from a temperature sensitive strain as was described before for preproteins. Lep assembly into these vesicles was reduced to 1.5 ± 1% (n=3) (lanes 7 and 8). It is therefore concluded that the *in vitro* integration reaction of Lep requires a functional Sec machinery.

Efficient translocation of precursor proteins depends on anionic phospholipids in the membrane (de Vrije *et al.*, 1988; Kusters *et al.*, 1991). To investigate the influence of anionic phospholipids on the assembly of Lep, vesicles were isolated from a lipid biosynthetic mutant strain, HDL11. In this strain, the *pgsA* gene is placed under control of the *lac* operon. This gene is responsible for the synthesis of the major negatively charged phospholipid in *E. coli*, phosphatidylglycerol. In the absence of the inducer IPTG, the anionic phospholipid content of the inner membrane is below 10 % (Kusters *et al.*, 1991)which is 2.5 times lower than in wild type cells. Lep assembles into vesicles isolated from HDL11, which was grown in the absence of IPTG, with efficiencies below 1 % (lanes 9 and 10). The importance of anionic phospholipids for the integration process was also tested by addition of polymyxin B which specifically interacts with anionic phospholipids (El Mashak and Tocanne, 1980).

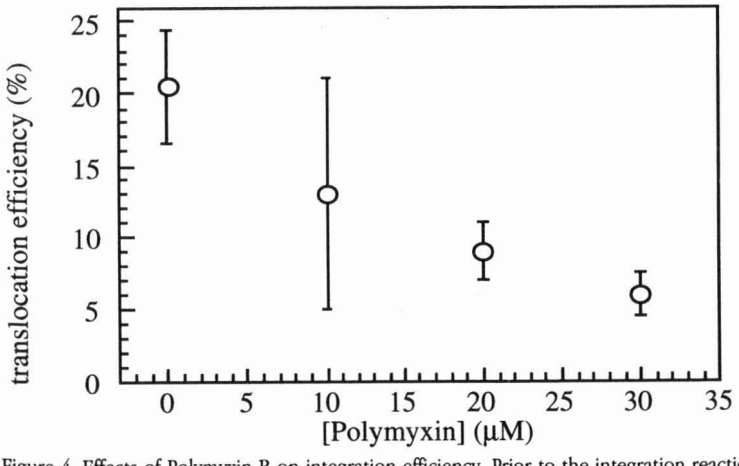

Figure 4. Effects of Polymyxin B on integration efficiency. Prior to the integration reaction ... with the indicated concentrations polymyxin B sulphate. Translocation was carried out and ... under materials & methods. Mean values of translocation efficiencies are depicted and ... standard deviation.

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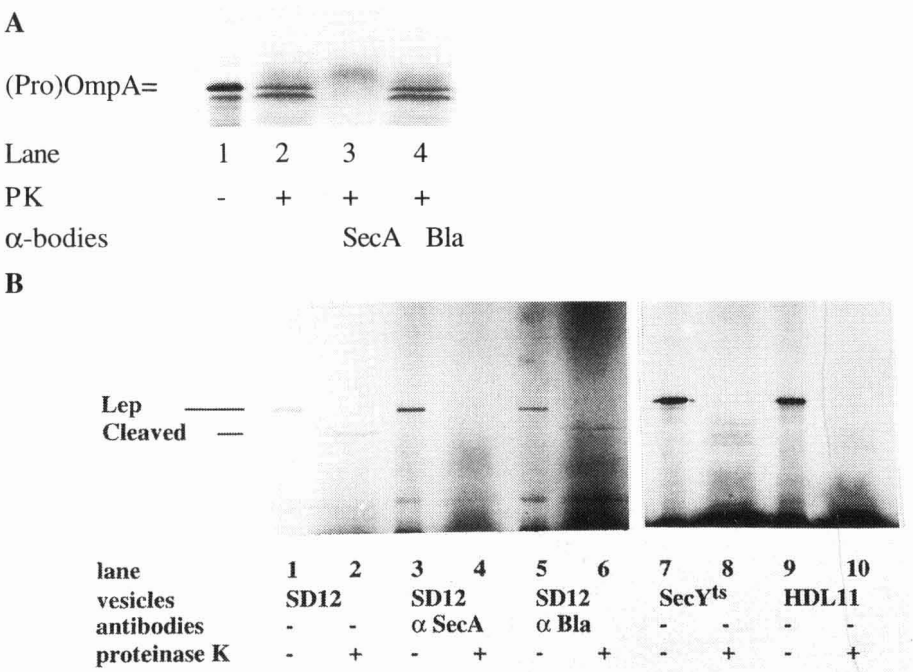

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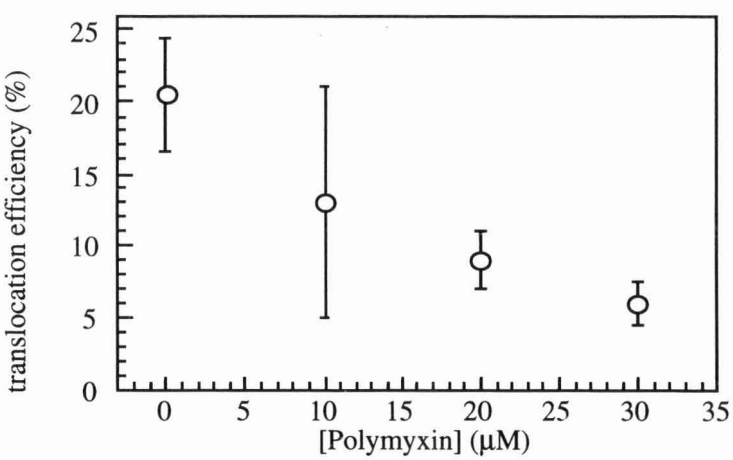
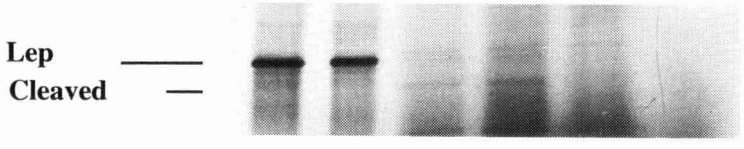


Figure 4. Effects of Polymyxin B on integration efficiency. Prior to the integration reaction vesicles were incubated with the indicated concentrations polymyxin B sulphate. Translocation was carried out and analysed as described under materials & methods. Mean values of translocation efficiencies are depicted and the error bars indicate standard deviation.

Prior to integration, the vesicles were incubated with different concentrations of polymyxin. Figure 4 shows that integration efficiencies decrease with increasing polymyxin concentrations. 20 μM Polymyxin causes a half maximal block of the integration reaction.

For precursor proteins it was shown that translocation could occur after completion of translation. Using the *in vitro* system the possibility of posttranslational integration of the integral membrane protein Lep could be investigated. Posttranslational integration was assayed as follows: 28 minutes after start of translation, 20 μM puromycin was added. After two minutes vesicles were added (lanes 3, 4, and 5) or not (lanes 1 and 2) . ATP was added to one incubation mixture (lane 4) to make sure that energy was not in short supply. After 20 minutes PK was added (lanes 3 and 4). Both in absence and presence of extra ATP, 3 ± 1 % integration was observed, which is 5-10 times less than in the cotranslational experiment. It was checked that under the integration conditions no protein synthesis takes place. In our *in vitro* system, synthesis of Lep reaches a maximum within 25-30 minutes (results not shown) and to ensure arrest of synthesis, puromycin was added to dissociate the ribosomes. The efficacy of this procedure was checked by first carrying out translation in the presence of unlabelled methionine for 28 minutes. Subsequently the translation mixture was incubated with 20 μM puromycin for 2 minutes, after which radioactively labelled methionine was added and the incubation prolonged for 20 minutes. No radioactive material was observed at the position of the full length Lep protein (Fig. 5, lane 6). It should therefore be concluded that under these conditions integration can occur posttranslationally albeit very inefficient.



lane	1	2	3	4	5	6
cold met.	-	-	-	-	-	+
puromycin	-	+	+	+	+	+
vesicles	-	+	+	+	+	-
ATP	-	-	-	+	-	-
protease	-	-	+	+	+	-
TX-100	-	-	-	-	+	-

Figure 5. Assay to study posttranslational membrane integration of leader peptidase. See text for details.Translation was carried out for 28 minutes in the presence of radiolabelled (lane 1-5) or non labelled methionine (lane6) after which samples were incubated with 20 μM puromycin for 2 minutes. Either ATP and labelled methionine (lane 6) or inverted inner membrane vesicles were added and the incubation was prolonged for 15 minutes. Assembly was determined as described in Materials and methods.

Discussion

In this paper a cell-free transcription-translation system is described to synthesize leader peptidase which can be used to study membrane integration. In the present system Lep is produced in a S-135 lysate to study co- and posttranslational membrane integration. During a cotranslational incubation with wild type vesicles 20% of the synthesized Lep became integrated in the vesicles. This seems reasonable for *in vitro* systems since translocation of precursor proteins in similar systems is usually in the same range, typically 25-40 % (Chen *et al.*, 1985; de Vrije *et al.*, 1987). In a previous paper (van Klompenburg *et al.*, 1995), the native population of Lep molecules in inverted inner membrane vesicles was studied. ProteaseK treatment of inverted membrane vesicles, followed by SDS-PAGE and western blotting with Lep antibody yielded also a band of 32 kDa (van Klompenburg, *et al.*,1995). This not only corroborates the assignment of the observed 32 kDa fragment in the present study, but also shows that the *in vitro* synthesized Lep resides in the same orientation in inverted inner membrane vesicles as the native population.

On the basis of *in vivo* experiments the integration of membrane proteins is thought to follow one of two possible pathways. Proteins with large periplasmic loops, such as Lep, depend fully on the activity of the Sec machinery, while proteins with smaller periplasmic loops, such as many transporter proteins, can integrate in the inner membrane independent of the Sec machinery. However, Sec-independence is most often only operationally defined as the ability to succesfully integrate in the membranes of mutant strains in which the Sec dependent precursor translocation is impaired (Andersson and von Heijne, 1993). Only *in vitro* systems allow to firmly address this point. In our system the integration of Lep was dependent on the Sec machinery just as was observed *in vivo* . Previously, another *in vitro* system was described to study membrane integration of Lep and which was focussed on identifying segments within Lep that were required for assembly (Moore *et al.*, 1988). The *in vitro* system from literature differs in two points from the present system. Our system does not require purified AraC protein and it makes use of a S-135 lysate instead of a S-30 lysate.

Our results indicate that the integral membrane protein Lep can integrate posttranslationally in inverted membrane vesicles using an *in vitro* system. There are also *in vivo* data which suggest that the protein can insert posttranslationally in the inner membrane. Andersson and von Heijne performed *in vivo* integration experiments in which the membrane passage of P2 of Lep is blocked by addition of the uncoupler CCCP. After inactivation of this compound, translocation of the P2 domain could proceed (Andersson and von Heijne, 1994). In addition, genetically engineered membrane proteins with four membrane spanning segments inserted *in vivo* in the membrane in a fashion which was not compatible

with a simple linear N- to C integration process. Such a linear integration was expected if insertion was to happen completely cotranslationally (Gafvelin and von Heijne, 1994). It should therefore be concluded that integration of membrane proteins can occur posttranslationally. For precursor proteins it was shown that their translocation could also occur after completion of translation. For instance, 8% of the prePhoE which was synthesized in an *in vitro* system translocated into the lumen of a vesicle in a posttranslational translocation experiment while during cotranslational incubations, 25% of the synthesized material was translocated (de Vrije *et al.*, 1987). In our experiments, posttranslational membrane integration of Lep is even less efficient (3 %) when compared to cotranslational experiments in which 20% was integrated. The relative low efficiency of posttranslational integration of Lep may be explained by properties of the protein. Lep contains three hydrophobic stretches, of which two assume a transmembrane configuration. It can be envisaged that these segments have a strong tendency to aggregate. Recently it was reported that posttranslational membrane insertion of the hydrophobic membrane protein lactose permease was possible in *in vitro* systems and stimulated by the presence of the molecular chaperone GroEL (Bochkareva *et al.*, 1996).

In this paper two methods were used to study the influence of anionic lipids on integration of Lep. Vesicles from the lipid biosynthetic mutant strain HDL11 which contain less than 10 % anionic phospholipids were employed and the polycationic antibiotic polymyxin B was used to shield negative charges on vesicles with wild type lipid composition. In both experiments, the integration efficiencies were decreased in comparison to the control situation, i. e. wild type vesicles and no polymyxin. Strain HDL11 was used before in several studies aimed at the effects of reduced anionic lipid on protein translocation and colicin action. It was shown there, that in this particular strain low anionic lipid concentrations did not affect internal ATP concentration nor the membrane potential, demonstrating that the effects of anionic lipids are not caused by a de-energized membrane. Polymyxin which carries 5 positive charges, was used to study the anionic lipid dependency of precursor proteins *in vitro* (de Vrije *et al.*, 1989). It was reported that polymyxin did not affect the membrane potential nor did it cause aggregation of vesicles (de Vrije *et al.*,1989). In our studies a half maximum inhibitory effect was found at approximately 20 μM which corresponds to the presence of 1 polymyxin molecule in the assay per 4-5 anionic phospholipid molecules which is close to a charge-stoichiometric complex. It is concluded that *in vitro* membrane insertion of Lep depends on anionic lipids. This is the first example of a membrane protein without cleavable signal sequence which requires anionic phospholipids for integration.

There are at least two different ways in which anionic phospholipids could affect the integration of Lep. It was shown that the translocation ATPase activity of SecA decreased upon lowering the amount

of anionic phospholipids (Lill *et al.*,1990). This could account for hampered precursor translocation or membrane protein integration at decreased anionic lipid contents. On the other hand, also direct interactions between anionic lipids and Lep are feasible.

There are a few examples ilustrating that hampered SecA function is at least not the only factor explaining decreased translocation efficiencies at low anionic lipid levels. Experiments using chimeric proOmpF-Lpp molecules with artificial signal sequences revealed that effects of anionic phospholipids on translocation depended on the amino acid composition of the signal sequence, but not on the SecA dependency of translocation. Translocation of ProOmpF-Lpp molecules with long hydrophobic polyleucine stretches in their signal sequence was independent of anionic phospholipids but still required SecA for translocation (Phoenix *et al.*, 1993a; Phoenix *et al.*, 1993b). In addition it was shown that SecA independent integration of the M13 procoat membrane protein had a similar requirement for anionic phospholipids as a construct in which the periplasmic domain was enlarged to become SecA dependent (Kusters *et al.*, 1994). These studies demonstrate that anionic lipids could also interact directly with precursors and membrane proteins. For the M13 procoat protein it was observed that positively charged amino acyl residues in the cytoplasmic regions flanking the two hydrophobic segments were required for insertion (Gallusser and Kuhn, 1990) and the results of binding studies with lipid vesicles indicated that electrostatic interactions between these positively charged residues and anionic phospholipids are involved in initiation of insertion(Gallusser and Kuhn, 1990). It is feasible that binding of positive charges to the negative surface of the membrane facilitates the insertion of hydrophobic segments into the hydrophobic core of the membrane. Charge interactions between anionic phospholipids and positively charged amino acids can play a general role in membrane protein insertion.

Chapter 6A

Discussion

General

Lipids and proteins are important constituents of membranes. Interactions between these components and the consequences for the assembly and function of the *E.coli* inner membrane protein leader peptidase (Lep) were investigated and described in this thesis. A His-tagged version of Lep was purified and used to calibrate a quantitative assay which was employed to determine the amount of Lep molecules per cell (chapter 2). The precursor processing activity of the purified Lep was studied and it was observed that the presence of phospholipids in the assay medium stimulated the activity (chapter 2). A truncated form of Lep, corresponding to the catalytic domain, binds both to *E. coli* inner membrane vesicles and to pure lipid vesicles (chapter 3). Moreover, monolayer experiments showed a remarkable specificity of this domain for phosphatidylethanolamine (chapter 3).

Investigations into the role of anionic lipids in membrane protein assembly were described in chapters 4 and 5. In chapter 4, the possible involvement of anionic phospholipids in the positive inside rule was investigated. Constructs with an excess of positive charges in different hydrophilic loops of Lep were used. In cells with wild type lipid composition, the positively charged loops always stayed in the cytoplasm. Whereas in cells with decreased anionic phospholipid contents, the orientation of some derivatives from Lep indicated passage of positive charges (chapter 4). It was therefore concluded that anionic lipids contribute to the cytoplasmic localisation of loops with positively charged amino acids. To allow a more detailed view on the factors playing a role in membrane protein integration, an *in vitro* system was set up (chapter 5). It was found that integration of Lep depended on the Sec machinery and on the presence of anionic phospholipids (chapter 5). The results of the latter two chapters will be discussed in a separate section (6B) dealing with the role of anionic phospholipids in protein translocation and membrane protein insertion.

Expression of leader peptidase

During normal growth of wild type *E. coli* cells, no pool of unprocessed precursor proteins is observed and from pulse chase experiments it is clear that the half-life of precursor proteins is within the range of seconds (Josefsson and Randall, 1981; de Vrije *et al.*, 1988; Kusters *et al.*, 1994). Therefore it is concluded that both translocation and processing are fast and efficient processes. It is not known whether the precursor is associated with any of the Sec proteins, or how far it is in the translocation process, when cleavage occurs.

Here, we will discuss two principally different ways to achieve fast processing of translocated precursors. Lep could be permanently associated with other components of the Sec machinery, such as SecY, SecE, or SecG. Alternatively, Lep is not associated with the Sec machinery but has to find its substrate by diffusion. In the first situation Lep would be at the right place to attack precursors during their translocation and this obviates the need for a high affinity for the substrate. If such an association exists, one would expect that there are as many Lep molecules in the cell as translocation sites. Various *E. coli* strains were shown to contain approximately 10^3 Lep molecules per cell (chapter 2), which is approximately twice the number of SecY molecules in the cell (Matsuyama *et al.*, 1992), and Lep is therefore in functional excess. Moreover, Lep was never found to copurify with any of the Sec proteins (Brundage *et al.*, 1990; Matsuyama *et al.*, 1990; Akimaru *et al.*, 1991; Tokuda *et al.*, 1991; Matsuyama, Fujita, 1992), arguing against a stable association. Finally, binding experiments with the catalytic domain of Lep showed no increased binding to inner membrane vesicles as compared to pure lipid vesicles (chapter 3) which suggests that this part of Lep has no specific affinity for any of the Sec proteins. The second possibility to achieve efficient processing of translocated precursor proteins is more in agreement with the facts mentioned above. The presence of Lep molecules in the membrane which are not associated to the Sec machinery, correlates well with the efficient processing of proteins which do not require the Sec machinery for translocation. One of these proteins, M13 procoat, is even correctly processed when only Lep is reconstituted in liposomes. This at least tells that association of the precursor with the Sec machinery is not required for processing. Additional support for the view that processing of precursors can take place after their dissociation from the Sec-machinery comes from studies with leader peptidase depletion strains (Dalbey and Wickner, 1985). Due to low levels of Lep, translocated precursors accumulate before they are slowly converted into their mature forms. Apparently these translocated precursors do not jam the Sec-machinery since translocation does not seem to be very much hindered in these cells. We therefore assume that they can diffuse away from their translocation side before they get processed.

The functional excess of Lep in *E. coli* cells may also obviate the need for tight regulation. At least during normal growth, the amount of Lep per cell remained constant (chapter 2). In other organisms this can be different, as was shown for *Bacillus subtilis*. SipS is one of the chromosomally encoded signal peptidases of *B. subtilis* with homology to Lep (van Dijl *et al.*, 1992) (Fig. 1). Transcriptional fusions using β-galactosidase as a reporter indicated drastically increased expression of SipS during stationary phase (Bolhuis *et al.*, 1996). This is most likely

related to the secretion of large amounts of proteins in the growth medium during the stationary phase. When large amounts of artificial precursors were expressed in *E. coli*, processing became limiting, suggesting that the amount of Lep was not adjusted. In accordance with this, simultaneous overexpression of Lep restored processing (van Dijl *et al.*, 1991).

Despite the excess of Lep in living cells, the efficiency of processing of precursors after translocation into inverted membrane vesicles is very variable and not complete (de Vrije *et al.*, 1987). There are several possible explanations for these observations. At the first place the trans side of the translocation process, formed by the outer membrane and periplasmic space, is different in the vesicles. That this difference with the natural situation may have consequences for translocation related processes is for instance demonstrated by SecD and SecF. Both proteins have large periplasmic domains and are required for translocation in living cells and spheroplasts, but not for translocation into vesicles. It is in principle conceivable that a factor present at the periplasmic side influences the processing efficiency. Another explanation stems from the fact that Lep most likely does not form a stable complex with the other Sec proteins. Therefore it is in principle possible that Lep is not evenly distributed over the vesicles containing the Sec-machinery. It is also interesting to note that leader peptidase activity was also found in outer membrane fractions (Zwizinski *et al.*, 1981) from which the inner membrane marker NADH oxidase was absent. This could point to the suggested heterogeneity after vesicle formation. Whatever the reason for the variability is, it is clear that it will interfere with interpretation of experiments aimed at determining the influence of different conditions on processing, when isolated inverted membrane vesicles are used.

Towards a structural model for signal sequence cleavage by Lep

Data about the activity of Lep accumulated from studies *in vivo* and *in vitro*. It was concluded that the active site residues reside in the periplasmic P2 domain and that the whole H1-P1 region and part of H2 did not carry any residues involved in activity (Bilgin *et al.*, 1990). Unlike most of the known serine proteases which use a catalytic tryad of serine, histidine and aspartate residues, the only residues Lep absolutely required for catalysis were serine 90 and lysine 145 (Black, 1993; Tschantz *et al.*, 1993). The special features of the action of Lep are also demonstrated by the lack of inhibition by standard protease inhibitors (Dalbey and von Heijne, 1992). Instead, the activity of Lep could be inhibited by a β-lactam (Kuo *et al.*, 1994). This points to similarities between the active site of Lep and that of β–lactamase which also uses a serine-lysine dyad for

are more proteases described which involve a serine/base dyad and they are classified as a special clan of serine proteases (Rawlings and Barrett, 1994). Apart from prokaryotic (protease family S26) and eukaryotic (S27) signal peptidases, also LexA-like proteases (S24) belong to this group (Rawlings and Barrett, 1994). The primary sequence of the regions surrounding the active site residues of these proteins are depicted in figure 1.

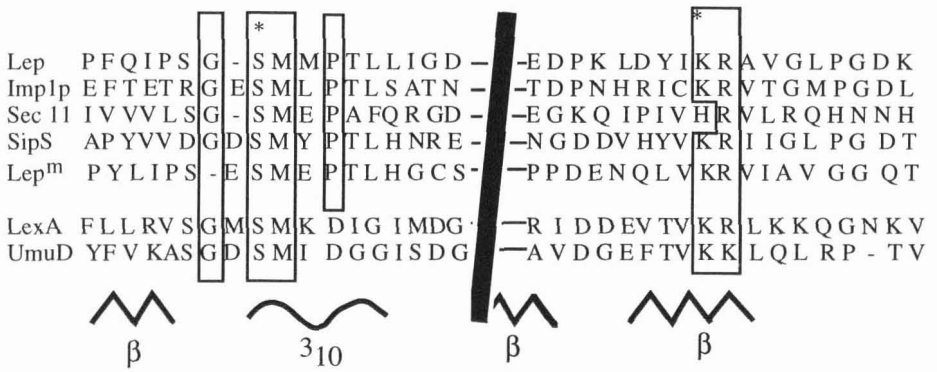


Figure 1. Sequence comparison of processing enzymes and LexA. The primary sequence of the regions around the (presumed) active site residues (indicated by asterisks) of the following enzymes are depicted: Lep = leader peptidase from *Escherichia coli,* (Wolfe *et al.*, 1983) Imp1p = inner membrane protease 1 (Behrens *et al.*, 1991) from *Saccharomyces cerevisiae*, Sec11 = Sec 11 from *S. cerevisiae* (Böhni *et al.*, 1988), SipS = signal peptidase from *Bacillus subtilis* (van Dijl *et al.*, 1992), Lep^m= leader peptidase *Mycobacterium tuberculosis* (Connor *et al.*, 1996), LexA = LexA from *E. coli* (Horii *et al.*, 1981). UmuD = UmuD from *E. coli*. Secondary structure elements (β-sheets and 3_{10} helix) as apparent from the UmuD' crystal structure are indicated under the sequence alignment. LexA and UmuD are aligned as indicated in Peat *et al.* (Peat *et al.*, 1996). Other sequences were aligned manually, using the catalytic site residues (ser 90 and lys 145 of the Lep sequence) as guide points.

Interestingly, the crystal structure of UmuD', one of the LexA like proteases was recently solved (Peat *et al.*, 1996). UmuD' adopts an unusual fold with a globular β structure. In absence of crystallographic data on Lep, it is attractive to speculate about the possible relevance of the UmuD' structure for Lep. In circular dichroism studies, Lep and Lep mutants display spectra which are indicative for high contents of β structures. (Tschantz *et al.*, 1993) The three dimensional structures of the catalytic sites of UmuD' and TEM1 β-lactamase could also be easily superimposed, which is in agreement with the observed inhibition of precursor processing by β-lactam antibiotics. The secondary structure elements surrounding the catalytic site residues of UmuD' are indicated under the alignment. However, it should be realised that there is only a weak sequence similarity between Lep and UmuD'. Moreover, the crystallized form UmuD' is the

inactive product of the autocatalytic protease action of UmuD and has most likely undergone conformational changes compared to the active form (Peat *et al.*, 1996).

The catalytic domain of Lep is much larger than UmuD' (247 versus 114 residues). It is possible that the large periplasmic domain of Lep is therefore also involved in substrate recognition, for instance by binding the mature part of precursors. Also the membrane anchors of Lep can in principle be involved in substrate recognition. The C-terminal half of H2 is somewhat conserved in all signal peptidases (Dalbey and von Heijne, 1992). Moreover, the second hydrophobic segment of Lep is a bit short to span the entire hydrophobic part of the bilayer and has approximately the same length as signal sequences. It is conceivable that the hydrophobic mismatch of H2 and the signal sequence with the lipid environment facilitates the formation of an enzyme-substrate complex.

To study the influence of lipids on the activity of Lep, a new purification protocol based on a His-tagged version of Lep was set up (chapter 2). Either zwitterionic or anionic lipids stimulated the activity of Lep towards one of its natural substrates, prePhoE. Slightly higher processing efficiencies were obtained after addition of anionic phospholipids (chapter 2). On basis of current knowledge it is not possible to distinguish between several possible reasons for the stimulation by lipids. The lipids could for instance prevent aggregation of Lep or the precursor protein. Alternatively, they could induce conformational changes in the precursor which are required for processing. It was shown that the conformation of signal sequences depended on their lipid environment (Keller *et al.*, 1992). It is also possible that lipids have their influence on the catalytic domain of Lep.

A construct (Δ2-75) corresponding to the catalytic P2 domain, lacking the membrane anchors, has been purified (Kuo *et al.*, 1993). The activity of Δ2-75 was stimulated by the presence of lipids (Tschantz *et al.*, 1995). It was observed that this construct was able to bind to lipid vesicles and monolayers (chapter 3). Monolayer experiments showed that Δ2-75 is able to penetrate between the lipids. The strongest interaction was observed with phosphatidylethanolamine (PE). It is most likely that two features of PE are important in this respect; PE is zwitterionic and it has a small headgroup. It was shown that penetration into monolayers of lipids with negatively charged headgroups was much decreased. Also with the zwitterionic lipid phosphatidylcholine, which has a more bulky headgroup, less penetration was observed. The small headgroup of PE may give Δ2-75 the opportunity to penetrate between the lipids and get involved in for instance hydrogen bonding or hydrophobic interactions. We consider

it most likely that the affinity of the catalytic domain for membranes, and especially PE, helps to position the catalytic site residues with respect to the plane of the membrane.

On basis of the current knowledge about Lep and the structure of signal peptides we propose a structural model for signal sequence cleavage which is shown in figure 2. We assumed that the hydrophobic thickness of the bilayer is about 30 Å. The primary sequence of Lep is depicted in the chapter 1. H1 of Lep comprises 22 hydrophobic residues (1-22) and assuming a linear distance of 1.5 Å between α carbons of adjacent amino acid residues, it seems likely that H1 spans the bilayer in α-helical conformation. Evidence that H1 and H2 adopt, at least partial, helical structures comes from disulphide mapping studies (Whitley *et al.*, 1993). These studies also revealed proximity of residues at the N-terminus of H1 and the C-terminus of H2 as examplified by the possibility of a disulphide bridge between positions 3 and 75. H2 seems too short to span the bilayer entirely in helical conformation since it comprises just 15 hydrophobic amino acids (residues 62-76). It is not known whether glutamate 61 resides in the hydrophobic core of the bilayer or whether the N-terminal half of H2 adopts a non-helical structure. In this thesis we provided evidence for lipid binding by the catalytic domain of Lep. Especially the hydrophobic H3 segment (residues 83-98) which carries the catalytic important serine at position 90, is likely to be involved (chapter 3). This segment is as long as the membrane spanning H2 segment and almost as hydrophobic (see figure 4, chapter 3). On basis of experiments with inverted Lep constructs it was suggested that in the absence of H2, H3 inserts into the membrane together with H1 as a helical hairpin (Nilsson *et al.*, 1993). However, H3 was not long or hydrophobic enough to permanently reside in the membrane. One point mutation immediately following H3 (D99L) was sufficient to turn H3 into a membrane spanning segment (Nilsson *et al.*,1993). Because of these and our own findings we draw H3 in our model in the bilayer but it is not known in which conformation or with what orientation. About the location of the rest of P2 not much is known but it seems likely that in the three dimensional structure, the other catalytic site residue, lysine 145 is close to serine 90.

Several investigations aimed at the elucidation of structures of signal peptides (Bruch *et al.*, 1989; Rizo *et al.*, 1993; Chupin *et al.*, 1995). The structure of signal peptides depend on their environment and this flexibility might play a role in their function. The 21 residues long signal peptide of prePhoE is used here as an example and its primary structure is given in the legend to figure 2. In membrane mimetic environments like SDS micelles, the α helix is the predominant

secondary structure element (Chupin *et al.* 1995). The N-terminal region and the hydrophobic core form a rather stable α helix up till glycine 12, after which the helix is less stable. No helix was observed beyond residue 18. It should be noted that since signal peptides are to some extent flexible structures, it is possible that interactions with Lep or translocase proteins induce structural changes we can not anticipate in our model.

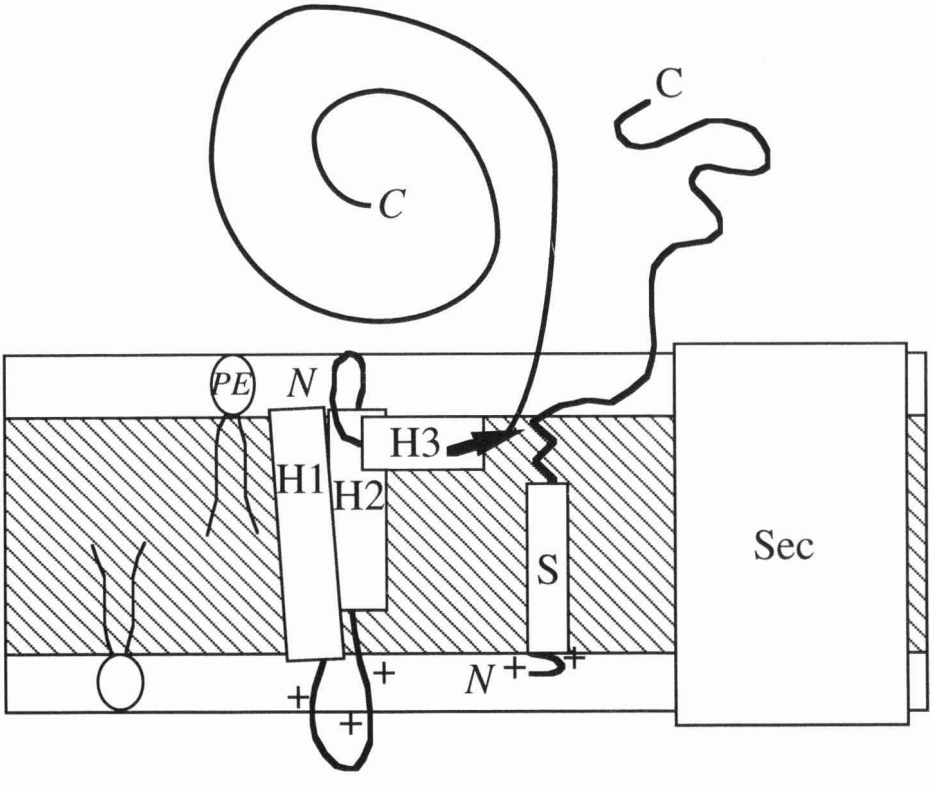


Figure 2. Structural model for the action of Lep. The presence of the hydrophobic segments of Lep (H1, H2 and H3) are indicated by white boxes. H1 comprises residues 1-22, H2 comprises residues 62-76 and H3 consists of residues 83-98. The positively charged N-terminus, the hydrophobic core and the C-terminal part of the signal sequence (S) are indicated by a line, a box and a line respectively. The arrow indicates approximately from where the active site serine in H3 can attack the cleavage site. For this model we made use of knowledge on the structure of the PhoE signal peptide (MKKSTLALVVMGIVASASVQA) with the cleavage site after the last alanine.

Also the presence of the mature part of the protein could in principle influence the conformation, but it was at least shown that also in the context of the precursor, the signal sequence has specific interactions with lipids in model membranes (Keller *et al.*, 1996; van Raalte *et al.*, 1996). In the

model proposed here, the first three residues of the signal sequence (MKK) are depicted in the headgroup region of the cytoplasmic half of the bilayer. Assuming a helical conformation until residue 18, the helix spans about two-third of the hydrophobic core of the bilayer. Even if the last three residues are in completely extended conformation, the cleavage site just about reaches the headgroup region of the periplasmic site. This means that if the precursor indeed assumes a transmembrane orientation before cleavage, processing should at least happen very close to or maybe even within the membrane.

Such a transmembrane orientation is likely because of at least two independent observations. When precursors are not processed, either because of alterations in the signal sequences or because of limited amounts of Lep, they remain bound to the membrane via their signal sequences (Koshland and Botstein, 1982; Dalbey and Wickner, 1985). When proteins were fused to the N-terminus of signal sequences, this did not interfere with processing, while the new extended N-terminus stayed in the cytosol during the translocation process (Kuhn, 1987). These observations make it very unlikely that the signal sequence leaves the membrane before processing occurs. Therefore it is concluded that processing indeed has to happen close to the membrane.

The presence of a hydrophobic segment within the catalytic domain of Lep is one of the ways to bring the catalytic site close to the membrane. Experiments performed *in vivo* indicated that signal sequences with very long hydrophobic cores were not cleaved. According to our model this probably means that the recognition site for Lep is moving out of the membrane. This model and the involvement of H3 may be special for processing by Lep from *E. coli*. While it is evident that processing requires proximity of the signal sequence cleavage site and the active site of the processing enzyme, it seems likely that different organisms employ different mechanisms to achieve this. While Lep from *E. coli* has a hydrophobic segment near its catalytic centre, in other organisms the active site of the processing enzyme can be closer to the end of a transmembrane helix or alternatively, the length of the signal sequence may be increased. In the primary sequence of Lep from *Mycobacterium tuberculosis* the active site serine is closer to the end of the transmembrane segment (Fig. 1) and this might in this case help to bring the active site closer to the membrane. The alternative strategy may be followed by SipS. Increasing the length of the hydrophobic core of the precursor of TEM-β-lactamase completely inhibited processing by Lep in *E.coli* while translocation was not affected, but when SipS from *B. subtilis* was expressed in these cells, processing was restored. This indicates that SipS can attack cleavage sites which are out of

reach of the *E. coli* enzyme. With respect to the model for *E. coli* Lep, it is notable that SipS does not contain a hydrophobic H3 segment and that signal sequences in *B. subtilis* are in general longer than in *E. coli*. (von Heijne and Abrahmsen, 1989).

Chapter 6B

The role of anionic phospholipids in protein insertion and translocation in bacterial membranes

based on publication (VII)

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. 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 outer membrane which 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 and 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 *pgsA* 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 *pssA* gene is disrupted, no PE is synthesized and the inner membrane contains only anionic phospholipids (DeChavigny *et al.*, 1991). However, the cells now require divalent cations and grow slowly .(DeChavigny *et al.*, 1991; Rietveld *et al.*, 1993) 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 table I.

Table I. Lipid composition of wild type and mutant *E. coli* cells. The lipid composition of the inner membranes of SD12 (de Vrije, 1989), 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 $MgCl_2$.

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

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 which have to function outside the cytosol. For overviews on this so called Sec-machinery the reader is referred to (Arkowitz and 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 and Beckwith, 1985; Kusters *et al.*, 1989; Randall *et al.*, 1990; Kumamoto, 1991) which also plays a role in targeting (de Cock and 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 and Beckwith, 1982; Hartl *et al.*, 1990; Economou and 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 and 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 and 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 and Beckwith, 1982). Apart from the anionic lipids which play a special role in translocation and which form the topic of this review, also several other aspects of the lipid bilayer should be mentioned here. It was stated above that the lipids from *E. coli* are organized in a dynamical liquid-crystalline bilayer. But also substantial and regulated amounts of non-bilayer lipids are present. These lipids are essential for survival of cells and required for dynamic processes such as protein translocation (Rietveld *et al.*, 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 and Izui, 1976; Ito *et al.*, 1977; Pages *et al.*, 1978; DiRienzo and Inouye, 1979).

It was recently established that many of the proteinaceous components involved in translocation in prokaryotes and eukaryotes are conserved (Ng and 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 *et al.*, 1991). Apparently, it is only the negative charge which 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 basis of vesicle aggregation studies and deletion mutagenesis that SecA contains two distinct lipid binding sites (Breukink *et al.*, 1993; Breukink *et al.*, 1995). Breukink *et al.* observed very efficient penetration of SecA into lipid monolayers made from anionic lipids but not into monolayers of zwitterionic lipids (Breukink *et al.*, 1992). Binding of SecA to vesicles, as determined by fluorescence quenching studies, was also stimulated by the presence of anionic lipids (Ulbrandt *et al.*, 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, London, 1992). It was proposed on basis of experiments employing monolayers of anionic phospholipids, that SecA undergoes a nucleotide dependent membrane insertion-deinsertion cycle (Breukink, Keller, 1993). Binding of non- hydrolyzable ATP analogs 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 *et al.*, 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 *et al.*, 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 *et al.*, 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 ten fold 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 *et al.*, 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 and Wickner, 1991). On 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 which 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 and Wickner, 1994). Upon ATP hydrolysis SecA de-inserts and the precursor is released from SecA. Than, the cycle can start again to move the next piece of the precursor across. It was shown by Lill *et al.* that the ATP-ase activity of SecA was stimulated by anionic phospholipids (Lill *et al.*, 1990).

It is therefore concluded that interactions between 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 required 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 did neither depend on the presence of positive charges at the N-terminus nor on anionic lipids in the membrane (Phoenix *et al.*, 1993a). 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.*,1993a). Strikingly, translocation of all these precursors with positive charges at the N-terminus required SecA (Hikita and 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.*, 1993b).

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 37^0C, but it was found that their growth was arrested at 30^0C (Nishiyama *et al.*, 1994). This arrest could be alleviated by extra synthesis of anionic lipids (Kontinen and Tokuda, 1995). It is unclear whether this is 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 and 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 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 figure 1.

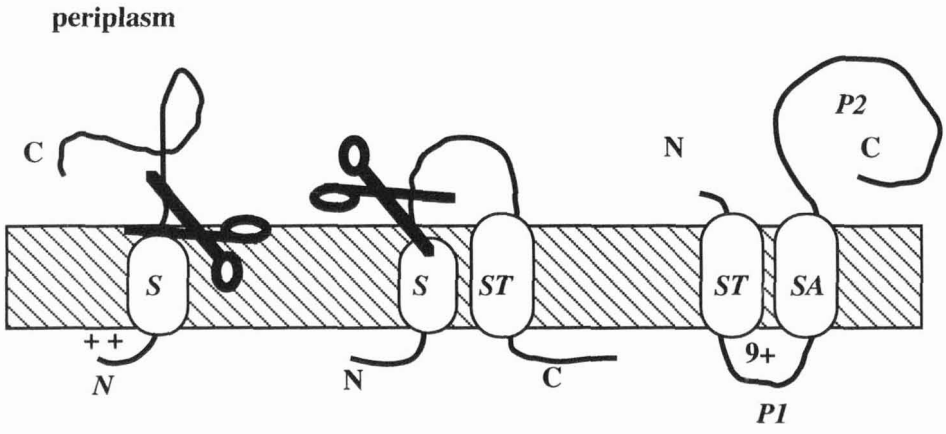


Figure 1. Schematic representation of the similarities between precursors and membrane proteins. Uncleaved forms of a secretory precursor protein (left) and M13 procoat (middle) are drawn. The cleavage site directly behind the signal sequence (S) is indicated by scissors. Presence of a stopt transfer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

Membrane integration of proteins can follow two pathways depending on the lengths of the hydrophilic segments which have to pass the membrane. It was shown *in vivo* that segments longer than approximately 60 amino acids depend on the function of the Sec- machinery including SecA while shorter loops do not require this (von Heijne, 1989; Lee *et al.*, 1992; Andersson and 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 *et al.*, 1988; Lee *et al.*, 1992; chapter 5). 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 (chapter 5).

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 *et al.*, 1981; Watts *et al.*, 1981; Watts *et al.*, 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 and Kuhn, 1990). Moreover, while wild-type procoat bound very efficiently to anionic lipid vesicles, the negatively charged mutants did hardly show any binding (Gallusser and Kuhn, 1990). This strongly suggest 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 and 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 possiblity very recent experiments combined the availability of the lipid biosynthetic mutant strain HDL11 and constructs derived from Lep with various charge distributions (chapter 4). 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 (chapter 4). This is the most direct proof thusfar 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 and 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. 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 and Vonheijne, 1994). It was not possible to translocate more positive charges across the membrane, regardless of 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 and 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).

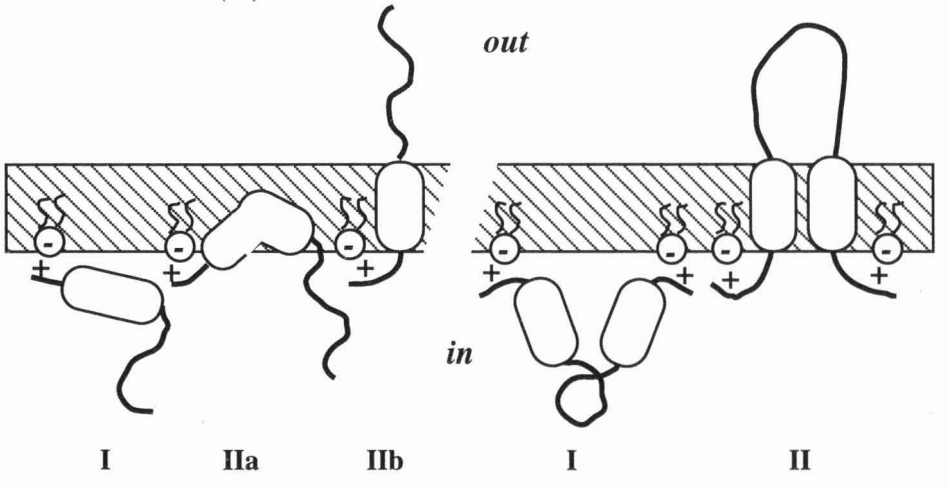


Figure 2. Model for the interactions between anionic lipids and precursor and membrane proteins in initial stages of translocation and insertion. For detailed description: see text.

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 *et al.*, 1996a; Nouwen *et al.*, 1996b). 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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Samenvatting

Inleiding

Alle levende wezens zijn opgebouwd uit cellen. Ieder mens bestaat uit zo'n honderdduizendmiljard van deze microscopisch kleine bouwsteentjes. Andere organismen, zoals bijvoorbeeld de darmbacterie *Escherichia coli* bestaan uit slechts één enkele cel. Ondanks de enorme verscheidenheid aan organismen op aarde, vallen op het niveau van de cel vooral de overeenkomsten op. Daarom worden veelal gemakkelijk te kweken eencellige organismen zoals bacteriën onderzocht om meer over onze eigen cellen te weten komen.

Een cel bestaat op zijn minst uit cytoplasma (celsap), waarin onder andere eiwitten en erfelijk materiaal ronddrijven, en een vlies (membraan) dat het cytoplasma van haar omgeving afscheidt en haar in staat stelt om haar specifieke samenstelling te behouden. Veel cellen, zoals bijvoorbeeld die van planten en dieren, bevatten niet alleen een celmembraan, maar ook membranen die interne compartimenten omgeven waar speciale biochemische reacties plaatsvinden. Veel processen waarbij de ene vorm van energie moet worden omgezet in een andere, vinden aan de membranen van zulke compartimenten plaats. Beroemd in dit opzicht zijn de membranen van de bladgroenkorrels in de plantecel waar de omzetting van lichtenergie in chemische energie gebeurt.

Membranen bevatten voornamelijk twee soorten molekulen; lipiden en membraaneiwitten. Lipiden zijn opgebouwd uit een hydrofiele (= waterminnende) kopgroep en twee hydrofobe (= waterafstotende) staarten. In de membraan zijn lipiden georganiseerd in een dubbellaag met de staarten in het midden van de membraan en de kopgroepen aan de buitenkant. Zo'n lipide dubbellaag is een zeer sterke barrière voor stoffen die in water oplosbaar zijn. Daarom moeten er om gecontroleerd transport van bijvoorbeeld voedingsstoffen mogelijk te maken ook eiwitten aanwezig zijn. Sommige eiwitten zitten helemaal in de membraan ingebed, terwijl anderen zich in het grensvlak van membraan en waterige omgeving bevinden.

De darmbacterie *E. coli* is omgeven door een envelop die bestaat uit twee membranen en een tussenliggende ruimte, het periplasma (hoofdstuk 1, figuur 1). Alle eiwitten van de cel worden in het cytoplasma gemaakt. Eiwitten die dus hun functie buiten het cytoplasma hebben, moeten over minstens een membraan getransporteerd worden. Om deze eiwitten (precursors) te kunnen onderscheiden van eiwitten die niet getransporteerd hoeven te worden, krijgen ze bij hun productie een extra stukje eiwit (signaal sequentie) mee, dat als adreslabel dient. Na het

membraantransport wordt dit adreslabel weer verwijderd. Dit wordt door het integrale membraaneiwit leader peptidase (Lep) gedaan. In alle levende organismen die we kennen, komen Lep-achtige eiwitten voor, wat nog eens de overeenkomst tussen de verschillende celtypen weergeeft. In dit proefschrift werd in hoofdstuk 2 en 3 gekeken naar interacties tussen Lep en lipiden en het belang daarvan voor het functioneren van Lep. Lep is een integraal membraaneiwit met een groot domein in het periplasma (hoofdstuk1, figuur 7), dat dus ook de membraan moet passeren. In hoofdstuk 4 en 5 werd daarom gekeken naar de inbouw van Lep in de membraan en de rol die speciale lipiden in dit proces kunnen spelen.

Praktische hoofdstukken en discussie

Om de activiteit van Lep goed te kunnen bestuderen, werd het eerst gezuiverd. In hoofdstuk 2 wordt beschreven hoe aan één uiteinde van Lep eerst een zogenaamde histidine vlag werd gezet waarmee Lep aan koper of nikkel-ionen kan binden. Vervolgens werd dit Lep gezuiverd middels zogenaamde metaal affiniteits chromatografie. Het zuivere Lep werd in verschillende experimenten gebruikt. Zo werd gevonden dat dit Lep ook in zuivere vorm in staat is om signaalsequenties van precursors te verwijderen. Deze activiteit werd gestimuleerd door de aanwezigheid van lipiden. Uit eerdere experimenten met gezuiverd Lep bleek dat één Lep molecuul zo'n vijftig signaalsequenties per minuut kon verwijderen. Met het gezuiverde Lep kon ook worden bepaald dat er ongeveer duizend Lep moleculen in één *E. coli* cel aanwezig zijn. Per cel moeten ongeveer tienduizend signaalsequenties per minuut worden verwijderd en blijkbaar is Lep dus geen beperkende factor voor het transport van eiwitten over de binnenmembraan.

Het periplasmatische domein van Lep (P2, figuur 1 hoofdstuk 3) bevat dat deel van het eiwit dat verantwoordelijk is voor de afsplitsing van signaalsequenties (het zogenaamde catalytisch centrum) en wordt voorafgegaan door twee membraanankers (H1 en H2). Ook zonder deze ankers is Lep actief en ook de activiteit van het ankerloze Lep wordt gestimuleerd door lipiden. Daarom werd in hoofdstuk 3 onderzocht of P2 ook zonder ankers aan membranen kon hechten. Dat bleek zo te zijn en bovendien werd gevonden dat de lipiden in de membraan hiervoor verantwoordelijk waren. P2 bond met name sterk aan het veel voorkomende lipide phosphatidylethanolamine. Eén van de belangrijkste conclusies was dat P2 niet alleen kon binden aan lipiden maar ze zelfs een beetje opzij kon drukken. Zodoende komt het catalytisch centrum heel dichtbij, of mischien wel in de membraan te liggen. Dit lijkt voor het mechanisme van Lep

zeker van belang. Signaal sequenties voelen zich door hun chemische aard heel erg thuis in de membraan en zullen zich voor het afknippen grotendeels daar bevinden. Het lijkt dus voor Lep noodzakelijk om zich naar de membraan toe te buigen om de signaalsequentie-knipplaats te kunnen vinden. Dit werd in een model gepresenteerd in hoofdstuk 6A, en schematisch weergegeven in figuur 2 van dat hoofdstuk.

Lep is niet alleen zelf een interessant eiwit, maar wordt bovendien vaak gebruikt als model voor andere membraaneiwitten. De orientatie van eiwitten in de membraan is belangrijk voor hun functie. Een membraaneiwit dat ondersteboven in de membraan zit kan over het algemeen zijn werk niet doen. Daarom wilden wij weten welke factoren de oriëntatie bepalen. Een belangrijke rol lijkt weggelegd voor electrische ladingen. Van membraaneiwitten is bekend dat die delen die over de membraan heen moeten (dus aan de buitenkant van de cel, of in het periplasma zitten), verarmd zijn in positieve ladingen. Terwijl dat niet geldt voor die delen die in het cytoplasma blijven. De vraag is dus eigenlijk: Wat houdt die positieve ladingen tegen? In hoofdstuk 4 werd gekeken naar lipiden met negatieve ladingen. Het bleek dat wanneer het gehalte aan negatief geladen lipiden van 25% tot 10% werd teruggebracht, positieve ladingen makkelijker de membraan konden oversteken. In dit onderzoek werd gebruik gemaakt van Lep constructen met verschillende verdelingen van de positieve ladingen. Sommige constructen konden onder gegeven omstandigheden dan ook op de kop in de membraan worden aangetroffen. De belangrijkste conclusie uit dit hoofdstuk was dat negatief geladen lipiden meehelpen aan het bepalen van de oriëntatie van membraaneiwitten.

Om goed na te kunnen gaan welke factoren betrokken zijn bij het inbouwen van eiwitten in de membraan, werd in het laatste praktische hoofdstuk (hoofdstuk 5) van de cel een bouwpakket gemaakt. Van de binnen membraan van *E. coli*werden omgekeerde blaasjes (inverted vesicles) geperst met de cytoplasmatische kant naar buiten wijzend en de periplasmatische zijde naar binnen. Aan de buitenkant konden dan DNA dat voor Lep codeert, en bepaalde cytosolische componenten worden toegevoegd. Met dit systeem kon specifiek Lep gemaakt worden en dit Lep bleek geschikt om in de inverted vesicles te worden ingebouwd. Zoals verwacht kwam het periplasmatische (P2) domein dan in het binnenste van de vesicles terecht (hoofdstuk 5, figuur 2). Door verschillende soorten vesicles te gebruiken en door verschillende componenten toe te voegen werd de inbouw gekarakteriseerd. Inbouw kon plaats vinden nadat de synthese gestopt

was, hetgeen duidelijk maakt dat membraan eiwit productie en inbouw niet gelijktijdig plaats hoeven vinden. Verder bleek inbouw afhankelijk te zijn van een complexe eiwitmachinerie (Sec machinerie) die ook meehelpt bij het membraan transport van precursor eiwitten. Bovendien bleek de inbouw afhankelijk van het negatief geladen lipiden gehalte. Verlaging van dit gehalte of afscherming van de negatieve lading door een positief geladen antibioticum ging efficiënte inbouw tegen. De overeenkomsten die hier gevonden werden tussen membraan transport van precursors en de inbouw van membraaneiwitten, kwamen ook weer naar voren in de discussie in hoofdstuk 6B. In dit hoofdstuk worden de structurele overeenkomsten tussen membraaneiwitten en precursoreiwitten op een rij gezet en gekoppeld aan de negatief geladen lipiden afhankelijkheid van membraan inbouw en transport. Hier werd een model gepresenteerd voor de werking van negatief geladen lipiden in beide processen (figuur 2). Dit model verklaart hoe negatief geladen lipiden zowel de inbouw en oriëntatie van membraaneiwitten als het membraantransport van precursors kunnen beinvloeden.

Nawoord

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Curriculum vitae

De schrijver van dit proefschrift werd op 8 mei 1968 iets voor middernacht te Zutphen geboren. In deze plaats in het oosten van het land werd aan het Baudartius college HAVO en VWO doorlopen, zodat in september 1987 kon worden begonnen met een universitaire studie Biologie aan de Universiteit Utrecht. In september 1992 werd de studie afgesloten met het doctoraalexamen met als specialisaties het bijvak biochemie (Prof. de Kruijff) en het hoofdvak moleculaire genetica (Prof. Weisbeek). Op 1 oktober 1992 begon een vierjarige aanstelling als Assistent In Opleiding bij de vakgroep Biochemie van Membranen van de faculteit Scheikunde aan de Universiteit Utrecht onder supervisie van Prof. Dr. B. de Kruijff. Het eerste jaar van het onderzoek werd verricht onder supervisie van Prof. von Heijne, destijds verbonden aan het Karolinska Instituut in Stockholm, Zweden. Vanaf 1 maart 1997 werkt de schrijver van dit proefschrift binnen het kader van een vierjarige post-doc aanstelling die gefinancierd wordt door de Nederlandse Kanker Bestrijding, aan membraaneiwitten die betrokken zijn bij multidrug resistentie. Dit vindt plaats aan de Rijksuniversiteit Groningen bij de vakgroep Moleculaire Microbiolobie van Prof. Konings.

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