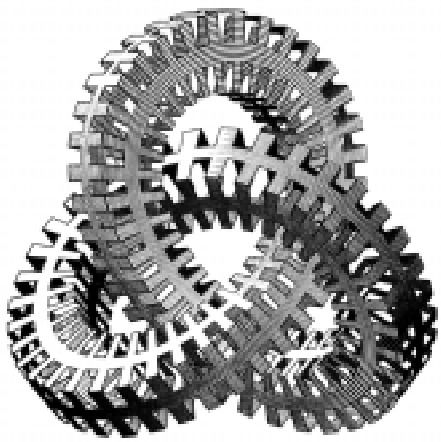
Folding and gating of the outer membrane porin PhoE of *Escherichia coli*



Folding and gating of the outer membrane porin PhoE of *Escherichia coli*

Vouwing en mechanisme van sluiten van de buiten-membraan porie PhoE van *Escherichia coli*

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. H.O. Voorma
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op woensdag 22 september 1999 des namiddags om 14.30 uur
door

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geboren op 27 mei 1970
te Groningen

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This research was supported by the Netherlands Organization for Scientific Research (NWO), sector Earth- and Life Sciences (ALW).

Vormgeving en lay-out
Grafico Delft

Druk
Grafico Delft

Omslagfiguur
M.C. Escher's "Knopen"

‘De natuur kent het grote geheim en glimlacht’
Victor Hugo

aan mijn ouders
en Frans

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CHAPTER 1

General introduction

General introduction

Architecture of the *Escherichia coli* cell envelope

The cell envelope of Gram-negative bacteria, such as *Escherichia coli*, consists of a double membrane (Fig. 1). Due to this architecture, the cell can maintain a microenvironment essential for cell viability. Whereas the physiological conditions inside and outside of the cell differ substantially, the membranes provide a suitable protection to the bacterium against fluctuations in the surrounding environment.

OUTER MEMBRANE. The outer membrane (OM) is a highly asymmetrical bilayer composed of phospholipids in the inner leaflet and lipopolysaccharides (LPSs) in the outer leaflet (Lugtenberg and van Alphen, 1983). Both are amphipathic molecules, the latter consisting of three covalently linked domains: lipid A, the core oligosaccharide and the O-antigen (Raetz, 1990). Lipid A anchors LPS in the OM and consists of a backbone of a glucosamine dimer to which five to seven saturated fatty acid chains are linked. The core oligosaccharide is composed of 6 to 10 sugars, whereas the O-antigen is a repeating oligosaccharide of 1 to 40 repeats, each consisting of 3 to 6 sugars. The O-antigen displays a great variability in composition among bacterial

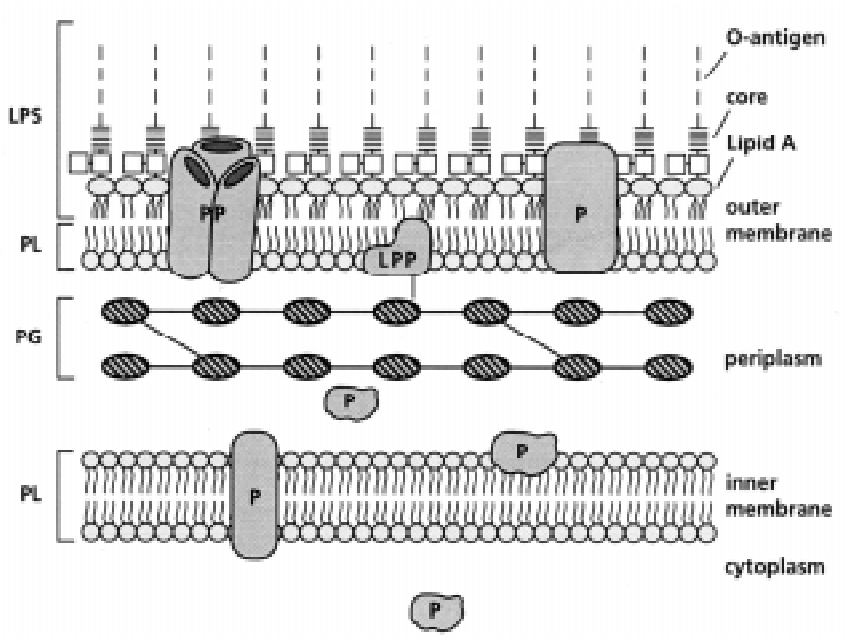

Fig. 1. Architecture of the cell envelope of Gram-negative bacteria, such as *Escherichia coli*. The cytoplasm is surrounded by the inner membrane, the periplasm and the outer membrane. The IM consists of phospholipids (PL) and proteins (P). The OM consists of LPS, PL, lipoprotein (LPP) and proteins. The most abundant proteins are the trimeric pore proteins (PP) and OmpA. LPS consists of three moieties, lipid A, the core and the O-antigen. The periplasm is filled with a highly hydrated peptidoglycan matrix (PG) and contains several different proteins.

species. The anionic head groups of core and lipid A have a strong affinity for divalent cations thereby forming an impermeable network, which acts as a barrier for the diffusion of both hydrophilic and hydrophobic molecules (Nikaido and Vaara, 1985). This lipidic barrier contains outer membrane proteins (OMPs) essential for the passage of hydrophilic solutes. The OM is surrounded in many Gram-negative bacteria by a capsular polysaccharide, which functions as an extra protection, for instance against stress conditions (Gottesman and Stout, 1991).

INNER MEMBRANE. The inner membrane (IM) is composed of phospholipids and membrane proteins. The latter can either be integral or peripherally associated with the membrane and function, amongst others, in regulating the flow of nutrients and metabolic products in and out of the cell. The

phospholipid composition of the IM and OM are similar, *i.e.* phosphatidylethanolamine (PE; 70-80%), phosphatidylglycerol (PG; 15-20%) and cardiolipin (CL; 5%), although an enrichment of PE in the OM has been reported (Lugtenberg and Peters, 1976).

PERIPLASM. The periplasm, sandwiched between IM and OM, is filled with a highly hydrated polymer, the peptidoglycan (Hobot *et al.*, 1984), which reduces the diffusional mobility of periplasmic proteins (Brass *et al.*, 1986). This peptidoglycan is essential for the cell to maintain its rigidity and shape and protects it from osmotic lysis. The glycan chains are composed of a disaccharide of N-acetylglucosamine and N-acetylmuramic acid, which are connected to each other via oligopeptides. The periplasm contains a variety of proteins involved in nutrient accumulation, detoxification, catabolism and protein targeting and folding.

Outer membrane proteins

The OM contains numerous proteins with a variety of functions. Most of these proteins form channels allowing the passage of nutrients across the OM. Three different types of channel-forming proteins involved in nutrient uptake, *i.e.* the general diffusion porins, the solute-specific porins and the receptors, can be distinguished each with specific characteristics. Some of these proteins are constitutively expressed while the synthesis of others is induced under certain growth conditions.

GENERAL DIFFUSION PORINS. The general diffusion porins allow for the uptake of hydrophilic molecules with molecular weights up to 600 Da and lack specific substrate-binding sites. In *E. coli*, OmpF, OmpC and PhoE, which show a high degree of sequence homology, belong to this type of proteins (Benz and Bauer, 1988; Nikaido, 1992). The production of the cation-selective porins OmpF and OmpC is controlled by environmental conditions. OmpC is preferentially expressed at higher temperature and osmolarity, while OmpF is expressed at lower temperature and osmolarity (van Alphen and Lugtenberg, 1977). OmpR and EnvZ act as activator and sensor proteins, respectively, of a two-component regulatory system controlling expression of these porins (Igo *et al.*, 1989). Under conditions of phosphate starvation, the synthesis of the anion-selective porin PhoE is induced (Overbeeke and Lugtenberg 1980). This porin allows for the preferential uptake of negatively charged solutes, including phosphate (Benz *et al.*, 1985). Expression of this

porin is under control of the PhoB/PhoR two-component regulatory system (Makino *et al.*, 1988).

SPECIFIC CHANNELS. Specific channels function as general diffusion pores as well, but they can be distinguished from this type of pores by the presence of a substrate-binding site (Nikaido, 1992). An example of such a specific pore-forming protein is LamB, which contains a binding site for maltodextrins and facilitates maltose- and maltodextrin-uptake at low sugar concentrations (Benz *et al.*, 1986; Klein and Boos, 1993). These solute-specific porins show no sequence similarity to the general diffusion porins but share some structural features with them (see next paragraph).

RECEPTORS. The OM also contains proteins that contain a high-affinity binding site for their ligands and are therefore called receptors. These receptors function as gated channels. This energy-costing process requires IM energy, which is transduced to the receptors via TonB and accessory proteins. The expression of these specific channels is very often induced under special growth conditions together with an IM uptake system for the ligand and a periplasmic substrate-binding protein. Examples of such receptors are FhuA and FepA, required for the uptake of the siderophores ferrichrome and ferric enterobactin, respectively (Postle, 1990).

LIPOPROTEINS/OmpA. In addition to proteins involved in nutrient uptake, the OM contains several other proteins, including lipoproteins. Lipoproteins are lipid-modified proteins, which are anchored via their lipid moiety, either in the IM or in the OM. Braun's lipoprotein (Lpp) is located in the OM and about one third of the molecules are covalently associated to the peptidoglycan, thus fixing the membrane to the underlying peptidoglycan. Lpp together with the major outer membrane protein OmpA are required for the structural stability of the membrane and for maintenance of the cell's morphology (Sonntag *et al.*, 1978). Besides its structural role, OmpA might form diffusion pores in the OM (Sugawara and Nikaido, 1992 and 1994; Saint *et al.*, 1993). However, its ionophore properties have not yet been established *in vivo* and are disputed on the basis of its resolved 3D-structure (Pautsch and Schulz, 1998).

OMPs INVOLVED IN PROTEIN SECRETION. The export of proteins across the OM requires the presence of large pores usually formed by multimeric proteins, *e.g.* TolC, which is involved in haemolysin secretion (Wandersman and Delepaire, 1990; Koronakis *et al.*, 1997), PapC involved in export of pilus components (Norgren *et al.*, 1987; Thanassi *et al.*, 1998) and the

secretins PulD and XcpQ, which are involved in the secretion of pullulanase by *Klebsiella oxytoca* and of many different proteins in *Pseudomonas aeruginosa*, respectively (d'Enfert *et al.*, 1989; Bitter *et al.*, 1998).

OTHER OMPs. The OM also contains a few proteins, the function of which is largely unknown. Among them are OmpX (Mecsas *et al.*, 1995), a phospholipase (OMPLA; Scandella and Kornberg, 1971) and the proteases OmpT (Grodberg and Dunn, 1988; Stumpe *et al.*, 1998) and OmpP (Kaufmann *et al.*, 1994).

Structure of outer membrane proteins

For integral membrane proteins, OMPs have a very special structure. Their amino acid compositions are even more polar than those of water-soluble proteins, and they contain predominantly β-structure, in contrast to the α-helical structures of most other integral membrane proteins. The first structure of a porin resolved, that of *Rhodobacter capsulatus* porin (Weiss *et al.*, 1991), revealed a construction principle that is likely to be valid for all others (Schulz, 1996). The presence of a β-barrel consisting of antiparallel β-strands was subsequently confirmed by the resolution of the crystal structures of *Rhodopseudomonas blastica* porin (Kreusch *et al.*, 1994), the *E. coli* porins OmpF and PhoE (Cowan *et al.*, 1992) and the porin from *Paracoccus denitrificans* (Hirsch *et al.*, 1997). These porins are homotrimers of intimately associated monomers each producing a channel (Fig. 2). The monomer spans the outer membrane by 16 antiparallel amphipathic β-strands in which all strands are hydrogen-bonded to their next neighbors along the chain. The strands are tilted by 30 to 60° relative to the trimer axis. The hydrophobic residues in the strands face outward to the hydrophobic core of the membrane or toward the subunit interface, while the hydrophilic residues face the pore interior.

LOOP L3. The strands are connected via short turns on the periplasmic side and longer loops on the surface-exposed side. One of these loops, L3, folds into the β-barrel forming a constriction or "eyelet", which determines the diffusion properties of the porin. The position of L3 is determined by interactions between the loop and the barrel wall via a hydrogen-bonding network between the tip of L3 and adjacent residues in the barrel wall and via salt bridges at the root of L3 (Karshikoff *et al.*, 1994). This loop is characterized by the presence of a short α-helical segment and a highly

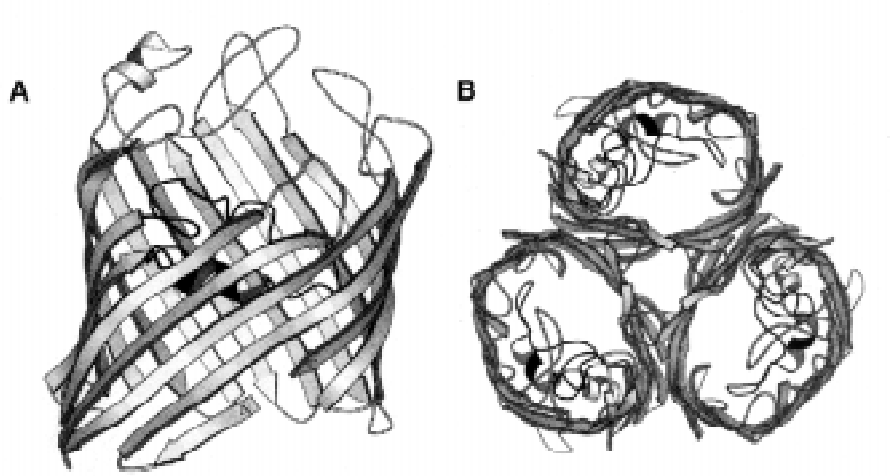

Fig. 2. Structure of *E. coli* porins. (A) General fold of the PhoE monomer (Cowan *et al.*, 1992). The large hollow β-barrel structure is formed by the antiparallel arrangement of 16 β-strands. The strands are connected by short regular turns on the periplasmic side (bottom), whereas long irregular loops face the cell exterior (top). The N- and C-terminus are connected via a salt-bridge. (B) Schematic representation of the PhoE trimer. The view is from the extracellular space along the molecular threefold symmetry axis. The internal loop, which connects β-strands 5 and 6 and constricts the pore in the barrel, is loop L3 (Schirmer, 1998).

conserved PEF(G/V)G motif at the tip of the third loop (Fig. 3) (Jeanteur *et al.*, 1991). Folding of the α-helix is suggested to occur early in the assembly process and is considered to be essential for stabilizing the β-barrel (Van Gelder *et al.*, 1997b). The PEF(G/V)G sequence is conserved in enterobacterial and neisserial porins but not in those of *R. capsulatus* and *R. blastica*. While the Pro residue initiates the turn, the Glu and Phe residues are involved in interactions with the barrel wall (Cowan *et al.*, 1992; Karshikoff *et al.*, 1994). The glycine residues are probably important because of their high flexibility, which may be essential for optimal pore functioning (Van Gelder *et al.*, 1997b). A strong traverse electrical field exists at the constriction zone produced by the presence of basic residues (mainly arginines) on the barrel wall facing negatively charged residues on L3. This electrical field might facilitate the dehydration of polar solute molecules that traverse the channel and may be important for the expulsion of hydrophobic molecules (Karshikoff *et al.*, 1994). Charged residues present in the constriction zone and at the

extracellular mouth determine the ion-selectivity of the general porins. The anion-selective PhoE porin contains in the constriction zone two additional positively charged residues as compared with the cation-selective porins OmpF and OmpC. One of these residues, Lys125, plays a dominant role in the selectivity as was demonstrated after mutagenesis (Bauer *et al.*, 1988).

SUBUNIT INTERFACE. The trimer is stabilized by both hydrophobic and hydrophilic interactions between the subunits. The subunit interface is build up by a structure of alternating bulky and small side chains that form together a very rigid hydrophobic core (Schulz, 1992). The hydrophilic interactions mainly involve loop L2, which reaches into the pore of a neighboring monomer where it extensively makes hydrogen bonds and a few salt bridges with barrel wall residues and residues of loop L3. Besides forming a salt bridge, Glu66 at the tip of L2 is also part of a H-bonding network with Arg93, Asp120 and Arg126 in the adjacent monomer (Karshikoff *et al.*, 1994; Phale *et al.*, 1998). These interactions proved to be important for the stability of the trimer as indicated by site-directed mutagenesis. Replacement of Glu66 by an Arg weakened the subunit interactions in the PhoE trimer resulting in

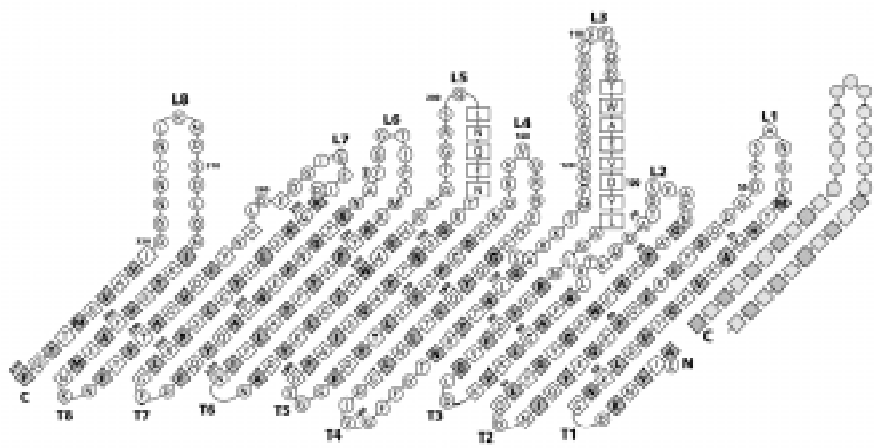

Fig. 3. Topology of the PhoE monomer with the amino acid sequence in the one-letter code. Shaded and open diamonds indicate β-strand residues with their side chains directing toward the exterior or to the interior of the β-barrel, respectively. Secondary structure assignments are diamonds for β-strands, rectangles for α-helices and circles for turns and loops. The loops at the cell surface are denoted L1 to L8, and the turns at the periplasmic side T1 to T8. The connection between N- and C-terminus via a salt-bridge is indicated.

dissociation of trimers into folded monomers (Van Gelder *et al.*, 1996). These tight interactions between the monomers explain the extreme heat-stability of the trimers and their resistance to high concentrations of detergents and chaotropic agents. At the subunit interface of the *E. coli* porins, the N- and C-terminus are linked together by an intrasubunit salt-bridge (Fig. 3). The termini of the *R. capsulatus* and *P. denitrificans* porins do not form intra- but inter-subunit salt-bridges (Weiss *et al.*, 1991).

INTERACTION WITH LIPIDS. The trimer is tightly associated with the lipid bilayer. Two rings of aromatic residues at the water-lipid interface might protect the trimers from damage during mechanical movements in the membrane, whereby the protein can be shielded by the rotation of the aromatic side chains (Schulz, 1992). Furthermore, ionogenic groups on the surface-exposed and the periplasmic side interact with the lipid A/core moieties of LPS and with the headgroups of the phospholipids, respectively.

3D-STRUCTURE OF OTHER OMPs. Recently, the structures of several other OMPs have been solved. All share the common β-stranded barrel motif but with a varying number of β-strands. In the case of the monomeric siderophore receptors FhuA (Locher *et al.*, 1998) and FepA (Buchanan *et al.*, 1999), 22 β-strands traverse the OM and the channel is sealed off from the periplasmic side by a plug formed by an N-terminal domain. The N-terminal half of the monomeric OmpA protein forms an eight-stranded antiparallel β-barrel (Pautsch and Schulz, 1998), whereas the C-terminal domain is located in the periplasm. The structures of the specific channel-forming protein LamB of *E. coli* (Schirmer *et al.*, 1995) and of *Salmonella typhimurium* (Meyer *et al.*, 1997) and of the sucrose-specific porin SrcY (Forst *et al.*, 1998) revealed 18 stranded β-barrels and a smaller eyelet than in the general diffusion porins. One side of the eyelet is lined with aromatic side chains, which participate in sugar binding. SrcY is closely homologous to LamB but contains about 70 additional N-terminal amino acids on the periplasmic side. This extension may form a regulating domain that reduces sucrose loss (Schulz, 1996; Forst *et al.*, 1998). The outer membrane phospholipase A (OMPLA) was crystallized as a monomer but is only active as dimer (Dekker *et al.*, 1997). The monomers form a β-barrel consisting of 12 antiparallel β-strands but, in contrast to the porins, the β-barrel is closed at both sides by loops, and no pore activity has been reported for OMPLA (Snijder *et al.*, manuscript submitted for publication).

Functional characteristics of porins

The properties of the porin channels have been studied by various methods both *in vivo* and *in vitro*. The permeation of solutes through the OM of intact cells was studied by antibiotic sensitivity assays, by measuring the rates of uptake of β-lactam antibiotics and of radiolabelled substrates into the cells and by determining the growth rate of the cells on sugars of various molecular weights (Benson *et al.*, 1988; Misra and Benson, 1988; Klebba *et al.*, 1994; Nikaido *et al.*, 1983; Bauer *et al.*, 1988; Todt and McGroarty, 1992). These methods allowed for the determination of the apparent channel size and of ion selectivity. *In vitro*, the permeability rates of sugars, antibiotics and amino acids into liposomes containing reconstituted channels have been used to determine size and selectivity (Luckey and Nikaido, 1980; Nikaido and Rosenberg, 1983; Lee *et al.*, 1992; Trias and Nikaido, 1990).

ELECTROPHYSIOLOGICAL STUDIES. Electrophysiological studies, using either planar lipid bilayers or patch-clamp analysis, demonstrated that the pores are not permanently open but can be closed upon application of a membrane potential above a certain threshold value (Lakey *et al.*, 1985; Dargent *et al.*, 1986; Morgan *et al.*, 1990). This phenomenon is known as voltage gating. Subsequently, a variety of factors have been shown to modulate the opening/closing of porins. Polyamines (delaVega and Delcour, 1995 and 1996; Iyer and Delcour, 1997; Liu *et al.*, 1997; Samartzidou and Delcour, 1999a and b) and the periplasmic membrane-derived oligosaccharides (Delcour *et al.*, 1992) promoted pore closing. Other modulators of porin closure are a low pH (Todt *et al.*, 1992), hydrostatic pressure (Le Dain *et al.*, 1996) and lipids (Ishii and Nakae, 1993). It is unclear whether voltage-dependent gating has a biological function, because the magnitude of the Donnan potential that exists across the OM *in vivo* is lower than the threshold potential that is required to induce pore closing *in vitro* (Senn *et al.*, 1988). However, the presence of any of the other factors mentioned that modulate pore-activity may decrease the membrane potential that is required for pore closing. Anyhow, the direct regulation of porin activity at the molecular level, in addition to the substitution of one porin species by the other by transcriptional regulation, presents an attractive possibility for the control of OM permeability as proposed by Le Dain *et al.* (1996).

MECHANISM OF GATING. The mechanical mechanism of voltage gating is unclear. Molecular dynamics studies suggested that closure could be caused

by a large conformational change of the L3 loop (Björkstén *et al.*, 1994; Soares *et al.*, 1995; Watanabe *et al.*, 1997). However, Tieleman and Berendsen (1998) did not observe any movement of L3 in their molecular dynamics study. An alternative explanation was offered by atomic force microscopy, which suggested that the large extracellular loops may fold toward the center of the pores, thereby closing them (Schabert *et al.*, 1995; Müller and Engel, 1999). It has also been suggested that gating is actually caused by large cations that block the pore under influence of the electrical field (Schulz, 1996). Bainbridge *et al*. (1998b) concluded that voltage gating is a general property of the β-barrel pore structure rather than of any particular substructure.

CHANNEL SIZE AND SELECTIVITY. L3 determines the channel size and selectivity of the pores, and contributes to the stability of the trimer. Insertion of amino acids in L3 of PhoE narrowed the pore (Struyvé *et al.*, 1993), whereas deletions in L3 of OmpF (Benson *et al.*, 1988) and OmpC (Misra and Benson, 1988; Rocque and McGroarty, 1990) widened the channel. Deletions within L3 also affected the stability of the trimers (Rocque and McGroarty, 1990; Van Gelder *et al.*, 1997b). OmpC (Benson *et al.*, 1988) and OmpF (Misra and Benson, 1988) mutants that allowed for the passage of maltodextrins were located within the eyelet. The charged residues within the eyelet that create the transversal electrical field determine the ion-selectivity of the pore (Bauer *et al.*, 1988) and act as voltage sensors (Lakey *et al.*, 1991; Saint *et al.*, 1996; Van Gelder *et al*., 1997a).

Biogenesis of outer membrane proteins

During the past decades, much research was focused on the targeting and translocation of OMPs and periplasmic proteins to and through the IM. These proteins are synthesized in the cytoplasm as precursor proteins with N-terminal signal sequences. This signal sequence is essential for translocation, since it directs the precursor to the Sec proteins, which catalyze the passage through the IM. During translocation, the signal sequence is cleaved off, resulting in the generation of the mature protein (for a review, see Danese and Silhavy, 1998). Periplasmic proteins and OMPs use the same pathway for transport across the IM, since the signal sequences of both types of extracytoplasmic proteins are exchangeable (Tommassen *et al.*, 1983). After cleavage of the signal sequence, the pathways diverge: periplasmic proteins fold into their native conformation, whereas OMPs have to be sorted to the OM. The de-

tails of this sorting process remain elusive. It has been proposed that the contact sites between the two membranes are involved or, alternatively, the proteins could pass freely through the periplasm (Fig. 4). The unfolded protein may insert into the membrane or, alternatively, folding of the monomer and even trimerization could precede membrane insertion (Fig. 4). Possible roles for periplasmic chaperones and/or folding catalysts during the assembly of OMPs will have to be investigated.

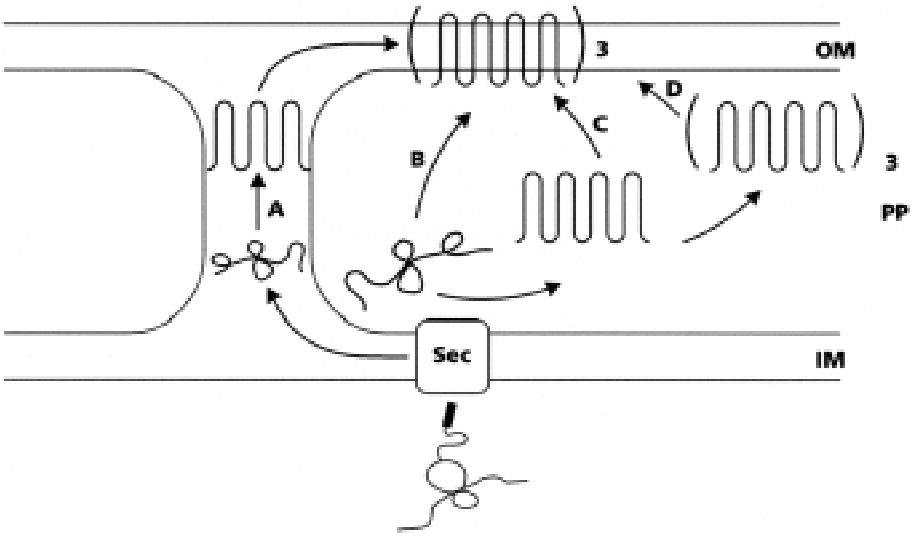

Fig. 4. Models for the biogenesis of OMPs. Precursors of OMPs are targeted to the translocation apparatus (Sec), located in the inner membrane (IM). During or after translocation, the signal sequence (thick line) is removed, and the mature protein is either released in the periplasm (B-D) or sorted to the outer membrane (OM) via the contact sites between the IM and OM (A). After release in the periplasm, the mature protein will adopt tertiary (C) or even quaternary (D) structure prior to insertion, or, alternatively, will directly insert into the OM before adopting its native state (B). The final structure of the porin is a trimer.

Bayer's junctions versus periplasmic intermediates

Two different pathways have been proposed for the transport of OMPs from the IM to the OM, *i.e.* either via contact sites between the two membranes, whereby the OMPs remain in a membranous environment during transport,

or via a free periplasmic intermediate, which is sorted to the OM.

ADHESION SITES. Adhesion sites between the membranes have been visualized by electron microscopy (EM) after plasmolysis of *E. coli* cells in 10-20% sucrose (Bayer, 1968). During plasmolysis, the OM and peptidoglycan are separated from the IM due to shrinkage of the cytoplasm, except at approximately 100-200 sites per cell, where IM and OM adhere to one another (Bayer, 1994). The existence of these sites has been disputed. They were considered to be artifacts of sample preparation, since improved EM methods were not able to visualize these sites (Kellenberger, 1990). However, Bayer (1991) demonstrated that only rapid fixation preserves the *in vivo* state of the cell and thereby the fragile contact sites. The transport of OMPs via the adhesion sites is supported by EM studies, which localize the insertion of newly exported porins to the adhesion sites (Smit and Nikaido, 1978). Additionally, fractionation studies of cell envelopes revealed a fraction of a density, intermediate between those of IMs and OMs, which contained most of the newly made OMPs (de Leij *et al.,* 1979), and these OMPs were incorporated into the OM even before synthesis was completed (de Leij *et al.,* 1978). In mitochondria, close contacts between IM and OM have also been observed. Their role in protein import was demonstrated by the facts that (i) cytoplasmic polysomes are attached to mitochondria at these sites, (ii) the sites are enriched in protein components of the import machinery, and (iii) translocation intermediates fractionate with the contact sites (Hines, 1992).

PERIPLASMIC INTERMEDIATE. The periplasmic intermediate pathway is supported by data obtained from several studies. (i) Deletions within the mature sequences of OmpA, PhoE and FhuA prevented the proper assembly in the OM and caused these OMPs to accumulate in the periplasm (Bosch *et al.,* 1986; Freudl *et al.,* 1985; Coulton *et al.,* 1988). However these "dead-end" forms cannot be chased into the OM and could therefore not be considered as intermediates. (ii) Overproduction of OmpA caused some overproduced protein to localize in the periplasm (Freudl *et al.,* 1986), but this could also be explained as mislocalization due to the high expression level. (iii) FhuA has been reported to proceed through a membrane-free pool after cleavage of the signal sequence and before insertion into the OM (Jackson *et al.,* 1986). However, for OmpF, no soluble or membrane-free intermediate could be detected (Boyd and Holland, 1980). (iv) Treatment of cells with phenethyl alcohol prevented incorporation of OmpA into the OM and caused the protein to co-fractionate with periplasmic proteins (Halegoua and Inouye,

1979). (v) A metastable trimer of OmpF was detected in the periplasm using a repertoire of conformation-specific monoclonal antibodies (Fourel *et al.*, 1992). (vi) Spheroplasts secreted a water-soluble OmpF monomer, which could trimerize and insert into the OM *in vitro*, but only after adding low levels of detergents (Metcalfe and Holland, 1980; Sen and Nikaido, 1990). (vii) Finally, *in vitro* synthesized PhoE proteins could insert into the OM (de Cock and Tommassen, 1996). However, the requirement of Triton X-100 questions the relevance for the *in vivo* situation. Obviously, additional research will be required to settle definitely whether the OMPs are transported via the adhesion sites or via the periplasm.

Outer membrane protein assembly intermediates

Translocation of OMPs across the IM and cleavage of the signal sequence yields a mature protein, which has to adopt its tertiary structure and to insert into the OM. Several orders of events can be proposed (Fig. 4). The identification of assembly intermediates and their subcellular localization have been used to shed light on the pathway. Presently, four assembly intermediates have been described for one or more of the porins: (i) the mature, processed unfolded monomer, (ii) the folded monomer, which has a higher mobility on SDS-PAGE than the denatured form, (iii) the dimer, and (iv) the metastable trimer, which is less resistant to heat and SDS than the native trimer. For the monomeric OMPs, one assembly intermediate has been observed: the immature processed (imp) intermediate, which is the unfolded monomer.

MATURE UNFOLDED MONOMER. After translocation of the OMP across the IM, the unfolded mature form emerges from the IM. This unfolded monomer has been observed for OmpF, LamB and OmpA. In the case of LamB, this intermediate was extracted from the IM suggesting that it is IM-associated at least very early in the biogenesis process (Stader and Silhavy, 1988; Stader and Justice, 1994). Misra *et al.* (1991) studied the assembly of a temperature-sensitive mutant LamB protein and detected monomers fractionating with the OM, indicating that trimerization is not a prerequisite for OM localization. Pulse-chase experiments revealed a processed assembly intermediate of OmpA, designated imp-OmpA, which had not yet acquired the characteristic heat-modifiability and protease-resistance of native OmpA and was localized in the IM (Freudl *et al.*, 1986). After overproduction, imp-OmpA was detected in the periplasm. The fact that the addition of LPS converted imp-OmpA into

native OmpA indicated that imp-OmpA is a true assembly intermediate.

FOLDED MONOMER. Whereas α-helical membrane-spanning segments of polytopic IM proteins can insert independently of other membrane-spanning segments into the membrane, a β-strand of an OMP with its amphiphilic character is incompetent to insert independently into the OM. This suggest that tertiary folding of OMPs in the periplasm precedes membrane insertion. Indeed, large deletions, which are expected to destroy tertiary structure formation in PhoE (Bosch *et al.,* 1986 and 1988) and OmpA (Freudl *et al.,* 1985; Klose *et al.,* 1988a) prevented OM insertion of these proteins. Folded monomers have been detected *in vivo* as assembly intermediates in the case of OmpF, LamB and PhoE (Fourel *et al.,* 1992; Rouvière and Gross, 1996; Van Gelder and Tommassen, 1996). In all three cases, pulse-chase experiments revealed that this folded intermediate was chased into higher molecular weight intermediates. The folded monomer of OmpF was localized in the periplasm, whereas in the other studies, no localization experiments were performed.

DIMER. The subsequent step in the assembly of porins might be dimerization. Dimers have indeed been detected in the case of OmpF (Reid *et al.,* 1988). In pulse-chase experiments, this dimer was chased into trimers indicating that it is a true assembly intermediate (Reid *et al.,* 1988).

METASTABLE TRIMER. The final step in the assembly process is the formation of the trimer. A trimeric assembly intermediate that is less stable than the native trimer has been detected in the case of LamB and OmpF. Metastable OmpF trimers have been detected in the periplasm using mAbs recognizing conformational epitopes (Fourel *et al.,* 1992). The kinetics of the assembly process of LamB have been studied in pulse-chase experiments (Vos-Scheperkeuter and Witholt, 1984). The monomers disappeared with a half-time of 20 s and assembled into metastable trimers. These trimers were converted into stable trimers with a half-time of about 5.7 min. Since LamB was already detected after 30-50 s at the cell surface, the metastable trimers must have been inserted into the OM. Apparently, the trimerization process is a multistep process with at least one intermediate. However, the studies mentioned above are not in agreement with respect to the subcellular localization of this intermediate.

SORTING SIGNAL. Independent of the order of events (Fig. 4), some kind of a sorting signal is expected to be present in OMPs for their targeting to the correct membrane. Sequences of unrelated OMPs have been compared to identify a putative sorting signal. A conserved motif of hydrophobic residues

in the last membrane-spanning segment with a Phe (or Trp) in the ultimate C-terminal position was detected in the porins, OM enzymes and receptors of unrelated bacteria, including *E. coli*, *Pseudomonas* and *Neisseria* (Struvyé *et al.*, 1991). Substitution or deletion of residues within this motif in PhoE and OmpA affected the efficiency of the OM localization (Struyvé *et al.*, 1991; Klose *et al.*, 1988b and 1989). Although, it has been demonstrated that this motif contributes *in vitro* to the efficiency of the formation of an assembly-competent folded monomer (de Cock *et al.*, 1997), an additional role in targeting, possibly as a recognition site for a chaperone, remains a distinct possibility.

Assembly of OMPs in vitro

To understand the process of folding and assembly of OMPs in detail, molecular biological, biochemical and biophysical analyses *in vitro* are required. Such *in vitro* analyses may circumvent some of the problems that are encountered in *in vivo* experiments, such as proteolytic degradation of misfolded or mistargeted proteins and feedback inhibition of OMP synthesis by accumulating OMPs at intermediate stages in the biogenesis. Various *in vitro* systems have been developed to study the folding and assembly of OMPs. The OMPs can be (over)expressed *in vivo*, purified and denatured with chaotropic agents and the refolding process can be studied. Alternatively, these OMPs are synthesized *in vitro* in cellular extracts of *E. coli*, containing all cytoplasmic and periplasmic components, and the requirements for folding and insertion into the OM can be studied (Van Gelder *et al.*, 1997c).

***IN VITRO* REFOLDING.** Purified and denatured OmpA and OmpF have been refolded upon dilution in either detergent micelles or in membrane vesicles (Dornmair *et al.*, 1990; Eisele and Rosenbusch, 1990; Surrey and Jähnig, 1995; Kleinschmidt and Tamm, 1996). In the presence of membrane vesicles, native monomeric OmpA was obtained, all of which was inserted in the same oriented manner, and the refolding of the β-barrel occurred simultaneously with translocation of all four β-hairpins into the lipid bilayer (Kleinschmidt *et al.*, 1999). The membrane vesicles used had to be small. Small vesicles are characterized by a highly curved lipid bilayer, due to which the lipid packing is not optimal. This feature may even allow the insertion in the absence of detergent. Freeze fracturing EM showed that the outer fracture face of the OM is densily occupied with particles while the periplasmic side

of the OM is covered with highly-curved micelle-like structures (Verkleij *et al.,* 1977; van Alphen *et al.,* 1978). These particles might enable the insertion of OMPs, similar to the small lipid vesicles in the *in vitro* reconstituted system for OmpA assembly. Moreover, PE, which is the most abundant phospholipid present in the OM, forms non-lamellar structures thereby stabilizing local concave surface curvature (de Kruijff *et al.,* 1997), which might be required for insertion of OMPs into the OM. During the *in vitro* refolding, aggregation of OmpA had to be prevented, which could reflect the involvement of a chaperone *in vivo* (Surrey and Jähnig, 1992). Refolding of OmpF in the presence of membrane vesicles resulted in insertion of dimers and trimers (Surrey *et al.,* 1996). The monomers remained trypsin-sensitive, indicating that they were not stably inserted into the membrane.

***IN VITRO* SYNTHESIS AND FOLDING.** The folding and assembly of *in vitro* synthesized PhoE and OmpF has been studied extensively. An assembly-competent folded monomer of PhoE could be formed in the presence of very low amounts of Triton X-100 (0.015%), LPS and divalent cations (de Cock and Tommassen, 1996). The LPS properties required for this folding are the negative charges in the inner core region and a non-lamellar structure of lipid A (de Cock *et al.,* 1999a), and, consistently, LPS of deep rough mutants was much less efficient in folding of PhoE (de Cock and Tommassen, 1996). Similarly, the imp-OmpA (see section "Outer membrane protein assembly intermediates"), which accumulated in cells overproducing this protein, could be converted into a heat-modifiable form after addition of LPS to cell envelopes (Freudl *et al.,* 1986). The role of LPS in the folding of PhoE *in vitro* is consistent with *in vivo* data, which showed a severe defect in the biogenesis of porins in deep-rough mutants (Tommassen and Lugtenberg, 1981; Reid *et al.,* 1990; Laird *et al.,* 1994). Furthermore, the biogenesis of the porins, but not of OmpA, was strongly affected *in vivo* by the drug cerulenin, which inhibits fatty acid synthesis, consistent with an important role of LPS (or phospholipids) in porin biogenesis (Bocquet-Pages *et al.,* 1982; Pages *et al.,* 1982; Bolla *et al.,* 1988; Fourel *et al*., 1992). Additionally, the density of the particles at the outer surface of the OM was reduced in a strain expressing defective LPS molecules (Verkleij *et al.,* 1977). The reduced number of particles might explain the effect of LPS mutations on the biogenesis of OMPs. Finally, the observation that an assembly defect of an OmpF mutant protein could be suppressed *in vivo* by mutations that affect the LPS/phospholipid ratio (Laird *et al.,* 1994; Misra and Miao, 1995; Deng and Misra,

1996; Kloser *et al.,* 1996 and 1998; Danese and Silhavy, 1998) suggests an important role for LPS and/or phospholipids in OMP biogenesis. Trimerization and OM insertion of the folded monomers of PhoE generated *in vitro* could be achieved by the addition of outer membranes and increasing the Triton X-100 concentration to 0.08% (de Cock and Tommassen, 1996). The role of Triton X-100 in this *in vitro* folding and assembly process remains elusive, but it might mimic the role of a periplasmic chaperone, since Triton X-100 affected the assembly-competent state of PhoE: *in vitro* synthesized PhoE became assembly-incompetent with a half-life of 12 min and 90 s in the absence and presence of Triton X-100, respectively (de Cock *et al.,* 1996). Furthermore, it was demonstrated that trimerization could precede OM insertion *in vitro* (de Cock and Tommassen, 1996). Indications for the existence of a periplasmic assembly intermediate of OmpF were obtained by Sen and Nikaido (1990). Spheroplasts secreted a water-soluble monomeric form of OmpF, which could trimerize upon addition of cell envelopes and trace amounts of Triton X-100. The resulting trimeric OmpF co-fractionated with the cell envelopes, indicating its association with the membranes. *In vitro* synthesized OmpF could be converted into trimers in the presence of cell envelopes or purified LPS even in the absence of Triton X-100 (Sen and Nikaido, 1991a and b). This is remarkable, since PhoE could not be converted into trimers in the presence of purified LPS. Sen and Nikaido (1991b) noticed that trimerization of *in vitro* synthesized OmpF was less efficient than that of OmpF secreted by spheroplasts, indicating that passage through the IM may be necessary for the protein to achieve an assembly-competent state.

Periplasmic folding catalysts

The periplasm is a folding compartment, which contains molecular chaperones and folding catalysts, which could contribute to the folding of OMPs, if these OMPs indeed pass through the periplasm on their way to the OM. Whereas chaperones usually act by preventing aggregation and misfolding, folding catalysts accelerate specific rate-limiting steps in the folding reaction (Ellis and van der Vies, 1991). Two types of folding catalysts have been identified in the cell, *i.e.* disulfide oxidoreductases and peptidyl prolyl *cis/trans* isomerases (PPIases).

DISULFIDE BONDS. Disulfide bonds can play different roles in protein structure and activity. For many proteins, disulfide bonds are permanent features of

the final folded structure, which enhance the stability of the protein. The formation of these bonds may be an essential step in the folding pathway (*e.g.* BPTI) (Darby *et al.,* 1995) and promote a folding pathway even when the native folded protein contains no such bonds (*e.g.* P22 tailspike protein) (Robinson and King, 1997). In the case of oxidoreductases, the formation of a disulfide bond is a consequence of the enzymatic activity, and their reduction is essential for this activity (*e.g.* thioredoxin reductase). The oxidation state of OxyR (Zheng *et al.,* 1998) and Hsp33 (Jakob *et al.,* 1999) varies according to environmental conditions and serves as a defense mechanism to oxidative stress (Åslund and Beckwith, 1999b).

DISULFIDE BONDS IN CYTOPLASM. Cytoplasmic proteins do generally not contain structural disulfide bonds. Initially, cysteines in cytoplasmic proteins were considered to remain in the reduced state by the presence of the thioredoxin/thioredoxin reductase and the glutathione/glutaredoxin pathways (Rietsch and Beckwith, 1998; Åslund and Beckwith, 1999a). However, there are indications that suggest that the lack of an enzyme that promotes disulfide bond formation in the cytoplasm is responsible for the maintenance of reduced cysteines. In a thioredoxin reductase null mutant strain, thioredoxin 1 and 2 accumulated in an oxidized state and were capable of disulfide bond formation in the normally periplasmic enzyme PhoA, which was mislocalized due to a deletion of the signal sequence (Derman and Beckwith, 1991; Derman *et al.,* 1993; Prinz *et al.,* 1997; Stewart *et al.,* 1998). The function of thioredoxin 1 solely depends on the redox balance of its environment, since the fusion of thioredoxin 1 to a signal sequence resulted in its export to the periplasm, where it functioned as an oxidant (Debarieux and Beckwith, 1998).

Dsb PROTEINS. Sofar, six Dsb proteins, involved in periplasmic disulfide bond formation, reduction and isomerization, have been identified and characterized. These proteins lack overall sequence homology, but they have two properties in common: they all contain the active site Cys-X-X-Cys and, with the exception of DsbB, they all have the thioredoxin fold (Martin, 1995).

DsbA. The periplasmic DsbA protein, which was the first discovered Dsb protein (Bardwell *et al.,* 1991; Kamitani *et al.,* 1992), proved to catalyze disulfide bond formation very efficiently in unfolded substrates (Joly and Schwarz, 1994; Darby and Creighton, 1995; Frech *et al.,* 1996). The catalyzing capacity of the protein is caused by the destabilizing effect of the active site disulfide (Wunderlich *et al.,* 1993a and b; Zapun *et al.,* 1993) and the abnormal low pK_a of Cys30 (Grauschopf *et al.,* 1995; Guddat *et al.,*

1998), which is the essential residue of the Cys-X-X-Cys motif of DsbA (Wunderlich *et al.*, 1995).

DsbB. After catalysis, DsbA is reduced, and reoxidation is performed by the integral membrane protein DsbB (Missiakas *et al.*, 1993; Kishigami *et al.*, 1995). DsbB has two pairs of essential cysteines (Jander *et al.*, 1994). The disulfide bond between Cys104 and Cys130 in the C-terminal periplasmic loop acts directly to oxidize DsbA (Guilhot *et al.*, 1995). The second pair of cysteines, Cys41 and Cys44, which are located in the N-terminal loop, reoxides Cys104 and Cys130. Oxidation of this second pair requires the presence of a functional respiratory chain (Kobayashi *et al.*, 1997; Kobayashi and Ito, 1999).

DsbC. When a protein contains only two cysteines, DsbA will inevitably form the correct disulfide bond. However, DsbA may promote incorrect disulfide bonds in substrate proteins with multiple cysteines. Incorrect pairing must be corrected for the protein to assume its native conformation. This isomerization of disulfide bonds is catalyzed by the periplasmic DsbC protein (Missiakas *et al.*, 1994; Rietsch *et al.*, 1996; Joly and Schwarz, 1997; Sone *et al.*, 1997a and b). The active site cysteines of the dimeric DsbC are normally in the reduced state (Zapun *et al.*, 1995).

DsbD. After isomerization, oxidized DsbC is reduced by the inner membrane protein DsbD (Sambongi and Ferguson, 1994; Missiakas *et al.*, 1995; Crooke and Cole, 1995). DsbD also reduces DsbE and is involved in copper tolerance (Fong *et al.*, 1995). The reduction of DsbD depends on the cytoplasmic proteins thioredoxin and thioredoxin reductase (Rietsch *et al.*, 1997).

DsbE. The DsbE protein is required to maintain the heme-binding site of apo-cytochrome c reduced and, therefore, the active site cysteines are in the reduced state (Throne-Holst *et al.*, 1997; Fabianek *et al.*, 1998; Reid *et al.*, 1998). This protein has been localized in the periplasm (Raina and Missiakas, 1997) and in the inner membrane with its active site exposed to the periplasm (Throne-Holst *et al.*, 1997; Fabianek *et al.*, 1998).

DsbG. Recently, DsbG has been identified, but it remains under debate whether it functions as an oxidant or displays isomerase activity (Andersen *et al.*, 1997; van Straaten *et al.*, 1998; Bessette *et al.*, 1999).

DISULFIDE BONDS IN OMPs. Since disulfide bonds are scarce in *E. coli* OMPs, studies on the involvement of the Dsb proteins in the assembly of OMPs have been limited to DsbA. DsbA catalyzes the formation of a disulfide bond in OmpA, but this bond is located in the periplasmic C-terminal domain of

OmpA and may be formed even after the protein has inserted into the membrane (Bardwell *et al.*, 1991). The expression of OmpF was drastically reduced in a *dsbA* mutant at the transcriptional level, possibly as a consequence of feedback inhibition (Pugsley, 1993). However, this protein is devoid of disulfide bonds. Possibly, DsbA has additional functions and might act as a chaperone in the biogenesis of OmpF. Alternatively, it might catalyze disulfide bond formation in an as yet unidentified chaperone required for the OmpF assembly or in an as yet unidentified envelope protein involved in the regulation of *ompF* expression.

PEPTIDYL-PROLYL *CIS/TRANS* ISOMERASES. The second type of slow conformational rearrangements is the *cis/trans* isomerization of peptidyl-prolyl bonds (Schmid, 1993), which is catalyzed by PPIases. *In vitro* experiments have shown that the (re-)folding of proteins can be accelerated by PPIases from various sources. However, the exact role of these PPIases in protein folding *in vivo* remains to be established. PPIases have been identified both in the cytoplasm and in the periplasm, and they have been divided into three subfamilies: (i) the cyclophilins with RotA as a periplasmic member (Liu and Walsh, 1990), (ii) FK506-binding proteins with FkpA as periplasmic member (Horne and Young, 1995) and (iii) the parvulins with SurA in the periplasm and the inner membrane protein PpiD, the catalytic domain of which faces the periplasm (Rouvière and Gross, 1996; Dartigalongue and Raina, 1998).

RotA. RotA (also designated PpiA) is not essential for viability, and no changes in the protein composition of the outer membrane of a *rotA* mutant relative to that of the wild-type strain were detected (Kleerebezem *et al.*, 1995). This suggests that either RotA is not essential for the assembly of OMPs or another PPIase can take over its function. However, no residual PPIase activity could be detected in the periplasmic fraction of the *rotA* mutant, as tested with an artificial substrate in a protease-coupled enzyme assay. However, since PPIases of different families have different subsite specificities (Stoller *et al.*, 1995), this finding does not exclude the presence of residual PPIase activity.

SurA/PpiD/FkpA. Recently, a role for SurA and PpiD in the folding and assembly of OMPs has been demonstrated. SurA was initially identified as a protein essential for stationary phase survival (Tormo *et al.*, 1990). This survival defect of the *surA* mutants was due to an improper assembly of the cell wall-synthesizing apparatus at elevated pH in the absence of σ^S (Lazar *et al.*, 1998). By using a *surA* mutant strain, a role of SurA in the assembly of

OMPs, but not of periplasmic proteins, was demonstrated in pulse-chase experiments (Rouvière and Gross, 1996; Lazar and Kolter, 1996). In the absence of SurA, the conversion of the unfolded to the folded monomer of LamB appeared to be affected. SurA, which contains two parvulin-like domains, displayed only a very low PPIase activity as compared with the cytoplasmic Parvulin (Rouvière and Gross, 1996; Missiakas *et al.*, 1996a). The inner membrane protein PpiD was isolated as a multicopy suppressor of a *surA* mutant (Dartigalongue and Raina, 1998). Its role in OMP folding was demonstrated by the fact that the OMP levels were reduced in a *ppiD* mutant, although less severe than in a *surA* null mutant. A *ppiD/surA* double mutant proved to be lethal. PpiD showed significantly higher PPIase activity *in vitro* than SurA, in spite of the less pronounced phenotype of the *ppiD* mutant. Hence, SurA and PpiD might have another role in addition to their PPIase activity in OMP folding. A role for FkpA in OMP folding has so far not directly been demonstrated.

Other facilitators of outer membrane protein biogenesis

Skp. The periplasmic, cationic protein Skp (Skp stands for **s**eventeen **k**Da **p**rotein) appears to function in the assembly of OMPs. It has been observed that purified Skp binds *in vitro* to denatured OMPs (Chen and Henning, 1996; de Cock *et al.*, 1999b) and to LPS (Geyer *et al.*, 1979). Additionally, an *skp* mutant strain, although viable, contained lower concentrations of OMPs (Chen and Henning, 1996). However, the role of Skp in OMP assembly remains elusive. Since the *skp* gene is located in a cluster of genes involved in LPS synthesis (Thome *et al.*, 1990) and since various mutations affecting the LPS structure reduce the level of OMPs in the OM, Skp might only indirectly function in OMP biogenesis and have a primary role in LPS biogenesis. Missiakas *et al*. (1996a) proposed a role late in the assembly process, based on the observation that Skp overexpression, unlike that of SurA, did not suppress the phenotypic defects conferred by the expression of altered LPS. Skp may help to remove or exchange the original LPS molecule associated with folded monomers or pro-trimers of OMPs, thus facilitating their assembly. An opposing view was presented by de Cock *et al*. (1999b), who suggested a very early role of Skp in the biogenesis of OMPs, since Skp interacts specifically with non-native OMPs and has a high affinity for phospholipids. Skp might therefore be required for the efficient release of

OMPs from the Sec machinery in the IM or for targeting of the unfolded monomer directly after translocation to assembly sites at the periplasmic side of the IM.

LolA/LolB. For some OMPs, specific targeting pathways seem to have evolved. A role for the periplasmic protein LolA in the biogenesis of OM lipoproteins has been established. Spheroplasts retained the major lipoprotein (Lpp) in its mature form in the IM (Matsuyama *et al.*, 1995). However, Lpp was released in the soluble fraction, when LolA was added to the spheroplasts. LolA released specifically OM-destined lipoproteins (Tajima *et al.*, 1998), which differ from IM lipoproteins by the absence of an Asp at position + 2. The Lpp-LolA complex was specifically targeted to the OM, in which a lipoprotein, LolB, appeared to function as a receptor for the Lpp-LolA complex (Matsuyama *et al.*, 1997). The Lpp was transferred from LolA to LolB and subsequently incorporated into the OM.

PROTEINS INVOLVED IN SORTING SECRETINS. Recently, proteins have been characterized that are essential for OM localization of proteins of the secretin family, such as PulD, OutD and YscC of *K. oxytoca*, *Erwinia chrysanthemi* and *Yersinia enterocolitica*, respectively (Hardie *et al.*, 1996; Shevchik *et al.*, 1997; Koster *et al.*, 1997). The secretins require a small lipoprotein for protection against proteolysis and for insertion into the OM. For example, PulS appears to be a membrane-bound chaperone that facilitates the localization of PulD into the OM. PulS itself is likely to depend for its localization on the LolA/B system. In that case, PulD might recruit PulS to exploit the lipoprotein-targeting pathway to the OM.

PapD. The subunits of Pap pili require a specific chaperone for their targeting to the OM (Jones *et al.*, 1997). The release of processed pilus subunits from the IM into the periplasm requires the presence of the periplasmic PapD protein, since spheroplasts only released pilus subunits after addition of purified PapD. The pilus subunits contain a conserved C-terminal β-zipper motif. Deletion of this motif in one of the subunits, PapG, was demonstrated to facilitate its release in the periplasm in the absence of PapD, indicating that this motif functions as a membrane-anchor and that binding of PapD to this segment is required for release of the wild-type protein from the IM. The pilin/PapD complex is subsequently targeted to an OM protein, designated "usher", which facilitates the translocation of the pilins across the OM and their assembly into a pilus structure (Hung and Hultgren, 1998).

Monitoring envelope-protein physiology

The intracellular accumulation of misfolded proteins triggers responses to rescue these proteins. The cell, confronted with heat shock or other stresses leading to protein denaturation, rapidly induces the synthesis of heat-shock proteins. This stress response is compartmentalized in *E. coli* into cytoplasmic and extracytoplasmic responses that are controlled by distinct alternative sigma factors. Cytoplasmic stress triggers the σ^{32}-dependent heat-shock response directing the expression of chaperones, such as GroEL/GroES and DnaK/DnaJ/GrpE, or proteases, such as Lon and the members of the Clp family. These proteins target the misfolded protein for refolding or proteolysis, eventually leading to downregulation of the σ^{32} (encoded by *rpoH* gene) - activity upon relief of the cytoplasmic stress (for a review, see Missiakas *et al.,* 1996b). The extracytoplasmic stress response is controlled by at least two, partially overlapping signal transduction systems: the Cpx two-component system and the σ^{E}-mediated system (Fig. 5) (Connolly *et al.,* 1997; Missiakas and Raina, 1998; Raivio and Silhavy, 1999).

CPX. The Cpx two-component regulatory system consists of the IM sensor histidine kinase CpxA and the cytoplasmic response regulator CpxR (Raivio and Silhavy, 1997). Triggers that have been reported to activate the Cpx pathway are overproduction of the outer membrane lipoprotein NlpE leading to an increase in IM-associated NlpE (Snyder *et al.,* 1995), alterations in the phospholipid content of the IM (Mileykovskaya and Dowhan, 1997), overproduction of P pilus subunits in the absence of their chaperone, which causes their aggregation in the IM (Jones *et al.,* 1997) and an elevated pH (Danese and Silhavy, 1998a). Based on these observations, Raivio and Silhavy (1999) suggested that aggregated, misfolded proteins associated with the IM are the actual signal that induces the Cpx-response, and its activation is meant to relieve cell envelope-associated stresses. Additionally, the Cpx pathway can combat protein-mediated stresses due to expression of the mutant fusion proteins LamB-LacZ, LamBA23D and LamB-LacZ-PhoA, which most likely exerted their effects in the inner membrane (Cosma *et al.,* 1995). The Cpx system controls the expression of the periplasmic proteins CpxP, with unknown function (Danese and Silhavy, 1998a), the protease DegP (Danese *et al.,* 1995) and the folding catalysts DsbA, PpiD and RotA (Danese and Silhavy, 1997; Pogliano *et al.,* 1997). Furthermore, expression of the *cpxAR* operon is controlled by autoregulation (Danese and Silhavy, 1997).

σ^E. The activity of σ^E (encoded by *rpoE* gene) (Raina *et al.*, 1995; Rouvière *et al.*, 1995) is specifically modulated by fluctuations in the expression levels of OMPs (Mecsas *et al.*, 1993), by inactivating the gene for any of the periplasmic chaperones or folding catalysts, such as the Dsb proteins, SurA, FkpA or Skp, or by mutations in the *htrM* gene leading to production of

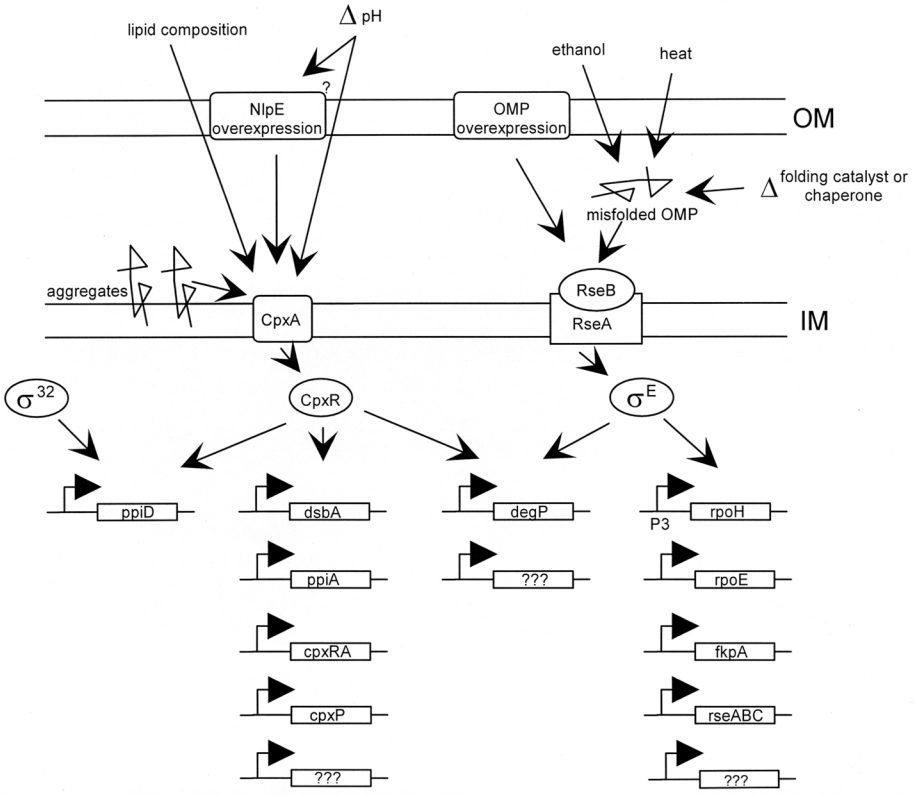

Fig. 5. The extracytoplasmic stress responses in *E. coli*. The extracytoplasmic stress response is controlled by partially overlapping pathways. The Cpx pathway controls the expression of several resident periplasmic folding proteins (DegP, PpiA and DsbA), of CpxP, of the regulators CpxRA and of the inner membrane protein PpiD, in response to the presence of inner membrane aggregates due to overproduction of NlpE, overexpression of pilus subunits in absence of the chaperone and structural or physical alterations in the envelope, induced by changes in pH or lipid composition. Expression of *ppiD* is also controlled by σ^{32}. The alternate σ-factor σ^E controls the expression of *degP, rpoH, rpoE, fkpA* and the regulators *rseABC* and several other unidentified genes in response to the accumulation of misfolded OMPs in the periplasm.

altered LPS. These triggers of the σ^E-response suggest that the accumulation of assembly intermediates of misfolded OMPs constitutes the actual trigger. The observation that the σ^E activity was unaffected by the overproduction of periplasmic proteins supports this supposition (Mecsas *et al.*, 1993). However, several exceptions to this rule have been reported, *i.e.* overproduction of penicillin-binding protein 2 (PBP2) and of two mutant proteins, asparaginase B and MalE31, also induced the σ^E-response (Missiakas and Raina, 1998). Whereas overproduction of PBP2 causes an alteration of the peptidoglycan structure, the latter two proteins were suggested to activate the σ^E pathway by titrating folding factors required for OMP assembly. Overproduction of some pilin subunits in the absence of the chaperone can also lead to activation of the σ^E pathway. This activation is less than three-fold, whereas activation of Cpx by overproduction of pilin subunits can be greater than 10-fold. Perhaps, this can be explained by the observation that misfolded pilin subunits not only accumulate in the IM when produced in the absence of their chaperone, but a small amount is also found in the periplasm (Raivio and Silhavy, 1999). Additionally, strains lacking σ^E are hypersensitive to detergents and hydrophobic agents, suggesting an OM permeability defect.

Sofar, only seven members of the σ^E regulon have been characterized, *i.e.* the protease DegP, the folding catalyst FkpA, σ^{32}, σ^E and the regulators RseABC (Danese and Silhavy, 1997). The signal for σ^E induction is generated outside of the cytoplasm, and this extracytoplasmic information is transmitted via the Rse proteins to the cytoplasm. RseA and RseC are both inner membrane proteins and function as negative and positive modulators, respectively, of σ^E. RseB, the periplasmic component, might be the sensor favoring either binding to RseA or RseC (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997).

OVERLAP OF STRESS RESPONSES. The Cpx and σ^E pathways overlap in the control of the expression of the periplasmic protease DegP. Furthermore, there is overlap with the σ^{32} cytoplasmic heat shock response, since the P3 promoter of σ^{32} is a target for σ^E, whereas *ppiD* expression is regulated both via Cpx and via σ^{32}. Additional overlap might be detected in the future, since sequences complying with the consensus CpxR-binding site have been detected in the promoter regions of potentially relevant genes, such as *rpoE* and *rpoH* (Raivio and Silhavy, 1999). Finally, the presence of misfolded OMPs in the extracytoplasm not only induces a σ^E response but also the synthesis of the capsular polysaccharide, leading to a highly mucoid pheno-

type, thereby strengthening the cell envelope. The synthesis of capsular polysaccharides is usually induced in strains producing defective LPS or in *dsbA, dsbB* or *surA* mutants with defects in OMP folding (Missiakas and Raina, 1997b).

The objective of this thesis

In this thesis, the *E. coli* outer membrane porin PhoE is used as a model protein to study the late steps in the assembly process and the role of loop L3 in pore functioning. The initial steps in the biogenesis of OMPs, *i.e.* the translocation of the precursor protein across the IM and the cleavage of the signal sequence by leader peptidase, have been studied extensively in the past. Much less is known concerning the later steps in the biogenesis process. In chapter 2, we addressed the question whether the mature protein is sorted to the OM via the contact sites between the IM and OM or via a periplasmic intermediate. Whereas the latter model was favored, direct evidence for it was lacking. To test this model, we investigated whether a periplasmic enzyme can modify PhoE on its way to the OM, which would prove that the protein is at least exposed to this compartment during its biogenesis. Furthermore, we reasoned that if this periplasmic protein creates an element of tertiary structure in PhoE, this would prove that PhoE is at least partially folded before it inserts into the OM. To study whether a periplasmic enzyme can modulate PhoE on its way to the OM, an intramolecular disulfide bond was engineered within the PhoE monomer and the role of DsbA in the formation of this disulfide bond was investigated.

Along similar lines, we investigated in chapter 3 whether trimers are formed prior to membrane insertion. To this end, a disulfide bond was engineered at the subunit interface and the role of the periplasmic Dsb proteins in the formation of the disulfide bond was investigated.

Meanwhile, a role for the periplasmic peptidyl-prolyl *cis/trans* isomerase (PPIase) SurA in the OMP assembly was described (Lazar and Kolter, 1996; Missiakas *et al.*, 1996a; Rouvière and Gross, 1996). However, because of its low PPIase activity, we hypothesized that SurA might have another, or at least an additional role in OMP biogenesis than the *cis/trans* isomerization of peptidyl-prolyl bonds. To investigate this possibility, a proline-less mutant PhoE protein was constructed and the role of SurA in the assembly of this protein was studied in chapter 4.

Porins are not permanently open but can be closed upon applying a membrane potential. The mechanism involved in pore closure is poorly understood. The mutant PhoE proteins with the artificial disulfide bonds, tethering loop L3 to the barrel wall (chapter 2), provided an excellent tool to test the hypothesis that a large physical movement of loop L3 into the lumen of the pore is the mechanical basis of pore closing. Voltage gating and other properties of these mutant porins were measured in the experiments described in chapter 5.

In chapter 6, the results obtained in these studies are discussed and compared with other studies in this field.

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CHAPTER 2

Folding of a bacterial outer membrane protein during passage through the periplasm

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Folding of a bacterial outer membrane protein during passage through the periplasm

Summary

The transport of bacterial outer membrane proteins to their destination might either be a one-step process via the contact zones between the inner and outer membrane or a two-step process, implicating a periplasmic intermediate that inserts into the membrane. Furthermore, folding might precede insertion or *vice versa*. To address these questions we have made use of the known 3D-structure of the trimeric porin PhoE of *Escherichia coli* to engineer intramolecular disulfide bridges into this protein at positions that are not exposed to the periplasm once the protein is correctly assembled. The mutations did not interfere with the biogenesis of the protein, and disulfide bond formation appeared to be dependent on the periplasmic enzyme DsbA, which catalyzes disulfide bond formation in the periplasm. This proves that the protein passes through the periplasm on its way to the outer membrane. Furthermore, since the disulfide bonds create elements of tertiary structure within the mutant proteins, it appears that these proteins are at least partially folded before they insert into the outer membrane.

Introduction

In Gram-negative bacteria, such as *Escherichia coli*, outer membrane proteins (OMPs) are synthesized in the cytoplasm as precursor proteins with an N-terminal extension, the signal sequence. These precursors, like those of periplasmic proteins, are translocated across the inner membrane via the Sec machinery, and the signal sequence is cleaved off. Intensive research over the past decade has led to the unraveling of the mechanism of this transport process into much molecular detail (Schatz and Beckwith, 1990; Pugsley, 1993a; Driessen, 1994). In contrast, the knowledge concerning the subsequent steps in the biogenesis of OMPs is very scarce. Two pathways for the sorting of OMPs to their destination have been proposed. On the one hand, these proteins might directly be inserted into the outer membrane via the contact sites between the two membranes (Bayer *et al.*, 1982). This model is supported by electron-microscopic studies, which reveal the presence of newly exported porins above the fusion sites (Smit and Nikaido, 1978). Furthermore, pulse-chase experiments suggested that nascent OMPs pass through a membrane fraction of intermediate density (de Leij *et al.*, 1978), which is supposed to contain the fusion sites, and that they are incorporated into the outer membrane even before their synthesis is completed (de Leij *et al.*, 1979). Alternatively, OMPs might pass through the periplasm on their way to the outer membrane. In this type of model, periplasmic folding might precede membrane insertion. Whereas the latter type of model is presently being favored (Nikaido, 1992; Tommassen and de Cock, 1995), direct evidence is lacking. The fact that mutant OMPs that fail to assemble correctly accumulate in the periplasm favors the periplasmic pathway (Freudl *et al.*, 1985; Bosch *et al.*, 1986), but could also be explained by a default pathway of defective OMPs. The observation that OMPs, synthesized *in vitro* or secreted by spheroplasts, can be assembled into isolated outer membranes (Sen and Nikaido, 1990; de Cock *et al.*, 1996) is also consistent with a free soluble assembly intermediate, but could also reflect an artificial pathway, especially since low amounts of detergents were required to stimulate insertion.

The periplasm contains molecular chaperones and folding catalysts, such as disulfide bond isomerases and peptidyl-propyl *cis/trans* isomerases (Liu and Walsh, 1990; Bardwell *et al.*, 1991; Rudd *et al.*, 1995) which assist in protein folding. If any of these proteins were directly involved in the folding of OMPs, this would demonstrate that these OMPs pass through the periplasm

on their way to the outer membrane and that folding precedes insertion. However, unequivocal evidence for such an involvement is lacking. Whereas it has been reported that mutations in genes encoding putative periplasmic chaperones or folding catalysts can influence the amount of OMPs assembled into the outer membrane, such effects could be indirect. For example, a mutation in the *dsbA* gene, which encodes an enzyme that catalyzes disulfide bond formation, drastically affected the expression of OmpF, even though this porin does not contain any cysteines (Pugsley, 1993b). Similarly, the observation that an *skp* mutation affects the expression of several OMPs (Chen and Henning, 1996) might be explained by a role of the *skp* gene product in the biogenesis of lipopolysaccharides (LPSs), since the *skp* gene is located in a cluster of LPS biosynthesis genes and LPS is involved in the biogenesis of OMPs (de Cock and Tommassen, 1996).

In the present study, we have investigated whether a periplasmic enzyme, DsbA, can modulate the folding of an OMP on its way to the outer membrane. As a model OMP, the trimeric porin PhoE is used in our laboratory. This protein does not contain any cysteines, but its structure has been resolved (Cowan *et al.*, 1992), which enabled us to introduce cysteines at positions that are in sufficiently close proximity to allow disulfide bond formation when the protein folds into its correct conformation. Each monomer of PhoE consists of a 16-stranded β-barrel with short turns at the periplasmic side and long loops at the cell surface. One of these loops, L3, forms a constriction within the barrel at half the height of the membrane. The sequence PEFGG (residues 109-113) at the tip of L3 is highly conserved in a superfamily of bacterial porins (Jeanteur *et al.*, 1991). Residues Glu110 and Phe111 are at hydrogen-bonding distance from Lys18 and Asp302, respectively, in the barrel wall (Karshikoff *et al.*, 1994). By constructing a series of double mutants, we were able to engineer disulfide bridges within PhoE. We demonstrate that disulfide bond formation in the mutant PhoE proteins is strongly stimulated by the periplasmic DsbA protein. Hence, it appears that PhoE passes through the periplasm on its way to the outer membrane. Furthermore, since the DsbA protein creates an element of tertiary structure within the mutant PhoE proteins, these proteins can at least be folded partially prior to their insertion into the outer membrane.

Results

Expression of cysteine-containing mutant PhoE proteins

Site-directed mutagenesis was applied to substitute cysteines for Lys18, Glu110, Phe111 and Asp302 in PhoE protein and double mutants with cysteines at positions 18 and 110, 110 and 302, and 111 and 302 were constructed by combining appropriate restriction fragments. The mutant plasmids (Table 1) were introduced in *phoR* strain CE1265 by transformation, and cell envelopes were isolated and analyzed by SDS-PAGE. All mutant proteins were efficiently expressed (Figure 1A). To study whether the mutant proteins were properly inserted into the outer membrane, their sensitivity to trypsin was assessed. Wild-type PhoE protein is resistant to proteases when it is correctly assembled into the outer membrane (Tommassen and Lugtenberg, 1984). Similarly, all the mutant proteins were protected when cell envelopes were treated with trypsin (Figure 1A), indicating that they

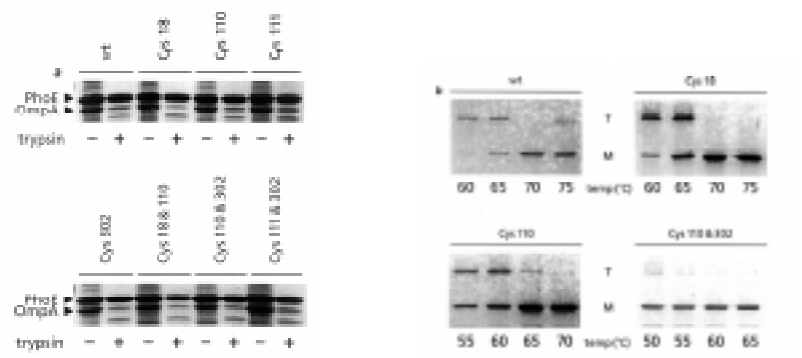

Fig. 1. Expression and assembly of cysteine-containing mutant derivatives of PhoE protein. (A) Cell envelopes were isolated from plasmid-containing derivatives of strain CE1265 expressing mutant PhoE proteins. Samples were treated with trypsin when indicated, after which the samples were analyzed by SDS-PAGE. The sample buffer was supplemented with 20 mM DTT. (B) Trimers of (mutant) PhoE proteins were isolated and incubated for 10 min at the indicated temperatures in the presence of DTT. T and M indicate the positions of the trimeric and the denatured monomeric forms of PhoE, respectively.

were correctly assembled. Furthermore, wild-type PhoE functions in the outer membrane as the receptor for phage TC45 (Chai and Foulds, 1978). CE1265

cells expressing either one of the mutant PhoE proteins were sensitive to this phage (data not shown), which confirms that the mutant proteins were correctly assembled. This conclusion was further substantiated by the observation that all mutant proteins could be isolated from the membranes as native trimers (see Figure 1B for examples). However, the stability of some of the mutant trimers was slightly affected. The wild-type trimer denatured into monomers only after incubation for 10 min at 70°C in 2% SDS (Figure 1B). The PhoE mutant trimers with substitutions at position 18 or at position 302 were equally stable as the wild-type trimer. The mutations at the tip of the third loop, *i.e.* at the positions of residues 110 and 111, affected the stability of the trimers since the mutant proteins denatured at 60-65°C (Figure 1B). All three double cysteine PhoE mutants denatured at 60°C. Together, these results show that all mutant proteins are correctly assembled into the outer membrane but that the stability of some of the proteins was slightly affected by the mutations.

Disulfide bond formation

Frequently, proteins with an intramolecular disulfide bond have a higher electrophoretic mobility than the reduced form of these proteins, probably because of their more compact shape (see, for example, Derman and Beckwith, 1995). To examine whether disulfide bonds are formed between the two cysteines in the mutant PhoE proteins trimers isolated from the outer membrane, were denatured at 95°C in the presence or absence of dithiothreitol (DTT) and analyzed by SDS-PAGE. For all double mutants, faster migrating forms were detected when they were analyzed in the absence of the reducing agent, although with varying efficiency (Figure 2A). In the case of the mutant with cysteines at positions 18 and 110, the faster migrating form was formed with an efficiency of 80-100%. The difference in the mobility of the reduced and the oxidized form was small and could only be visualized when the proteins were separated on gels containing 8 M urea. Also for the mutants with cysteines at positions 110 and 302, or 111 and 302, faster migrating forms were detected on the gel (Figure 2A). In these cases, the efficiency varied from experiment to experiment between 30 and 70%. The mobility differences were more pronounced (Figure 2A). In all cases, both bands reacted on a Western blot with a PhoE-specific monoclonal antibody (Figure 2B), which confirms that they both represent different forms of the mutant PhoE proteins.

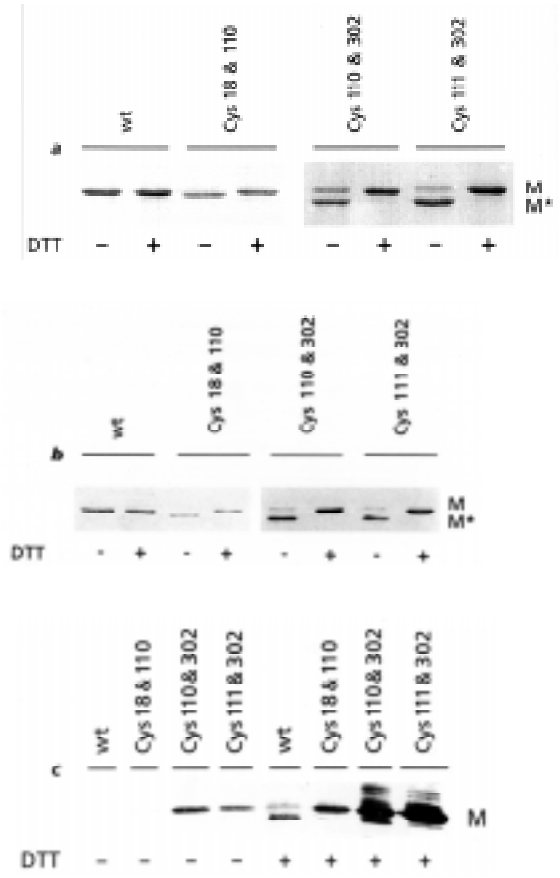

Fig. 2. Formation of disulfide bonds in cysteine-containing derivatives of PhoE protein. (A) Trimers were isolated and dissolved in sample buffer either supplemented or not with DTT, and the samples were boiled for 10 min and analyzed by SDS-PAGE. (B) Western blot analysis of the same samples, using a monoclonal antibody directed against PhoE. M and M* indicate the positions of the denatured form of PhoE and the faster migrating form with an intramolecular disulfide bridge, respectively. In both (A) and (B), the wild-type PhoE and the mutant with cysteines at positions 18 and 110 were analyzed on gels containing 8 M urea and the other proteins on gels without urea. (C) Isolated trimers of (mutant) PhoE were heated at 95°C in the presence or absence of DTT, after which a buffer was added which contained the thiol-specific reagent biotin-HPDP. After incubation for 1 h at room temperature, samples were loaded onto gels, blotted to nitrocellulose membranes and a streptavidin-peroxidase conjugate was used to detect biotin-labeled PhoE forms. The band underneath PhoE in the DTT-treated samples represents trace amounts of OmpA that were present in the samples, as was evidenced by Western blotting with an OmpA-specific monoclonal antibody (data not shown).

Since the bands with higher electrophoretic mobilities disappeared upon treatment of the samples with DTT, whereas those with the lower mobilities increased in intensity (Figure 2A), we assumed that they represent the oxidized and the reduced forms, respectively, of the mutant PhoE proteins. The presence of free SH groups in the protein samples was assessed with the thiol-specific reagent *N*-[6-(biotinamido)]hexyl-3'(2'pyridyldithio)-propionamide (HPDP) to which a biotin was attached. Trimers isolated from the outer membrane were denatured at 95°C either in absence or presence of DTT and subsequently incubated with biotin-HPDP. The proteins were separated by SDS-PAGE, blotted onto nitrocellulose paper, and proteins that had bound biotin were detected by using a streptavidin-horseradish peroxidase conjugate. The mutant protein with cysteines at positions 18 and 110 could only be labeled with biotin-HPDP after the protein had been denatured in the presence of DTT (Figure 2C). This result confirms that the cysteines in this mutant protein were efficiently oxidized into a disulfide bond. In the case of the PhoE mutants with cysteines at positions 110 and 302, or 111 and 302, only a single band was detected, which increased in intensity when the samples were denatured in the presence of DTT (Figure 2C). The monomers with the higher mobility on gels (Figure 2A) were never visualized with the biotin-labeled, thiol-specific reagent. Together, the results show that the anticipated disulfide bonds were indeed formed in the mutant proteins.

Time course of disulfide bond formation

The time course of the disulfide bond formation in the double cysteine PhoE mutants was determined in pulse-chase experiments. Plasmid-containing derivatives of strain CE1224 expressing the varying (mutant) PhoE proteins were pulse-labeled, immunoprecipitated and the formation of the disulfide bond was followed during the chase. In all three PhoE mutants, the disulfide bond was formed very rapidly since the oxidized forms were detected on the autoradiogram of SDS-polyacrylamide gels in the samples taken directly after the pulse. During the subsequent chase, no or hardly any increase in the amount of the oxidized forms was observed (Figure 3A). To study the kinetics of the assembly of the mutant PhoE proteins into their native conformation, samples from the same pulse-chase experiments were treated with trypsin. Small amount of trypsin-resistant PhoE forms were already detected directly after the pulse, but these amounts drastically increased during

the chase (Figure 3B). Apparently, disulfide bond formation in the mutant PhoE proteins is very rapid and precedes the maturation into the trypsin-resistant form.

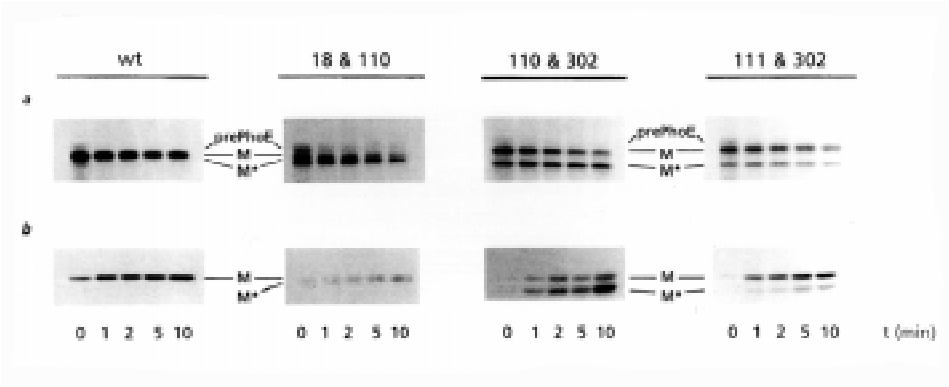

Fig. 3. Time course of the formation of the disulfide bonds and the assembly of the mutant PhoE proteins into a trypsin-resistant form *in vivo*. (A) The time course of disulfide bond formation in CE1224 cells expressing the PhoE mutants was studied in pulse-chase experiments. Cells were starved for phosphate and pulse-labeled for 30 s with [^{35}S]methionine, followed by chase periods as indicated. After immunoprecipitation with a polyclonal antiserum directed against PhoE, samples were boiled in sample buffer without DTT and analyzed by SDS-PAGE, followed by autoradiography. (B) The assembly of the PhoE mutants into a trypsin-resistant form. After various chase periods, samples of the cells were lysed and incubated in buffer containing trypsin. After adding Trypsin Inhibitor, the samples were boiled in sample buffer without DTT and analyzed by SDS-PAGE, followed by autoradiography. Indicated are the positions of the reduced monomeric (M), oxidized monomeric (M*) and precursor (prePhoE) forms of PhoE.

Involvement of DsbA in disulfide bond formation

To determine whether disulfide bond formation in the mutant PhoE proteins is stimulated by the periplasmic enzyme DsbA, the proteins were expressed overnight in P_i-limited medium (Levinthal *et al.*, 1962) in strain CE1224 and in its *dsbA* mutant derivative CE1442. Iodoacetamide (IAA) was added after isolation of the cells to prevent oxidation of the cysteines during the following procedures. The cell envelope protein patterns of the cells were analyzed by SDS-PAGE (Figure 4). The oxidized form of the mutant proteins with cysteines at positions 18 and 110, or at 110 and 302 could not be detected after expression in the *dsbA* mutant. A disulfide bond was still formed in the

case of the mutant protein with cysteines at positions 111 and 302 although with a reduced efficiency. The residual disulfide bond formation in this case might be explained by spontaneous oxidation or by the activity of other enzymes that catalyze disulfide bond formation, such as DsbC (Missiakas *et al.*, 1994). These results demonstrate that the periplasmic enzyme DsbA stimulates the formation of the disulfide bonds in the mutant PhoE proteins.

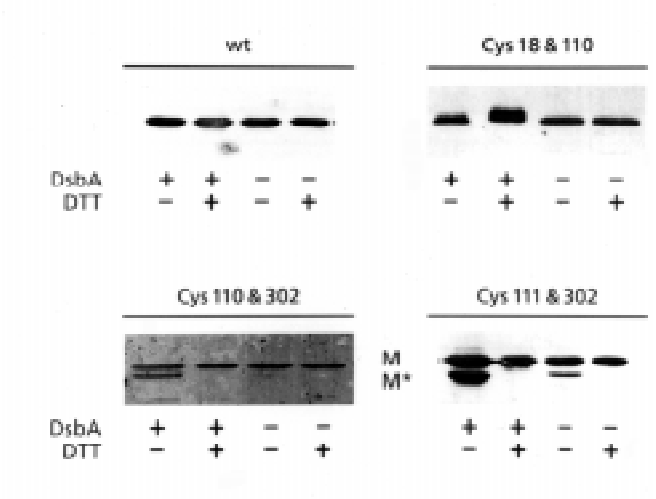

Fig. 4. Involvement of DsbA in the formation of the disulfide bonds in the mutant PhoE proteins. After overnight growth, cell envelopes were isolated from derivatives of strain CE1224 and of *dsbA* mutant CE1442 expressing (mutant) PhoE proteins, and dissolved in sample buffer either with or without DTT. After heating for 10 min at 95°C, samples were loaded onto gels, which in the case of wild-type PhoE and the mutant with cysteines at position 18 and 110 contained 8 M urea, and analyzed by Western blotting using a monoclonal antibody directed against PhoE. The reduced and oxidized forms of the mutant PhoE proteins are indicated by M and M*, respectively.

Discussion

OMPs are sorted to their final destination after cleavage of the signal sequence. Repeatedly, it has been postulated that these proteins pass through the periplasm on their way to the outer membrane, and that folding precedes outer membrane insertion (*e.g.* Tommassen, 1986). However, definite proof for this model has so far not been described. Here, by making use of the known 3D-structure of the outer membrane protein PhoE (Cowan *et al.*,

1992), we were able to engineer artificial disulfide bonds into this protein. Our results show that the formation of the disulfide bonds depends on the periplasmic enzyme DsbA. Previously, it has been demonstrated that DsbA catalyzes the formation of a disulfide bond in another OMP, *i.e.* OmpA (Bardwell *et al.*, 1991). However, the two cysteines in OmpA are located in the C-terminal part of OmpA, which is supposed to extend in the periplasm (Morona *et al.*, 1984; Vogel and Jähnig, 1986). Hence, from those experiments it cannot be concluded that DsbA modifies the OmpA protein on its way to the outer membrane. In contrast, we created the disulfide bonds in PhoE at a position that is not expected to be accessible from the periplasm, once the protein is correctly assembled into the membrane. Since DsbA nevertheless stimulated disulfide bond formation in the mutant PhoE proteins, our results prove that the protein passes through the periplasm on its way to the outer membrane. Furthermore, since the DsbA protein creates an element of tertiary structure within the mutant PhoE proteins, these proteins are at least partially folded before they insert into the outer membrane.

It was important to establish whether the mutant proteins were properly assembled and localized into the outer membrane. This condition was met by demonstrating that all the mutant proteins formed trimers, that they were trypsin-resistant and functional as phage receptors. However, the temperature stability of the trimers of the mutant proteins with substitutions of Glu110 or Phe111 was somewhat decreased. These residues belong to a highly conserved sequence motif, PEFGG, at the tip of loop L3 (Jeanteur *et al.*, 1991). They are probably contributing to a hydrogen-bonding network with residues in the barrel wall (Karshikoff *et al.*, 1994). Disruption of these interactions by the mutations might affect the stability of the trimers.

A study of the time course of disulfide bond formation in pulse-chase experiments revealed that this process was very rapid. A large number of the mutant proteins were oxidized during the pulse, and the proportion of proteins with a disulfide bond did not or only marginally increased during the chase. Previously, pulse-chase experiments have revealed a similarly rapid rate of disulfide bond formation in alkaline phosphatase, β-lactamase and OmpA in a *dsbA*$^+$ strain, whereas this rate was severely retarded in a *dsbA* mutant (Bardwell *et al.*, 1991). In contrast, the maturation of the (mutant) PhoE proteins into their trypsin-resistant form was a slower process. Apparently, disulfide bond formation occurred early in the folding process, which is consistent with the idea that DsbA has no access to the cysteines once the protein

is correctly folded.

Our observations prove the existence of periplasmic intermediates of OMPs during their biogenesis. However, these periplasmic intermediates are not necessarily soluble in the periplasm but may remain associated with the periplasmic side of the inner membrane and fold by interaction with nascent LPS (Bolla *et al.*, 1988; de Cock and Tommassen, 1996). Since the DsbA protein fixates an element of tertiary structure in the mutant PhoE proteins, our results demonstrate that these proteins are indeed at least partially folded when they insert into the outer membrane. This is in agreement with the hydrophilic amino acid composition of PhoE (Overbeeke *et al.*, 1983) as well as of other OMPs, which expose hydrophobic domains only after folding into their tertiary structure. It will be interesting to determine, along similar lines as described here, whether also quaternary structure formation (trimerization) also precedes insertion into the outer membrane.

Experimental procedures

Strains, phages and growth conditions

The *E. coli* K-12 strains CE1224 and CE1265 (Tommassen *et al.*, 1983) are deleted for the *phoE* gene and do not produce the related OmpF and OmpC proteins as a result of *ompR* mutations. Strain CE1265 carries in addition a *phoR* mutation, resulting in constitutive expression of the *pho* regulon. Strain CE1442 is a *dsbA::kan* derivative of CE1224 and was constructed by P1 transduction (Miller, 1972) using strain JCB572 (Bardwell *et al.*, 1991) as a donor. Sensitivity of strains to the PhoE-specific phage TC45 (Chai and Foulds, 1978) was determined by cross-streaking. Bacteria were grown at 37°C under aeration in L-broth (Tommassen *et al*., 1983), in a synthetic medium in which the phosphate concentration could be varied (Tommassen and Lugtenberg, 1980) or in the phosphate-limited medium described by Levinthal *et al.* (1962). When necessary for plasmid maintenance, the antibiotics chloramphenicol (25 μg ml^{-1}), ampicillin (100 μg ml^{-1}), or kanamycin (25 μg ml^{-1}) were added to the media.

Plasmids and DNA manipulations

Plasmid pJP29 is derived from the cloning vector pACYC184 and carries

the *phoE* gene (Bosch *et al.*, 1986). Plasmid pVG1 is derived from the vector pBluescriptII SK(+)/T7 and also carries the *phoE* gene but lacks the signal sequence-encoding part (Van Gelder *et al.*, 1994). Plasmid DNA was isolated as described (Birnboim and Doly, 1979), followed by anion-exchange chromatography on Qiagen columns (Diagen, Düsseldorf, Germany). PCR fragments were isolated from low melting point agarose by using Qiaex (Diagen). Standard DNA manipulations were performed according to Maniatis *et al.* (1982). Restriction endonucleases, T4 DNA ligase and *Pfu* DNA polymerase were used according to the manufacturers' protocols (Fermentas and Stratagene). To obtain PhoE mutants, site-directed mutagenesis was used according to the three-primer PCR method described by Landt *et al*. (1990). Oligonucleotides were purchased from Isogen (Amsterdam). Either pJP29 or pVG1 were used as templates for the PCR-based mutagenesis, and restriction fragments of the PCR products containing the mutations were

Table 1. Plasmids used

Plasmids	Relevant characteristics[a]	Reference
pJP29	Cam^r, wild-type *phoE* gene	Bosch *et al*. (1986)
pVG1	Amp^r, wild-type *phoE* gene lacking signal sequence	Van Gelder *et al*. (1994)
pEE1	Cam^r, mutant *phoE* with K18C substitution	This study
pEE2	Cam^r, mutant *phoE* with E110C substitution	This study
pEE3	Cam^r, mutant *phoE* with F111C substitution	This study
pEE4	Cam^r, mutant *phoE* with D302C substitution	This study
pEE5	Cam^r, mutant *phoE* with K18C and E110C substitution	This study
pEE6	Cam^r, mutant *phoE* with E110C and D302C substitution	This study
pEE7	Cam^r, mutant *phoE* with F111C and D302C substitution	This study

[a]Cam^r and Amp^r indicate resistance to chloramphenicol and ampicillin, respectively.

substituted for the wild-type fragments in pJP29. The resulting plasmids, pEE1, pEE2, pEE3 and pEE4 (Table 1), encode mutant PhoE proteins with a single cysteine. Double-mutant plasmids were obtained by exchanging appropriate restriction fragments. Plasmid pEE5 was constructed by substituting the *Mlu*I-*Bgl*II fragment of pEE2 for the corresponding fragment in pEE1. The plasmids pEE6 and pEE7 were constructed by substituting the *Bgl*II-*Xba*I fragment of pEE4 for the corresponding segments of pEE2 and pEE3, respectively. Successful mutagenesis was confirmed by DNA sequence analysis using the T7 DNA Polymerase Sequencing Kit (Pharmacia).

Isolation and characterization of cell fractions

After overnight growth, cells were collected by centrifugation and resuspended in 50 mM Tris-HCl, 5 mM EDTA pH 8.0, supplemented when indicated with 100 mM IAA (Sigma). Cell envelopes were isolated by centrifugation after ultrasonic disintegration of the cells (Lugtenberg *et al.*, 1975). To test the accessibility of proteins in the cell envelope fractions for trypsin, the cell envelopes were resuspended in 1 ml 10 mM Tris-HCl, 10 mM $MgCl_2$ pH 8.0 containing 50 µg trypsin (Struyvé *et al.*, 1991). The samples were incubated on ice for 20 min, after which the cell envelopes were reisolated by centrifugation. Protein patterns of cell fractions were analyzed by SDS-PAGE (Lugtenberg *et al.*, 1975) using the sample buffer described by Lugtenberg *et al.* (1975), except that the β-mercaptoethanol was omitted or replaced when indicated by 20 mM DTT.

Isolation of the PhoE trimers

Trimers were isolated as described previously (Agterberg *et al.*, 1990). Briefly, cell envelope fractions were incubated for 30 min at 40°C in a buffer containing 2% SDS as described, except that the β-mercaptoethanol was omitted. Peptidoglycan-protein complexes were pelleted by ultracentrifugation and PhoE was dissociated from the peptidoglycan by incubation for 30 min at 40°C in the SDS buffer supplemented with 0.6 M NaCl. After ultracentrifugation, the trimers were precipitated from the supernatant with 66% ethanol and dissolved in a buffer containing 20 mM Tris-HCl pH 8.0 and 0.1% SDS. When appropriate, the trimers were denatured into monomers by heating for 10 min at 95°C (or different temperatures when indicated) in the absence or presence of 20 mM DTT. Proteins were analyzed by SDS-PAGE and Western immunoblotting (Agterberg *et al.*, 1990). The monoclonal antibody used to detect PhoE protein was mE2-1, which recognizes the denatured protein.

Pulse-chase experiments

To study the time course of disulfide bond formation in the mutant PhoE proteins, cells were grown under phosphate limitation at 37°C as described (Bosch *et al.*, 1986) to induce the expression of mutant PhoE proteins. Cells

were labeled with [^{35}S]methionine (10 µCi ml^{-1}) for 30 s at 37°C and subsequently chased with an excess of non-radioactive methionine. At various time points during the chase, samples were taken after which they were mixed with 100 mM IAA and incubated for 20 min at 0°C. The proteins in the samples were precipitated with 5% trichloroacetic acid and PhoE forms were immunoprecipitated as described by Bosch *et al.* (1989) with slight modifications. Briefly, the pellet obtained after TCA precipitation was dissolved in 15 µl 2% SDS, 50 mM Tris-HCl, 1 mM EDTA pH 8.0 and boiled for 10 min. Subsequently, 485 µl Triton buffer containing 2% Triton X-100, 50 mM Tris-HCl, 0.15 M NaCl, 0.1 mM EDTA pH 8.0 was added. After centrifugation for 15 min in the Eppendorf centrifuge, a polyclonal antiserum directed against denatured PhoE was added to the supernatant. After overnight incubation under gentle shaking at 4°C, 2.5 mg protein A-sepharose Cl-4B (Pharmacia), dissolved in Triton buffer, was added and incubated for 1 h at room temperature under gentle shaking. The pellet obtained after centrifugation for 1 min in an Eppendorf centrifuge was washed twice in Triton buffer and once in 10 mM Tris-HCl pH 8.0, and finally resuspended in sample buffer without DTT, boiled and analyzed by SDS-PAGE and autoradiography. To analyze the time course of the assembly of the mutant PhoE proteins into a trypsin-resistant form, samples from the pulse-chase experiments were mixed with IAA and frozen at -20°C. Subsequently, the samples were thawed in the presence of 5% Triton X-100, 10 mM EDTA, 125 mM Tris-HCl pH 8.0 to which 15 mg ml^{-1} trypsin (Serva) was added. These mixtures were incubated at 0°C for 30 min. After adding a 3-fold excess of Trypsin Inhibitor (Serva) in 50 mM Tris-HCl, 20 mM $MgCl_2$ pH 8.0, membranes were pelleted by 30 min centrifugation at 15000 rpm in an Eppendorf centrifuge. The pellet was resuspended in sample buffer and boiled for 10 min before SDS-PAGE and autoradiography.

Detection free thiols

To detect free thiols in the mutant PhoE proteins, isolated trimers were incubated for 10 min at 95°C in the presence or absence of DTT as described. Subsequently, 20 mM phosphate-buffered saline (PBS), 10 mM EDTA, supplemented with 0.8 mM biotin-HPDP (Pierce) was added, and the samples were incubated for 1 h at room temperature. Subsequently, the proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters (Schleicher

and Schuell, 0.45 µm) using a semi-dry electroblotting apparatus (2117 Multiphor II, LKB). Streptavidin-horseradish peroxidase was used to detect biotin-labeled proteins. The peroxidase activity was developed with a solution of 4-chloro-1-naphtol (0.5 mg ml^{-1}) in 15% MeOH, 85% PBS and 0.01% H_2O_2.

Acknowledgements

We thank J.C.A. Bardwell and M. Kleerebezem for providing strains and monoclonal antibodies, respectively, and H. de Cock and P. Van Gelder for stimulating discussions. The investigations were supported by the Life Sciences Foundation (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

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CHAPTER 3

Insertion of pre-assembled trimeric porins into the bacterial outer membrane *in vivo*

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Insertion of pre-assembled trimeric porins into the bacterial outer membrane *in vivo*

Summary

Trimerization is a late step in the biogenesis of bacterial outer membrane porins and might either precede membrane insertion or occur in the outer membrane after insertion of the subunits. To distinguish between these possibilities, we have engineered an intermolecular disulfide bridge between the subunits of the porin PhoE of *Escherichia coli* at a position that is not exposed to the periplasm once the protein is correctly assembled. The mutations did not interfere with the biogenesis of the protein, and disulfide bond formation appeared to depend on the periplasmic oxidoreductase DsbG. Therefore, we conclude that the protein trimerizes in the periplasm and inserts as an assembled trimer into the outer membrane.

Introduction

The outer membrane of *Escherichia coli* protects the cell against harmful agents, such as antibiotics, bile salts and phospholipases. Its permeability largely depends on a number of abundant trimeric proteins that form non-

specific channels and are designated porins. One of these porins is the PhoE protein, the synthesis of which is induced under phosphate limitation. The 3D-structure of PhoE has been resolved (Cowan *et al.*, 1992). Each monomer of PhoE consists of a 16-stranded β-barrel with short turns at the periplasmic side and long loops at the cell surface. One of these loops, L3, forms a constriction within the barrel at half the height of the membrane. The subunits are connected via numerous hydrophobic and hydrophilic interactions, which determine the extreme stability of the trimers (Cowan *et al.*, 1992). Large hydrophobic residues pack together along the symmetry axis of the trimer. Loop L2 contributes to the stability of the trimer by making numerous hydrophilic intersubunit interactions (Van Gelder *et al.*, 1996; Phale *et al.*, 1998). This loop bends over to a neighboring subunit filling the gap in the barrel wall left by the internal loop L3.

Outer membrane proteins (OMPs) are synthesized in the cytoplasm as precursors with an N-terminal signal sequence, which is required for the translocation across the inner membrane via the Sec machinery (for a review, see Danese and Silhavy, 1998). After translocation, the signal sequence is cleaved off by the inner membrane-located leader peptidase, and the mature protein appears on the periplasmic side of the membrane. The periplasm contains chaperones and folding catalysts, such as the disulfide bond isomerases (Dsb proteins) and the peptidyl-prolyl *cis/trans* isomerases (PPIases) (Missiakas and Raina, 1997). Indeed, some of these proteins, including Skp (Chen and Henning, 1996; de Cock *et al.*, 1999) and the PPIase SurA (Rouvière and Gross, 1996; Eppens *et al.*, submitted), have been shown to play a role in OMP biogenesis. However, the order of events after the appearance of OMPs in the periplasm is poorly understood. Somewhere along the pathway from inner to outer membrane, the proteins have to fold into the native β-barrel configuration, and oligomeric OMPs have to assemble into their oligomeric structure. Determination of the subcellular localization of assembly intermediates has been applied to gain insight in this process. However, so far, the results are not conclusive. A trimeric intermediate of OmpF has been detected in the periplasm in immunoprecipitation experiments using conformation-specific monoclonal antibodies (Fourel *et al.*, 1992). However, it has been demonstrated that the detergents that are used in immunoprecipitation experiments can induce the folding of OMPs (Van Gelder *et al.*, 1994), and therefore, it is not clear whether this periplasmic intermediate of OmpF was trimeric *in vivo*, or whether it folded *in vitro* during

the immunoprecipitation reactions. Misra *et al*. (1991) used a temperature-sensitive mutant form of LamB protein and detected a monomeric intermediate in the outer membrane, which could be converted into trimers upon a shift to the permissive temperature, suggesting that membrane insertion precedes trimerization. However, the possibility that these monomers arose from dissociation of metastable trimers can not be excluded. Finally, pre-assembled PhoE trimers inserted *in vitro* into outer membranes, supporting the presumption that trimerization precedes outer membrane insertion (de Cock and Tommassen, 1996), but the requirement for detergents in this assay questions the relevance of these results for the *in vivo* process.

In our laboratory, the porin PhoE is used as a model to study the biogenesis of OMPs. This protein does not contain any cysteines. Previously, we have addressed the question whether tertiary structure formation precedes the outer membrane insertion of PhoE protein (Eppens *et al.,* 1997). By making use of the known 3D-structure, disulfide bonds were introduced in PhoE at positions that are not accessible for periplasmic enzymes, once the protein is assembled into the outer membrane. Since the formation of these disulfide bonds was dependent on the periplasmic enzyme DsbA, the formation of (elements of) tertiary structure apparently preceded the membrane insertion of this protein (Eppens *et al.,* 1997). We have now applied the same approach to investigate whether quaternary structure is gained before the protein inserts into the membrane. To this end, cysteines were introduced at positions that are in close proximity at the subunit interface. Subsequently, we investigated whether disulfide bonds between the subunits of the PhoE trimer were formed, and whether this disulfide bond formation is stimulated by any of the periplasmic Dsb proteins. If so, this would demonstrate that PhoE is assembled into trimers in the periplasm prior to its insertion into the outer membrane.

Results

Construction and characterization of cysteine-containing mutant PhoE proteins

The crystal structure of PhoE (Cowan *et al.,* 1992) revealed that residue E66 of one subunit is at hydrogen bonding distance of D120 of another subunit within the trimeric complex (Fig. 1). Thus, the substitution of these

residues by cysteines could result in the formation of intersubunit disulfide bonds. PhoE mutant proteins with single cysteine substitutions for E66 and D120, respectively, were obtained by site-directed mutagenesis, and the double mutant E66C/D120C was constructed by exchanging the appropriate restriction fragments. Subsequently, the single and double mutant PhoE

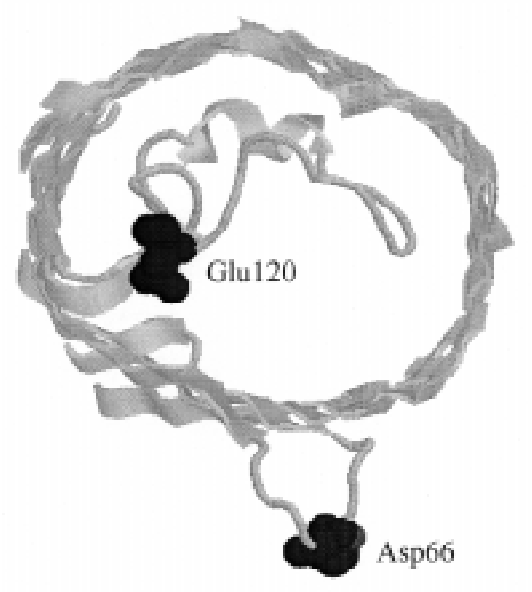

Fig. 1. Rasmol (Sayle and Milner-White, 1995) presentation of Swiss-model structure (Peitsch, 1995) of wild-type PhoE. Indicated are residues E66 at the tip of loop L2 and D120 at the root of the third loop, which were replaced by cysteines to create an intermolecular disulfide bond between two adjacent monomers. All loops except L2 and L3 are omitted for clarity, and the molecule is viewed from the surface-exposed side.

proteins were characterized. The expression level of the plasmid-encoded mutant proteins E66C and E66C/D120C in *phoR phoE* mutant strain CE1265 appeared unaffected as compared with that of the wild-type protein, whereas that of the mutant protein D120C was somewhat reduced (data not shown). The correct insertion of PhoE in the outer membrane was assessed in a phage-sensitivity assay. Cells expressing wild-type PhoE protein are sensitive to the PhoE-specific phage TC45, which requires residues in the surface-exposed loops L2 and L4 of PhoE as binding site (Bosch and Tommassen, 1987; van der Ley *et al.*, 1987). Cells expressing the single mutant PhoE proteins E66C and D120C were as sensitive toward phage TC45 as cells expressing the wild-type PhoE protein were, indicating that these mutant proteins were properly inserted into the outer membrane. Furthermore, even although E66 is located in loop L2, it does apparently not contribute to the phage-binding site. However, when TC45 was plated on cells expressing the double mutant protein E66C/D120C, an approximately 100-fold reduced efficiency of

plating was observed. The results show that at least a proportion of the total amount of each mutant protein was correctly assembled into the outer membrane. The decreased sensitivity of the double mutant might be caused by a slight conformational change due to disulfide bond formation between C66 at the tip of loop L2 and C120 at the root of loop L3 of the adjacent subunit.

Intersubunit disulfide bond formation

The correct assembly of the mutant PhoE proteins was further assessed in protease-sensitivity assays. Correctly assembled wild-type PhoE is resistant to trypsin (Tommassen and Lugtenberg, 1984). Cell envelopes were isolated from cells, which were preincubated with iodoacetamide (IAA) to prevent disulfide bond formation during sample preparation, and subjected to trypsin treatment. The samples were heated at 100°C in sample buffer in the absence or presence of DTT. The wild-type and all three mutant PhoE proteins appeared to be resistant to trypsin treatment (Fig. 2), demonstrating that they were correctly assembled into the outer membrane. In the case of the double mutant, two major bands of approximately 120 kDa and 80 kDa, as well as

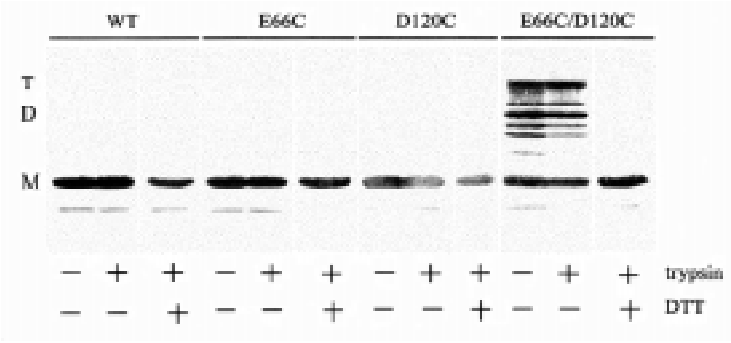

Fig. 2. Western blot analysis of cysteine-containing mutant derivatives of PhoE protein. Cell envelopes were isolated from plasmid-containing derivatives of strain CE1265 expressing mutant PhoE proteins. Samples were treated with trypsin when indicated and, subsequently, incubated for 10 min in sample buffer with or without DTT at 100°C prior to SDS-PAGE. After electrophoresis, the proteins were blotted and PhoE was detected with a PhoE-specific monoclonal antibody and alkaline phosphatase-conjugated goat-anti-mouse IgG (GAMAP). T, D, M indicate trimers, dimers and monomers of PhoE, respectively.

some minor bands, were detected in addition to the 40 kDa denatured monomeric PhoE. Since these bands disappeared upon incubation with DTT (Fig. 2), the 120 kDa and 80 kDa bands most likely represent trimeric and dimeric forms of PhoE, connected by intersubunit disulfide bonds. Both of these oligomeric bands were protected against the trypsin digestion (Fig. 2), showing that they were correctly assembled in the outer membrane. The disulfide bonds were formed with an efficiency of 30 to 40%. The amount of the additional bands varied from sample to sample. These bands most likely represent degradation products, which arose during sample preparation.

Role of DsbG in formation disulfide bond in mutant PhoE protein

To study whether the formation of the disulfide bonds between the monomers was catalyzed by any of the periplasmic Dsb proteins, the plasmid encoding the double-cysteine mutant PhoE was introduced in *dsbA*, *dsbC* and *dsbG* mutant strains and in their corresponding parental strains. Cell envelopes were isolated after growth of the cells under phosphate-limitation and analyzed by SDS-PAGE and Western blotting after boiling the samples in the presence or absence of DTT (Fig. 3). The absence of the periplasmic

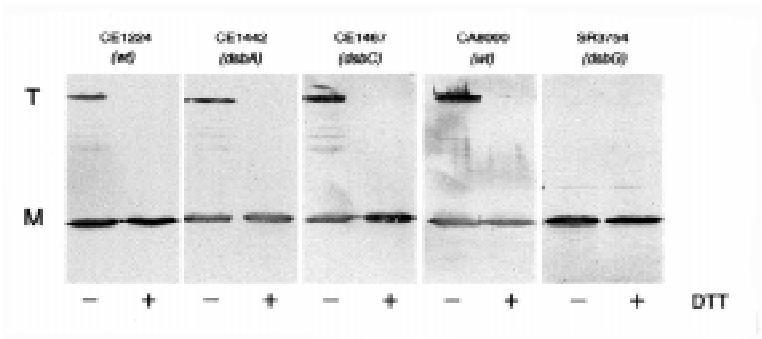

Fig. 3. Involvement of DsbG in the formation of the disulfide bond in the E66C/D120C mutant PhoE protein. After overnight growth, cell envelopes were isolated from the parental strains CE1224 and CA8000 and from *dsb* mutant derivatives of these strains, each expressing the E66C/D120C mutant PhoE protein. The samples were dissolved in sample buffer with or without DTT and heated for 10 min at 100°C. The samples were subsequently analyzed by SDS-PAGE and blotted, after which PhoE was detected by incubation with a PhoE-specific monoclonal antibody and GAMAP. The denatured trimers and monomers are indicated by T and M, respectively.

DsbA and DsbC proteins had no effect on the formation of the disulfide bonds between the PhoE subunits. However, the absence of the DsbG protein strongly affected the efficiency of the formation of the disulfide bonds (Fig. 3). Since the formation of the intersubunit disulfide bonds was apparently catalyzed by a periplasmic enzyme, we conclude that the assembly of the trimeric conformation of PhoE precedes outer membrane insertion.

Discussion

After the translocation of porin molecules across the inner membrane, the mature protein has to insert into the outer membrane and to adopt its native trimeric structure. Formation of the β-barrel structure has always been suggested to be a prerequisite for outer membrane insertion. Whereas α-helical membrane-spanning segments of polytopic inner membrane proteins can insert independently of other membrane-spanning segments into the membrane, a β-strand of an OMP with its amphiphilic character is expected to be incompetent to insert independently into the outer membrane. Indeed, by creating an intersubunit disulfide bond in PhoE and demonstrating that the formation of this bond is dependent on the periplasmic enzyme DsbA, we have been able to demonstrate that tertiary structure formation precedes membrane insertion (Eppens *et al.*, 1997). Consistently, the formation of a folded monomer of LamB has been reported to be assisted by the periplasmic peptidyl-prolyl *cis/trans* isomerase SurA (Rouvière and Gross, 1996). The subsequent transition from folded monomer to trimer might either precede outer membrane insertion or take place after insertion of the folded monomer. The results reported in the present study reveal that even quaternary structure is formed before the porins insert into the outer membrane. Based on the known 3D-structure of PhoE, we were able to engineer an artificial disulfide bond between the subunits of PhoE that is not accessible from the periplasm once the trimer is inserted into the outer membrane. The formation of this bond required the catalytic activity of the periplasmic DsbG protein. Hence, the disulfide bonds must have been formed in a periplasmic assembly intermediate, which demonstrates that trimerization precedes membrane insertion.

DsbG is an oxidoreductase, but its role in disulfide bond formation, reduction or isomerization remained so far unknown. It has been reported to act as a periplasmic disulfide isomerase like DsbC (Bessette *et al.*, 1999), but

oxidizing activity has been attributed to DsbG as well (Andersen *et al.,* 1997; van Straaten *et al.,* 1998). DsbA and DsbC proved not to be involved in the formation of the disulfide bond between the PhoE monomers. This is in agreement with the notion that DsbA preferentially binds to unfolded proteins (Frech *et al.,* 1996) and that DsbC is required for the isomerization of wrongly paired disulfide bonds (Sone *et al.,* 1997). DsbG might catalyze the disulfide bond formation between the folded subunits of the double mutant PhoE directly. Alternatively, DsbG could be involved in maintaining the proper redox state of a so far unknown Dsb protein, which catalyzes disulfide bond formation between folded structures.

This study revealed that PhoE trimerizes in the periplasm prior to membrane insertion. Nevertheless, trimerization could be a membrane-associated event. It is conceivable that the formation of this oligomeric conformation is localized at the periplasmic side of the outer membrane. *In vitro* studies reported the requirement of outer membranes during the trimerization (de Cock *et al.,* 1996). These membranes might function as a site for concentrating the folded monomers.

Experimental procedures

Strains, phages and growth conditions

The bacterial strains used are listed in Table 1. Sensitivity of strains to the PhoE-specific phage TC45 was determined by plague formation (Chai and Foulds, 1978). Bacteria were grown at 37°C under aeration in L-broth (Tommassen *et al*., 1983) or in the phosphate-limited medium described by Levinthal *et al*. (1962). For plasmid maintenance, the antibiotic chloramphenicol (25 μg ml^{-1}) was added to the media. For growth of the *dsbG* mutant strain SR3754 and, in control experiments, of its parental strain CA8000, the media were supplemented with oxidized DTT (5 mM; Sigma).

Plasmids and DNA manipulations

Plasmid pJP29 is derived from cloning vector pACYC184 and carries the *phoE* gene (Bosch *et al*., 1986). Plasmid DNA was isolated as described (Birnboim and Doly, 1979), followed by anion-exchange on Jetstar columns (Genomed Inc.). DNA fragments were isolated from agarose gel using the

Table 1. Strains

Strains	Relevant characteristics	Reference
CE1224	F-, *thr leu Δ(proA-proB-phoE-gpt) his thi argE lacY galK xyl rpsL supE ompR*	Tommassen *et al.,* 1983
CE1265	F-, *thi Δ(proA-proB-phoE) pyrF argG ilvA tonA phx rpsL sup recA56 vtr glpR ompR phoR18*	Korteland *et al.,* 1985
CE1442	CE1224 *dsbA::kan*	Eppens *et al.,* 1997
CE1467	CE1224 *dsbC::kan*	Braun *et al.,* submitted
CA8000	HrfH *thi*	Phabagen collection
SR3754	CA8000 *dsbGΩtet*	Raina laboratory collection

JetSorb Gel Extraction Kit 600 (Genomed Inc.). Standard DNA manipulations were performed according to Maniatis *et al*. (1982). Restriction endonucleases, T4 DNA ligase and *Pwo* DNA polymerase were used according to the manufacturers' protocols (Fermentas and Boehringer Mannheim). To obtain mutant *phoE* genes, site-directed mutagenesis was applied using the QuickChange site-directed mutagenesis kit (Stratagene). Oligonucleotides were purchased from Gibco BRL Life Technologies. Plasmid pJP29 was used as the template for the PCR-based mutagenesis. The resulting plasmids, pEE11 and pEE12, encode mutant PhoE proteins with E66C and D120C substitutions, respectively. The double-mutant plasmid pEE13 was constructed by substituting the *Pst*I-*Mlu*I fragment of pEE11 for the corresponding fragment in pEE12. Successful mutagenesis was confirmed by DNA sequence analysis using the Dye Terminator Cycle Sequencing Kit and the ABI 310 Automated DNA Sequencer (Perkin Elmer Co.) according to the manufacturers' instructions.

Isolation and characterization of cell fractions

After overnight growth, cells were collected by centrifugation and resuspended in 50 mM Tris-HCl, 5 mM EDTA pH 8.0, supplemented when indicated with 100 mM IAA (Sigma). Cell envelopes were isolated by centrifugation after ultrasonic disintegration of the cells (Lugtenberg *et al.*, 1975). To test the accessibility of proteins in the cell envelope fractions for trypsin, the cell envelopes were resuspended in 1 ml of 10 mM Tris-HCl, 10 mM $MgCl_2$ pH 8.0 containing 50 µg trypsin (Struyvé *et al.,* 1991). The samples were incubated on ice for 20 min, after which the cell envelopes were reisolated by centrifugation. Protein patterns of cell fractions were

analyzed by SDS-PAGE (Lugtenberg *et al*., 1975) using the sample buffer described by Lugtenberg *et al.* (1975), except that the β-mercaptoethanol was omitted or replaced, when indicated, by 20 mM DTT. Proteins were analyzed by SDS-PAGE and Western immunoblotting (Agterberg *et al.*, 1990). The monoclonal antibody used to detect PhoE protein was mE2-1, which recognizes the denatured protein.

Acknowledgements

We thank C. Ockhuijsen for technical assistance and F. Asma (N.C.C. Phabagen collection, Utrecht University) for providing strain CA8000. The continuous interest and helpful suggestions of H. de Cock and H. Adams were very much appreciated. The investigations were supported by the Life Sciences Foundation (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

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CHAPTER 4

Chaperone function of the periplasmic peptidyl-prolyl *cis/trans* isomerase SurA of *Escherichia coli*

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Chaperone function of the periplasmic peptidyl-prolyl *cis/trans* isomerase SurA of *Escherichia coli*

Summary

SurA is a periplasmic protein of *Escherichia coli* with peptidyl-prolyl *cis/trans* isomerase (PPIase) activity. It has been shown to be involved in the biogenesis of outer membrane proteins (OMPs). However, since SurA only marginally contributes to the total PPIase activity in the periplasm, the possibility that the role of SurA in OMP biogenesis is not correlated to its PPIase activity was investigated. To address this question a mutant form of the porin PhoE was constructed in which all prolines were replaced by alanines. The substitutions did not affect the assembly of the protein into the outer membrane. However, the substitution of Pro109, which is located in a highly conserved PEFGG motif at the tip of the constriction loop L3, affected pore characteristics, such as single conductance and voltage gating, in planar lipid bilayer experiments. The kinetics of the folding into a trypsin-resistant conformation of the wild-type and the proline-less mutant PhoE were studied in pulse-chase experiments in a *surA* mutant strain and its parental strain. The rate of folding of both proteins appeared to be similarly affected by the *surA* mutation. These results demonstrate that SurA has a function in addition

to its PPIase activity in the assembly of OMPs.

Introduction

The folding of a protein into its final 3D-structure is generally determined by its amino acid sequence. Nevertheless, protein folding is an assisted process *in vivo*. Two functionally entirely different types of proteins that assist in protein folding can be distinguished. The general chaperones, such as GroEL and DnaK, prevent misfolding and aggregation by binding to incompletely folded polypeptides. These proteins do not usually accelerate the folding kinetics (Ellis and van der Vies, 1991). The second type of helper proteins encompasses folding catalysts, which function by accelerating a specific, rate-limiting step in the folding process. In the periplasm of Gram-negative bacteria, two classes of folding catalysts can be distinguished. The Dsb catalytic system accelerates the formation of disulfide bonds in extracytoplasmic proteins. The Dsb proteins characterized sofar, *i.e.* DsbA, DsbB, DsbC, DsbD, DsbE and DsbG, are either involved in the formation, the isomerization or the reduction of disulfide bonds (Rietsch and Beckwith, 1998). The second class of folding catalysts consists of peptidyl-prolyl *cis/trans* isomerases (PPIases). Many different PPIases have been identified in the periplasm of *E. coli*, *i.e.* the cyclophilin RotA (Liu and Walsh, 1990), the FK506-binding protein FkpA (Horne and Young, 1995) and the parvulins SurA and PpiD, which is an inner membrane protein but its catalytic domain faces the periplasm (Lazar and Kolter, 1996; Missiakas *et al.*, 1996; Rouvière and Gross, 1996; Dartigalongue and Raina, 1998). SurA was initially identified as a protein required for survival during stationary phase (Tormo *et al.*, 1990; Lazar *et al.*, 1998). Subsequently, SurA was identified as a PPIase (Lazar and Kolter, 1996; Missiakas *et al.*, 1996; Rouvière and Gross, 1996) and *surA* mutants were found to be defective in the assembly of the outer membrane proteins (OMPs) OmpA, OmpF and LamB (Lazar and Kolter, 1996). More specifically, the conversion of the unfolded monomer of LamB into the folded monomer was found to be affected in *surA* mutant cells (Rouvière and Gross, 1996). In contrast, the folding of periplasmic proteins was not affected by the *surA* mutation. The observation that *surA* mutants have such a distinct phenotype was somewhat surprising to us, because of the presence in the periplasm of many other PPIases, which could potentially take over the SurA function. Moreover, the inactivation of *rotA*, which reduced

the total PPIase activity in the periplasm to barely detectable levels, did not result in any phenotype (Kleerebezem *et al.,* 1995). Therefore, we considered the possibility that SurA is not (only) functioning as a PPIase, but that it might have an additional function in the assembly of OMPs, which cannot be taken over by other PPIases. To address this question, we have constructed a mutant PhoE protein in which all prolines are replaced by alanines and studied the influence of a *surA* mutation on the biogenesis of this protein. Furthermore, since one of the proline residues substituted is located in the highly conserved PEFGG motif at the tip of the constriction loop L3 of the PhoE porin, the pore characteristics of this mutant protein were studied.

Results

SurA-dependency of PhoE biogenesis

The periplasmic PPIase encoded by the *surA* gene has been shown to be involved in the biogenesis of several OMPs, including OmpF, LamB and OmpA (Lazar and Kolter, 1996; Rouvière and Gross, 1996). To investigate whether this protein is also required for the folding of the porin PhoE, which is used as a model protein for studying OMP biogenesis in our laboratory, pulse-chase experiments were performed. The PhoE protein was expressed in strain CE1224 and in its *surA* mutant derivative CE1469 from plasmid pNN100, which contains the *phoE* gene under control of the inducible *tac*-promoter. After IPTG induction, cells were pulse-labeled and the kinetics of the assembly of PhoE into its native, trypsin-resistant conformation were studied. In strain CE1224, trypsin-resistant PhoE was already detected directly after pulse-labeling, and its amount increased during the chase and reached its maximum after a 2 min chase period (Fig. 1A). In the *surA* mutant strain, a delay in the formation of trypsin-resistant PhoE was observed and only after a 10 min chase period, the amount of trypsin-resistant PhoE was maximal (Fig. 1A). The delay in the formation of trypsin-resistant PhoE in the *surA* mutant strain most likely reflects a reduced rate of folding of the protein, but could also be explained by a delay in elongation during protein synthesis. To investigate this possibility, pulse-labeled samples were immunoprecipitated with an antiserum directed against PhoE in order to determine the total amount of full-length protein present at each stage. Evidently, less PhoE was synthesized in the *surA* mutant strain than in the wild-type strain, but in both

cases, the maximal amount of full-length protein was obtained after a 1 min chase period (Fig. 1B). Therefore, we conclude that the periplasmic PPIase SurA is required for the efficient folding of PhoE.

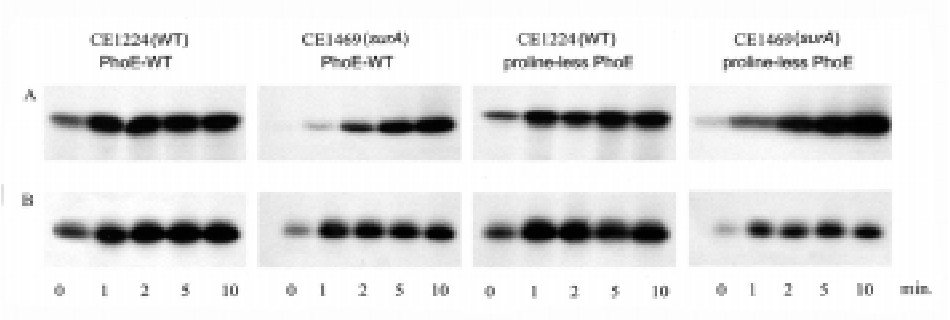

Fig. 1. Time course of the synthesis and of the formation of trypsin-resistant forms *in vivo* of PhoE and proline-less PhoE in wild-type and *surA* mutant strains. (A) The kinetics of the assembly into a trypsin-resistant form of the wild-type and the proline-less PhoE were studied *in vivo* in pulse-chase experiments in strain CE1224 and in its *surA* mutant derivative CE1469. Cells, containing either pNN100 (wild-type PhoE) or pEE26 (proline-less PhoE), were grown until mid-exponential phase and subsequently induced with IPTG and pulse-labeled for 45 sec with [^{35}S]methionine followed by chase periods as indicated. Samples of the cells were lysed and incubated in buffer containing trypsin. After adding trypsin inhibitor, the samples were boiled in sample buffer and analyzed by SDS-PAGE, followed by autoradiography. (B) The time course of the synthesis of wild-type and proline-less PhoE. After various chase periods, PhoE was immunoprecipitated from the samples with a polyclonal antiserum, and the immunoprecipitates were analyzed by SDS-PAGE, followed by autoradiography.

Construction and characterization of mutant PhoE proteins with alanine for proline substitutions

To determine whether PhoE biogenesis is dependent on the PPIase activity of SurA or on any other activity of this protein, alanines were substituted for all three prolines present in the amino acid sequence of PhoE. Thus, three single mutant PhoE proteins P109A, P235A and P261A were obtained and, subsequently, a triple mutant was constructed with alanines at the positions of all three prolines. All proteins were efficiently expressed in strain CE1224 and incorporated in the cell envelopes in a trypsin-resistant trimeric conformation (data not shown). Furthermore, expression of the mutant PhoE proteins in the *phoR phoE* mutant strain CE1265 conferred sensitivity to the

cells to the PhoE-specific phage TC45 (data not shown), which confirms the correct assembly of the proteins into the outer membrane. Only a slight decrease in the stability of the triple mutant protein was noticed upon incubation in sample buffer, containing 2% SDS. Whereas the wild-type PhoE trimers and the single mutants P109A, P235A and P261A denatured into monomers under these conditions only by incubation at temperatures above 65°C, the trimers of the triple mutant protein denatured already between 60-65°C.

Since the substitution of Pro109 by Ala does apparently not affect the biogenesis of the protein, even though it is part of a PEFGG motif, which is highly conserved in a large superfamily of bacterial porins (Jeanteur *et al.*, 1991), we considered the possibility that this residue is important for pore functioning, which would be consistent with its location at the tip of the constriction loop L3. Therefore, the pore characteristics of this mutant protein were studied *in vitro* in lipid bilayer experiments (Table 1). Indeed, replacement

Table 1. Electrical properties of wild-type and mutant porin channels in planar lipid bilayers

PhoE form	conductance (nS)		critical voltage (mV)	
wild-type	0.92 ± 0.07	(n=178)	139 ± 9	(n= 6)
P109A	0.15 - 2.30[a]	(n=184)	> 230	(n=18)
P235A	0.96 ± 0.21	(n= 58)	127 ± 7	(n=18)
P261A	0.94 ± 0.15	(n=167)	129 ± 10	(n=18)

[a]This mutant porin showed a huge heterogeneity in conductance steps.

of the P109 residue drastically affected the pore properties. Whereas the wild-type PhoE protein showed rather uniform conductance steps (Table 1), the P109A substitution resulted in a heterogeneous population of pores with conductances ranging from 0.15 nS to 2.3 nS (see Fig. 2 for examples). Furthermore, while the wild-type pores were closed at a high transmembrane potential (Table 1), as previously reported (Delcour, 1997), the P109A mutant pores could not be closed by applying transmembrane potentials of up to 230 mV (Fig. 3). No effects of the P235A and P261A substitution were anticipated and this was confirmed by the results of the lipid bilayer experiments, which showed similar single channel conductances and voltage gating of the P235A and P261A mutant porins as of the wild-type porin (Table 1).

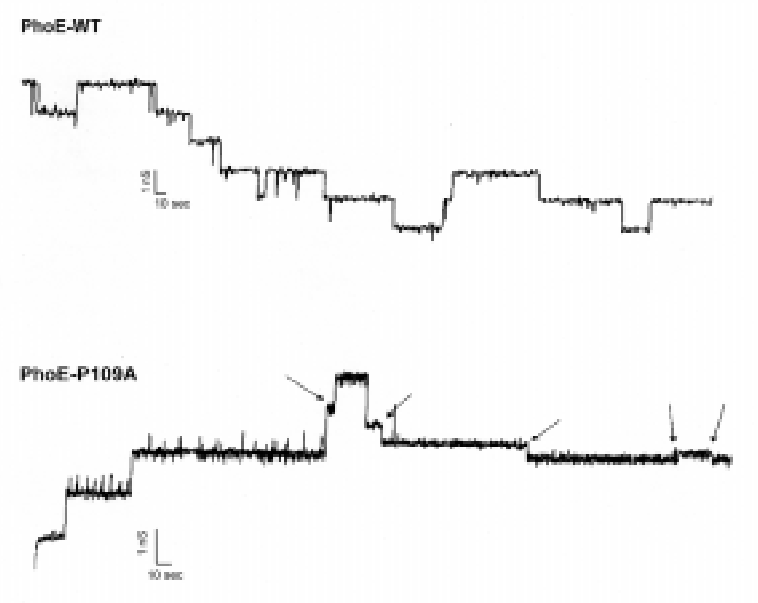

Fig. 2. Conductance recordings of wild-type PhoE pores and P109A mutant pores at a membrane potential of 100 mV. Whereas the wild-type pores show channel openings and closings of uniform size, the mutant pores show a large variety of conductance steps. Indicated by arrows are the smaller conductance steps.

SurA-dependency of the biogenesis of the proline-less mutant PhoE

If SurA is required for OMP biogenesis only because of its PPIase activity, the proline-less triple mutant PhoE would fold with similar kinetics into its final conformation in the presence or absence of SurA. To test the SurA-dependency of the triple mutant protein, this construct was expressed in the wild-type strain CE1224 and in its *surA* mutant derivative strain CE1469, and folding of the protein into its trypsin-resistant conformation was followed in pulse-chase experiments. In the wild-type strain, the triple mutant PhoE protein folded with similar kinetics into a trypsin-resistant conformation as the wild-type protein did, demonstrating that the mutations in the mutant protein do not affect its assembly (Fig. 1A). In the *surA* mutant strain, a delay was observed in the acquirement of the trypsin-resistant conformation of the triple mutant PhoE protein (Fig. 1A). This delay was not due to a reduced rate of protein elongation as revealed by immunoprecipitation, which showed that the total amount of radiolabeled PhoE did not further increase after a 1

min chase period (Fig. 1B). Since the PhoE mutant, in which all prolines are replaced by alanines, would not require any PPIase activity, the requirement for SurA during the assembly of the protein must reflect an additional function of SurA in OMP biogenesis.

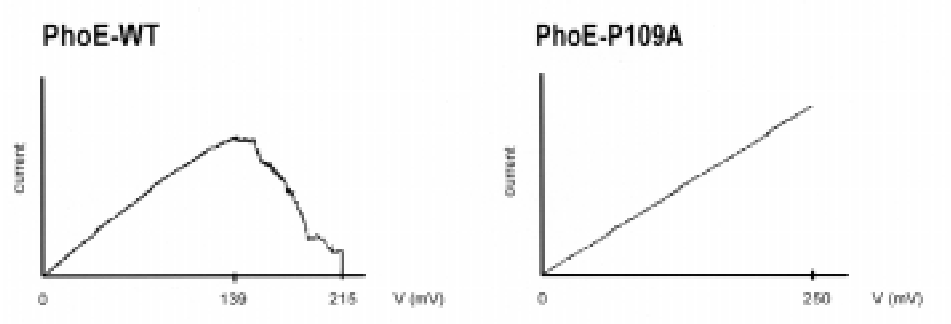

Fig. 3. Current to voltage curve of wild-type PhoE pores and P109A mutant pores. The wild-type pores close at a membrane potential of 139 mV, whereas the mutant pores could not be closed even if the potential was raised to 230 mV.

Discussion

The periplasmic protein SurA has been shown to be required during the biogenesis of the OMPs OmpF, OmpA and LamB (Lazar and Kolter, 1996; Rouvière and Gross, 1996). Here, we demonstrated that a *surA* mutation also affected the kinetics of the folding of the porin PhoE into a trypsin-resistant form. How could SurA exert its function in OMP biogenesis? It has been demonstrated previously that the protein stimulates the efficient conversion of the unfolded to the folded monomer of LamB (Rouvière and Gross, 1996). A slow step in the folding of proteins is the *cis/trans* isomerization of peptidyl-prolyl bonds, a process that is catalyzed by PPIases. Indeed, a low PPIase activity of SurA has been demonstrated *in vitro* (Missiakas *et al.*, 1996; Rouvière and Gross, 1996). However, SurA only marginally contributes to the total PPIase activity in the periplasm, and inactivation of the major PPIase, RotA, does not result in any detectable phenotype (Kleerebezem *et al.*, 1995). Hence, if the role of SurA in OMP biogenesis is correlated to its PPIase activity, the periplasmic PPIases have a high substrate specificity. Alternatively, SurA could have another function, in addition to its PPIase activity. To distinguish between a role for SurA as

PPIase or as chaperone, a mutant PhoE protein was constructed in which all three prolines were replaced by alanines. All single and the triple mutant proteins still formed trimers localized in the outer membrane, which were functional as pores and as phage receptor, although the temperature stability of the triple PhoE mutant was somewhat decreased. The substitution of the proline residue at position 109, which initiates the turn at the tip of the constriction loop L3 (Cowan *et al.,* 1992) and is part of the highly conserved PEFGG motif in a superfamily of bacterial porins, affected the single channel conductance and the voltage sensitivity of the pores in planar lipid bilayer experiments. The single channel conductances of the mutant pores were highly varying, suggesting structural heterogeneity. Furthermore, the channels could not be closed even if the potential was raised to 230 mV. Apparently, the replacement of the proline residue affects the architecture at the pore constriction site drastically, although the Swiss-model program did not predict a significant change in the backbone structure of the P109A mutant protein (data not shown). Previously, the deletion of the two glycine residues of the PEFGG motif was also shown to affect the single conductance and to result in a decreased voltage sensitivity (Van Gelder *et al.,* 1997).

In pulse-chase experiments, the kinetics of the assembly of the proline-less mutant PhoE protein into a trypsin-resistant conformation were determined. In wild-type cells, the mutant protein showed assembly kinetics comparable to the wild-type PhoE protein. Importantly, the assembly of the triple mutant was still affected by a *surA* mutation, demonstrating that an OMP, which is independent of PPIase activity for its folding, still requires the presence of SurA. Therefore, we propose that SurA, besides its activity as a PPIase, has an additional role as a chaperone in the folding of OMPs. Such a dual role of a folding catalyst, having an additional chaperone-like function, has previously been reported for enzymes involved in disulfide bond formation and reduction, including DsbA (Zheng *et al.,* 1997), thioredoxin (TrxA) (Russel and Model, 1986) and PDI (Song and Wang, 1995; Yao *et al.,* 1997). The combination of the properties of both a chaperone and a folding catalyst into a single protein might provide this protein with a high efficiency for folding (Wang and Tsou, 1998).

Experimental procedures

Strains, phages and growth conditions

The *E. coli* K-12 strains CE1224 (Tommassen *et al*., 1983) and CE1265 (Korteland *et al.,* 1985) are deleted for the *phoE* gene and do not produce the related OmpF and OmpC proteins as a result of *ompR* mutations. Strain CE1265 carries in addition a *phoR* mutation, resulting in the constitutive expression of the *pho* regulon. Strain CE1469 is a *surA::kan* derivative of CE1224 and was constructed by P1 transduction (Miller, 1972) using strain SR3199 (Raina laboratory collection) as a donor. Sensitivity of strains to the PhoE-specific phage TC45 (Chai and Foulds, 1978) was determined by cross-streaking. Bacteria were grown at 37°C under aeration in L-broth (Tommassen *et al.*, 1983) or in the phosphate-limited medium described by Levinthal *et al*. (1962). When necessary, the antibiotics chloramphenicol (25 μg ml^{-1}), ampicillin (100 μg ml^{-1}) or kanamycin (25 μg ml^{-1}) were added to the media.

Pulse-chase experiments

To study the time course of the biogenesis of wild-type and mutant PhoE proteins, cells were grown in L-broth at 37°C to an optical density at 660 nm of approximately 0.45. Cells were harvested (4000x*g*, 10 min), washed with methionine assays medium (Difco Laboratories) and finally resuspended in this medium. To induce the *tac*-promoter, 25 μM IPTG was added to the cells. After 45 min of induction, cells were labeled with Tran^{35}S-label (ICN Biomedicals Inc., Costa Mesa) for 45 s at 37°C and subsequently chased with an excess of non-radioactive methionine. At various time points during the chase, samples were taken and immediately frozen in liquid nitrogen. After defrosting, the samples were centrifugated for 30 min 15000 rpm in an Eppendorf centrifuge, and the pellet obtained was dissolved in buffer containing 5% (v/v) Triton X-100, 10 mM EDTA, 125 mM Tris-HCl, pH 8.0 to which 0.5 mg ml^{-1} trypsin (Serva) was added. These mixtures were incubated at 0°C for 45 min. After adding a 3-fold excess of Trypsin Inhibitor (Serva) in the same buffer, membranes were pelleted by 30 min centrifugation at 15000 rpm in an Eppendorf centrifuge. The pellet was resuspended in sample buffer and boiled for 10 min before SDS-PAGE and autoradiography. To determine the total amount of PhoE synthesized, at various time points

during the chase, samples were taken, precipitated with 5% trichloroacetic acid and PhoE forms were immunoprecipitated as described (Eppens *et al.*, 1997). Finally, the samples were resuspended in sample buffer, boiled and analyzed by SDS-PAGE. The gels were incubated for 30 min with Amplify (Amersham Corp.) and subsequently dried. X-ray films were exposed at -80°C.

Plasmids and DNA manipulations

The plasmids used are listed in Table 2. Plasmid DNA was isolated as described (Birnboim and Doly, 1979), followed by anion-exchange chromatography on Jetstar columns (Genomed Inc.). DNA fragments were isolated and purified from agarose gel using the JetSorb Gel Extraction Kit 600 (Genomed Inc.). Standard DNA manipulations were performed according to Maniatis *et al*. (1982). Restriction endonucleases, T4 DNA ligase and *Pwo* DNA polymerase were used according to the manufacturers' protocols (Fermentas and Boehringer Mannheim). To obtain mutant *phoE* genes, site-directed mutagenesis was applied according to the QuickChange site-directed mutagenesis kit (Stratagene). Oligonucleotides were purchased from Gibco BRL Life Technologies. Plasmid pJP29 was used as the template for the PCR-based mutagenesis. The resulting plasmids, pEE21, pEE22 and pEE23 (Table 2), encode mutant PhoE proteins with a single alanine for proline substitution each. Double-mutant plasmid pEE24 was obtained by an additional round of mutagenesis using pEE23 as the template. The triple-

Table 2. Plasmids used

Plasmids	Relevant characteristics[a]	Reference
pJP29	Cam^r, wild-type *phoE* gene	Bosch *et al.* (1986)
pNN100	Amp^r, wild-type *phoE* gene behind *tac* promoter	Nouwen *et al.* (1994)
pEE21	Cam^r, mutant *phoE* resulting in P109A substitution	This study
pEE22	Cam^r, mutant *phoE* resulting in P235A substitution	This study
pEE23	Cam^r, mutant *phoE* resulting in P261A substitution	This study
pEE24	Cam^r, mutant *phoE* resulting in P235A and P261A substitutions	This study
pEE25	Cam^r, mutant *phoE* resulting in P109A, P235A and P261 substitutions	This study
pEE26	Amp^r, mutant *phoE* resulting in P109A, P235A and P261A substitutions cloned behind *tac* promoter	This study

[a]Cam^r and Amp^r indicate resistance to chloramphenicol and ampicillin, respectively.

mutant plasmid pEE25 was constructed by substituting the *Mlu*I-*Cla*I fragment of pEE21 for the corresponding fragment in pEE24. Plasmid pEE26 was constructed by substituting the *Pst*I-*Bgl*II fragment of pEE25 for the corresponding fragment of pNN100. Successful mutagenesis was confirmed by DNA sequence analysis using the Dye Terminator Cycle Sequencing Kit and the ABI 310 Automated DNA Sequencer (Perkin Elmer Co.) according to the manufacturers' instructions.

Isolation and characterization of cell fractions

After overnight growth, cells were collected by centrifugation and resuspended in 50 mM Tris-HCl, 5 mM EDTA, pH 8.0. Cell envelopes were isolated by ultracentrifugation after ultrasonic disintegration of the cells (Lugtenberg *et al.*, 1975). To test the protease-sensitivity of proteins, the cell envelopes were resuspended in 1 ml of 10 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0 containing 50 µg trypsin (Struyvé *et al.*, 1991). The samples were incubated on ice for 30 min, after which the cell envelopes were reisolated by centrifugation. Protein patterns of cell fractions were analyzed by SDS-PAGE (Lugtenberg *et al*., 1975).

Isolation of the PhoE trimers

PhoE trimers were isolated from cell envelopes as described previously (Agterberg *et al.*, 1990). Briefly, cell envelope fractions were incubated for 30 min at 40°C in a buffer containing 2% SDS. Peptidoglycan-protein complexes were pelleted by ultracentrifugation, and PhoE was dissociated from the peptidoglycan by incubation for 30 min at 40°C in the SDS buffer supplemented with 0.6 M NaCl. After ultracentrifugation, the trimers were precipitated from the supernatant with 66% ethanol and dissolved in a buffer containing 20 mM Tris-HCl, pH 8.0 and 0.1% SDS. When appropriate, the trimers were denatured into monomers prior to SDS-PAGE by heating for 10 min at 95°C (or different temperatures to study the stability of the trimers) in sample buffer (Lugtenberg *et al.*, 1975). Proteins were analyzed by SDS-PAGE and Western immunoblotting (Agterberg *et al.*, 1990). The monoclonal antibody used to detect PhoE protein was mE2-1, which recognizes the denatured protein.

Reconstitution of porins into planar lipid bilayers

Lipid bilayer experiments to measure the single channel conductance and critical closing potential of the various PhoE forms were performed as described (Saint *et al*., 1996) with a slight modification. Briefly, planar lipid bilayers were formed across a pierced (Ø 150 µm) Teflon membrane, pretreated with a solution of *n*-hexadecane in *n*-hexane (1:40 v/v). Bilayer formation and conductance measurements were performed as described (Schindler, 1980; Montal and Mueller, 1979). Purified porins were added to the aqueous subphase, and their insertion into the bilayer was monitored by measuring the membrane current. All experiments were performed at room temperature. Measurements of channel conductances and of critical closing potential were exerted in 1 M KCl, 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4. The critical closing potential was determined by applying a potential ramp across the bilayer from 0 to +/- 250 mV over 100 s.

Protein modeling by e-mail

The Swiss-model program (Peitsch, 1995) was used to model the structures of the mutant PhoE proteins. The frame-structures for modeling were wild-type PhoE, OmpC, OmpF and the OmpF mutants D74A, D113G, R132P, G119D, R42C, R82C and Δ109-114. The graphic program Rasmol (Sayle *et al*., 1995) was used to visualize the modeled structures.

Acknowledgements

We thank C. Ockhuijsen for technical assistance. The continuous interest and helpful suggestions of H. de Cock were very much appreciated. The investigations were supported by the Life Sciences Foundation (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO), and by the European community EU grant BIO4-CT96-0119.

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CHAPTER 5

Role of the constriction loop in the gating of outer membrane porin PhoE of *Escherichia coli*

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Role of the constriction loop in the gating of outer membrane porin PhoE of *Escherichia coli*

Summary

Porins form voltage-gated channels in the bacterial outer membrane. These proteins are composed of three identical subunits, each forming a 16-stranded β-barrel. In this study, the role in voltage gating of a loop that forms a constriction within the pore was studied. The channel characteristics of mutant PhoE porins, in which the tip of the constriction loop was connected to the barrel wall, were determined. Whereas the properties of several mutant channels were changed, all of these channels could still be closed at high potential, showing that a gross movement of the constriction loop within the channel is not implicated in voltage gating.

Introduction

The outer membrane of *Escherichia coli* is an asymmetrical bilayer containing phospholipids and lipopolysaccharides in the inner and outer leaflet, respectively. This barrier protects the cell from damaging agents such as bile

salts, toxins and antibiotics. Several outer membrane proteins are implicated in providing the cell of its nutrients. The major porins of *E. coli*, OmpC, OmpF and PhoE, allow the passive diffusion of small hydrophilic molecules with molecular weights of up to 600 Da. Whereas the OmpF and OmpC pores are cation-selective, PhoE pores are anion-selective (Benz *et al.*, 1985). The three-dimensional structures of PhoE and OmpF have been determined (Cowan *et al.*, 1992). Each monomer of these trimeric proteins is folded as a β-barrel with 16 antiparallel β-strands. The strands are connected by short turns at the periplasmic side and long loops at the surface-exposed side of the membrane. The third loop (L3) is folded into the barrel, thereby forming a constriction at half the height of the membrane. The presence of clusters of negative and positive charges, located on L3 and opposite of L3 on the barrel wall, respectively, creates a strong transverse electrostatic field in the constriction zone (Cowan *et al.*, 1992; Weiss *et al.*, 1991; Karshikoff *et al.*, 1994), which determines to a large extent the permeability and the ion

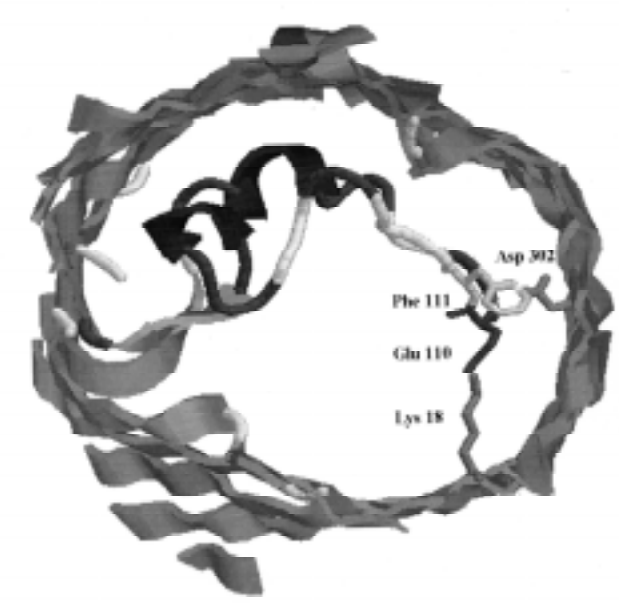

Fig. 1. Rasmol (Sayle and Milner-White, 1995) presentation of Swiss-model structure (Peitsch, 1995) of wild-type PhoE. Indicated are residues K18 and D302 in the barrel wall, and E110 and F111 at the tip of loop L3. These residues were replaced, two at a time, by cysteines to create intramolecular disulfide bonds. All loop except L3 are omitted for clarity and the molecule is viewed from the surface-exposed site.

selectivity of the pores (Bauer *et al.*, 1988; Saint *et al.*, 1996; Van Gelder *et al.*, 1997a). Reconstitution of porins into planar lipid bilayers revealed that the pores could be closed by application of a potential above a certain threshold value (Dargent *et al.*, 1986; Lakey, 1987). The physiological relevance of

this phenomenon, known as 'voltage gating', is unclear, since the Donnan potential that exists across the outer membrane was shown to be insufficient to close OmpF pores (Sen *et al.*, 1988). Possibly, voltage-dependent closing protects the cells after missorting of porin molecules to the cytoplasmic membrane. In this study, we investigated whether a gross movement of L3 into the channel lumen is implicated in voltage gating. Previously, we have described a series of PhoE mutants with artificial disulfide bonds between residues in the tip of loop L3 and residues in the barrel wall (Fig. 1) (Eppens *et al.*, 1997). Since, in these mutants, the tip of L3 is tethered to the barrel wall, a gross movement of the loop is prevented. The pore properties of these mutants were characterized in the present study.

Results

Pore formation of PhoE mutants in vivo

The residues E110 and F111 in the tip of loop L3 of PhoE are located in close proximity to residues K18 and D302 in the barrel wall (Fig. 1). Hence, we expected that the replacement of these residues by cysteines, two at a time, could lead to the formation of disulfide bonds. The construction of the PhoE double mutant proteins K18C/E110C, E110C/D302C and F111C/D302C and the corresponding single mutants was described previously (Eppens *et al.*, 1997). All mutant proteins were well expressed and correctly assembled into the outer membrane. The disulfide bonds in the double cysteine mutants were formed, although with varying efficiency (Eppens *et al.*, 1997). In the K18C/E110C mutant, the efficiency was near 100%, whereas it was approximately 50% for the two other double mutants.

To study whether the mutant proteins form functional pores *in vivo*, the rates of uptake of the β-lactam antibiotics cephaloridine and cefsulodin by intact cells were determined (Table 1). The uptake of both antibiotics was not or only marginally changed as compared to the wild-type in cells expressing the mutant PhoE proteins D302C and F111C/D302C. In the case of the F111C mutant protein, the uptake of both antibiotics was decreased, suggesting that the substitution decreased the channel size. The uptake of the neutrally charged cephaloridine was increased but that of the negatively charged cefsulodin was decreased in cells expressing the mutant protein K18C, suggesting that the anion selectivity of the pores is reduced by this

Table 1. Uptake of β-lactam antibiotics by CE1265 cells expressing mutant PhoE porins

Antibiotic	MW	Charge	Rate of uptake (%)							
			wild-type	K18C	E110C	F111C	D302C	K18C/ E110C	E110C/ D302C	F111C/ D302C
Cephaloridine	415	+ -	100	167 ± 1*	618 ± 19*	49 ± 12	98 ± 1	216 ± 4	624 ± 2	82 ± 4
Cefsulodin	550	+ - -	100	66 ± 1	322 ± 3	50 ± 4	119 ± 11	106 ± 3	388 ± 16	94 ± 3

The rate of uptake was calculated in nmol substrate/min per 10^8 cells. The value obtained for the cells expressing wild-type PhoE was set at 100% and those for the mutants are expressed in % relative to the wild-type. Experiments were performed at least three times independently and the standard deviations are given. Asterisks indicate data from Van Gelder *et al.* (1997a).

mutation. This result is consistent with the previously proposed role of K18 in the anion selectivity (Bauer *et al.,* 1988). The cells expressing the E110C mutant protein or the E110C/D302C double mutant protein showed an enormously increased uptake of both cephaloridine and cefsulodin. Interestingly, this effect of the E110C substitution was compensated for to a large extent by the K18C mutation in the K18C/E110C double mutant (Table 1).

Pore formation in vitro

To study the channel characteristics in further detail, the mutant PhoE proteins were reconstituted into planar lipid bilayers. Wild-type PhoE pores showed a single channel conductance of 0.63 nS and a symmetrical behavior with respect to the polarity of the potential (Table 2). Most of the mutant PhoE pores showed a similar conductance or a conductance that was slightly increased at one polarity of the potential only. For the E110C and E110C/D302C mutants, these results seem to contradict the increased uptake of antibiotics measured *in vivo* (Table 1; see further Discussion). The pores of mutant F111C showed a drastically decreased conductance (Table 2), in agreement with the reduced uptake of antibiotics. Interestingly, there appeared to be two different pore species, with conductances of 0.37 nS and 0.18 nS, respectively. The same was observed for the F111C/D302C double mutant. In this case, the single channel conductance could not exactly be determined at positive polarity of the potential, because of a noisy signal (Table 2).

Voltage gating

If the voltage-dependent closing of the PhoE pores is caused by sweeping of the entire loop L3 into the channel, this would be prohibited in the double cysteine mutants in which this loop is tethered to the barrel wall. The critical closing potential of the pores was determined by applying voltage ramps across the bilayers (Table 2). For the wild-type PhoE pores, the critical closing potential was 135 mV under our experimental conditions. The pores formed by the K18C/E110C double mutant showed a similar voltage sensitivity as the wild-type pores (Table 2). Since the disulfide bond in this mutant is formed with nearly 100% efficiency, it can be concluded that the gating behaviour is not changed when the tip of loop L3 is tethered to the barrel wall. As expected,

Table 2. Electrical properties of wild-type and mutant porin channels in planar lipid bilayers

PhoE form	conductance (nS)		critical voltage (mV)	
	+	-	+	-
Wild-type	0.63 ± 0.06 (170)	0.62 ± 0.03 (170)	135 ± 8 (26)	133 ± 10 (21)
K18C	0.61 ± 0.05 (35)*	0.74 ± 0.05 (33)	158 ± 8* (7)	206 ± 14 (7)
E110C	0.60 ± 0.05 (61)*	0.76 ± 0.05 (44)	88 ± 12* (10)	93 ± 1 (10)
F111C	0.37 ± 0.03 (38)	0.35 ± 0.02 (35)	121 ± 8 (10)	134 ± 9 (10)
	0.18 ± 0.02 (10)	0.17 ± 0.03 (17)		
D302C	0.61 ± 0.06 (49)	0.68 ± 0.05 (35)	103 ± 7 (4)	131 ± 10 (5)
K18C/E110C	0.55 ± 0.04 (39)	0.79 ± 0.03 (49)	145 ± 5 (4)	130 ± 11 (3)
E110C/D302C	0.65 ± 0.09 (34)	0.63 ± 0.08 (31)	96 ± 9 (10)	88 ± 5 (10)
F111C/D302C	~ 0.1 to 0.2	0.44 ± 0.02 (84)	112 ± 14 (8)	92 ± 9 (8)
		0.26 ± 0.03 (46)		

Single channel conductances and critical closing potentials were measured after reconstitution of the porins in planar lipid bilayers. The conductances are single channel conductances and correspond to approximately one third of the conductance observed for one inserted trimeric porin molecule. Measurements were performed at positive (+) and negative (-) polarity of the potential. Values in parentheses are the number of observations. Asterisks indicate data from Van Gelder *et al*. (1997a).

incubation of these mutant porins in 10 mM dithiothreitol (DTT) to reduce the disulfides did not change the gating behaviour (Table 2). A decreased voltage sensitivity was observed for the K18C mutant. In this case, the critical voltage was asymmetrical and dependent of the polarity of the potential. The mutant pores E110C, E110C/D302C and F111C/D302C showed an increased voltage sensitivity (Table 2). Despite these different characteristics, all mutant pores still showed voltage gating.

Discussion

Loop L3 of PhoE folds into the pore and forms a constriction that determines pore characteristics such as channel size and ion selectivity. The goal of this study was to determine whether this loop is also involved in voltage gating, by sweeping into the channel lumen and thus blocking the pore when a high transmembrane potential is applied. Such a mechanism was recently proposed on the basis of molecular dynamic simulation studies (Watanabe *et al.*, 1997). For this purpose, we employed a previously constructed set of PhoE mutants in which the tip of loop L3 is tethered to the barrel wall by disulfide bonds. In the K18C/E110C mutant, the disulfide bond was formed with nearly 100% efficiency. Still, these mutant pores closed at a high transmembrane potential. Hence, we conclude that pore closings are not mediated by a gross movement of L3 within the channel, but by more subtle rearrangements, involving only part of L3 (Soares *et al.*, 1995) or the side chains of the charged residues within the constriction zone (Van Gelder *et al.*, 1997a). Although the disulfide bonds in the other two double mutants, E110C/D320C and F111C/D320C, were formed with only approximately 50% efficiency, the results obtained with these mutants support our conclusion, since all the pores in the bilayers, and not only 50% of them, closed at transmembrane potentials above the critical closing potential. In this respect, it should be noticed that in each experiment typically 5-20 trimers were incorporated in the bilayers, and the experiments with these mutants were repeated 8-10 times (Table 2).

A number of additional properties of the mutant pores are worth noting. The residues E110 and F111 of PhoE are part of a PEFGG sequence, which is highly conserved in a superfamily of bacterial porins (Jeanteur *et al.*, 1991). Previously, we have shown that the deletion or the substitution of the two glycines in this sequence reduces the apparent pore size, both *in vivo* in

antibiotic uptake experiments and *in vitro* in single channel conductance measurements (Van Gelder *et al.,* 1997b). Now it appears that the substitution of the phenylalanine in this sequence by a cysteine has a similar effect. In the single channel measurements, two types of pores were detected, suggesting some structural heterogeneity in these mutant proteins. Such a structural heterogeneity was previously also observed in an OmpF mutant (Saint *et al.,* 1996). Replacement of the glutamate at position 110 by a cysteine resulted in a drastic increase in the uptake of the β-lactam antibiotics (Table 1; Van Gelder *et al.,* 1997a), suggesting a drastically increased pore size. Such an increased pore size was not observed in the single channel measurements. Such apparent discrepancies between the *in vivo* and *in vitro* assays have been noted before (Van Gelder *et al.,* 1997a and references therein). The increased uptake of antibiotics measured *in vivo* is not caused by a general leakiness of the outer membrane, caused by insertion of the mutant porin. In similar assays, the uptake of ampicillin (MW 371; one negative charge) was only marginally increased (1.4-fold) in the mutant, whereas that of nitrocefin (MW 520; two positive charges, three negative charges) was up to 400-fold increased (unpublished observations). Furthermore, whereas the sensitivity of the cells to some antibiotics, such as nitrocefin, was increased in filter disk assays, the sensitivity to other antibiotics (*e.g*. tetracycline, rifampicin) was not changed (data not shown). These observations strongly argue against an increased leakiness of the outer membrane of cells expressing the E110C mutant protein. Apparently, the substitution increases the permeability of the mutant pores for certain compounds, such as certain β-lactam antibiotics used in the *in vivo* assays, but not for others, such as the ions, measured in the planar lipid bilayer assays. Therefore, each of these assays gives only an apparent pore size, specific for the compound for which the permeability is measured. As argued before (Van Gelder *et al.,* 1997a), the permeability is determined not only by the size of the penetrating molecule, but also by its charges, which have to be oriented within the transverse electric field in the constriction zone.

How could the E110C substitution exert its drastic effect on permeability? Importantly, the effect of this substitution was largely reversed by the K18C substitution in the K18C/E110C double mutant. K18 and E110 are in very close proximity (Fig. 1) and, thus, the removal of the negatively charged glutamate could accentuate the positive charge of K18. This could certainly influence the orientation of the penetrating molecules within the transverse

electric field in the constriction zone. For an alternative explanation, it should be noted that E110 is probably hydrogen bonded to D302 in the barrel wall (Karshikoff *et al.*, 1994). Thus, the substitution of E110 might increase the flexibility of (the tip of) loop L3, which might increase the permeability for large, charged molecules (Van Gelder *et al.*, 1997b). By introducing the K18C mutation in the E110C mutant, a disulfide bond is formed (Eppens *et al.*, 1997) which tethers the tip of L3 again to the barrel wall and, thus, restores the original rigidity at the tip of L3.

Experimental procedures

Strains, plasmids and growth conditions

The *E. coli* K-12 strain CE1265 (Korteland *et al.*, 1985) is deleted for the *phoE* gene and does not produce the related OmpF and OmpC proteins as a result of an *ompR* mutation. The presence of a *phoR* mutation in this strain results in constitutive expression of the *pho* regulon. Bacteria were grown at 37°C under aeration in L-broth (Tommassen *et al.*, 1983). Plasmid pJP29 is derived from the cloning vector pACYC184 and carries the *phoE* gene (Bosch *et al.*, 1986). Plasmids pEE1, pEE2, pEE3, pEE4, pEE5, pEE6 and pEE7 are derivatives of pJP29 and carry mutations in the *phoE* gene, resulting in the substitutions K18C, E110C, F111C, D302C, K18C/E110C, E110C/D302C and F111C/D302C, respectively, in the PhoE protein (Eppens *et al.*, 1997).

Porin isolation and reconstitution into planar bilayers

(Mutant) PhoE trimers were isolated from strain CE1265 as described (Van Gelder *et al.*, 1997b), except that β-mercaptoethanol was omitted from the buffers to avoid reduction of the disulfide bonds. Lipid bilayer experiments were performed to measure the single channel conductance and critical closing potential of the various PhoE forms as described (Van Gelder *et al.*, 1997a). Briefly, planar lipid bilayers were formed across a pierced (Ø 150 μm) Teflon membrane, pretreated with a solution of *n*-hexadecane in *n*-hexane (1:40 v/v). Bilayer formation and conductance measurements were performed as described (Montal and Mueller, 1979; Schindler, 1980). Purified porins were added to the aqueous subphase and their insertion into the bilayer was

monitored by measuring the membrane current. All experiments were performed at room temperature. The sign of the membrane potential refers to the *cis*-side of the membrane. Measurements of channel conductivities and of critical closing potential were exerted in 1 M NaCl, 1 mM $CaCl_2$, 10 mM Tris-HCl pH 7.4. The critical closing potential was determined by applying a potential ramp across the bilayer from 0 to +/- 250 mV over 100 s.

Uptake of β-lactam antibiotics in vivo

The rates of permeation of β-lactam antibiotics through the outer membrane of CE1265 cells expressing the various PhoE mutants proteins were determined as described (Overbeeke and Lugtenberg, 1982). The cells carried plasmid pBR322 to obtain a high level of β-lactamase in the periplasm.

Acknowledgements

The investigations were supported by the Life Sciences Foundation (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

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CHAPTER 6

General discussion

General discussion

Outer membrane proteins, such as the porin PhoE of *Escherichia coli*, are synthesized in the cytoplasm as a precursor protein with an N-terminal signal sequence. This signal sequence targets the precursor to the Sec machinery, which mediates its translocation across the inner membrane. After translocation and cleavage of the signal sequence, the mature protein is sorted to its final destination, the outer membrane (Danese and Silhavy, 1998).

In the outer membrane, PhoE functions as a pore allowing for the passive diffusion of preferentially anionic compounds with molecular masses up to 600 Da. The pores are not constantly open, but can be closed, for example *in vitro* when a high transmembrane potential is applied (Morgan *et al.*, 1987). The trimeric PhoE porin is composed of three identical subunits (Cowan *et al.*, 1992). Each monomer spans the outer membrane by 16 antiparallel β-strands. The strands are connected by short turns on the periplasmic side and large loops on the surface-exposed side. Loop L3 folds into the pore thereby forming a constriction, which determines the exclusion limit of the pore. At the tip of this loop, the highly conserved sequence motif PEFGG is located (Jeanteur *et al.*, 1994). The presence of negatively charged residues on L3 and an opposing cluster of positively charged residues on the barrel wall

creates an electrical field within the pore. Loop L2 contributes to the trimer stability by latching into the adjacent subunit at the gap between L2 and L4 formed by L3. Residues in L2 interact with residues on both L3 and the barrel wall of the neighboring subunit. The trimer stability is also determined by numerous hydrophobic interactions at the subunit interface (Schulz, 1992).

PhoE is used in our laboratory as a model protein to study the biogenesis of OMPs and the functioning of outer membrane porins. This thesis specifically deals with late steps in the biogenesis of this protein, namely its translocation from the inner to the outer membrane and its folding into the native trimeric structure. In addition, the role of the third loop in voltage gating was studied.

Biogenesis of PhoE

Periplasmic folding: existence of a periplasmic intermediate

After sorting and translocation of PhoE across the inner membrane, the protein has to find its way to its destination, the outer membrane. Two pathways have been proposed for the translocation of OMPs from the inner to the outer membrane. One model postulates that the mature OMP is sorted to the outer membrane via the zones of adhesion between the membranes (Bayer *et al.,* 1994). Alternatively, the mature protein is released into the periplasm and inserts into the outer membrane after transit through the periplasm. To address this question, we studied whether a periplasmic folding catalyst could modify PhoE on its way to the outer membrane (Chapter 2). PhoE protein does not contain any cysteines. Based on the known 3D-structure (Cowan *et al.,* 1992), disulfide bonds were engineered in PhoE, linking loop L3 to the barrel wall. The cysteine residues were introduced at sites that are far apart in the primary structure but are at hydrogen-bonding distance in the native folded monomer. Therefore, the cysteines would be sufficiently close to allow for disulfide bond formation once the protein folds into a native-like structure. Moreover, the location of the disulfide bonds was chosen such that it would not be accessible from the periplasmic side of the membrane once the trimer is properly inserted, thereby excluding the possibility that the disulfide bond could be formed after insertion of the protein into the membrane. We demonstrated that intrasubunit disulfide bonds were formed and, moreover, that their formation required the periplasmic DsbA protein (Chapter 2). Since a periplasmic enzyme could apparently modify

PhoE on its way to the outer membrane, we concluded that PhoE passes through the periplasm during its biogenesis. Moreover, since DsbA created elements of tertiary structure in the mutant PhoE proteins, these proteins were at least partially folded before insertion into the outer membrane.

The existence of periplasmic intermediates in the assembly of OMPs has already been argued for many years. In an attempt to identify an outer membrane targeting signal within OMPs, overlapping deletions were constructed within the mature domain of several OMPs, including PhoE and OmpA (Bosch *et al.,* 1986; Freudl *et al.,* 1985). Since all the deletion mutant proteins accumulated in the periplasm, it was postulated that a periplasmic intermediate is involved in the biogenesis. More importantly, these studies indicated that the formation of the entire β-barrel is a prerequisite for outer membrane localization, thereby suggesting that folding precedes membrane insertion. This notion is supported by the very hydrophilic nature of OMPs. The amphipathic character of each individual β-strand would be incompatible with their insertion in the hydrophobic core of the membrane. Only after the folding of the OMPs into a β-barrel, a hydrophobic area is created which could be accommodated in the membrane. In accordance, in *in vitro* refolding experiments with OmpA, which forms an eight-stranded antiparallel β-barrel in the outer membrane (Pautsch and Schulz, 1998), all four β-hairpins were reported to cross the vesicle nearly synchronously (Kleinschmidt *et al.*, 1999). Furthermore, preassembled folded monomers of PhoE could be assembled *in vitro* as trimers in outer membrane vesicles (de Cock and Tommassen, 1996), although the requirement for detergents in these assays could question the relevance of these results in the *in vivo* assembly process. Possibly, the detergents might mimic the role of a chaperone *in vivo*.

Do our findings exclude the involvement of the zones of adhesion in the biogenesis of PhoE? Danese and Silhavy (1998) suggested that our results are not inconsistent with the adhesion zone model. For example, PhoE could travel through an adhesion zone with portions of the protein temporarily exposed to the periplasm and accessible to DsbA. In such a model, the β-strands would already have formed the β-barrel, which remains associated with the hydrophobic core of the phospholipids in the adhesion zone, while the loops connecting the strands would be exposed to the periplasm. However, Van Gelder *et al.* (1997a) reported that the formation of the α-helical segment in loop L3 is probably a very early event in folding and initiates the formation of loop L3, followed by folding of the β-barrel along loop L3.

Furthermore, we observed in pulse-chase experiments that the disulfide bonds were formed very rapidly and preceded the maturation into the trypsin-resistant form (Chapter 2). Indeed, it would be difficult to envisage how DsbA could have access to the cysteines at half the height of the β-barrel, once this barrel is closed. Thus, it seems unlikely that PhoE is temporarily embedded as a β-barrel in the adhesion zones, before the disulfide bonds are formed.

The existence of a periplasmic intermediate is consistent with the existence of a quality control mechanism, encoded by the σ^E regulon, which responds to overproduced or misfolded (outer membrane) proteins in the periplasm. The periplasm contains the σ^E-regulators RseB and the periplasmic domain of RseA, which are involved in sensing the folding of extracytoplasmic proteins (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997). The extracytoplasmic σ^E stress response causes an increased expression of periplasmic folding catalysts and proteases, which will rescue or degrade the misfolded proteins (Danese and Silhavy, 1998).

Trimerization of outer membrane proteins

In a subsequent step in the biogenesis pathway, trimeric outer membrane proteins, such as PhoE, have to oligomerize. The assembly of various OMPs has been studied previously, and several assembly intermediates with distinct biochemical and immunological characteristics have been detected. However, the results were not conclusive with respect to the subcellular localization of the trimerization process. The question whether trimerization precedes outer membrane insertion or whether membrane insertion precedes trimerization was addressed by a further exploitation of the approach as described in chapter 2: if the periplasmic Dsb proteins could create an intersubunit disulfide bond at a position that is not accessible from the periplasmic side of the membrane, this would mean that oligomerization takes place in the periplasm, prior to outer membrane insertion. Therefore, we engineered cysteines that are at hydrogen-bonding distance at the subunit interface of PhoE (Chapter 3). Indeed, intersubunit disulfide bonds were formed and the formation of the bonds was dependent on the periplasmic DsbG protein. Therefore, we concluded that the trimerization process precedes outer membrane insertion (Chapter 3).

Our results are consistent with an *in vitro* study, which revealed that pre-assembled PhoE trimers could insert in outer membranes (de Cock and Tommassen, 1996), although detergents appeared to be required in this assay.

Furthermore, when folded monomers were used in the *in vitro* insertion assay, trimeric forms were found to be inserted and the efficiency of trimerization was not reduced when excessive amounts of outer membranes were used (de Cock and Tommassen, 1996). If insertion of folded monomers would precede trimerization, such an inhibition of trimerization would be expected because of titration of individual monomers by different outer membrane vesicles. Based on the resolved structure of the porin of *Rhodobacter capsulatus* (Weiss *et al.*, 1991), it has been proposed that trimerization precedes membrane insertion of this protein. The β-strands of the monomers are not long enough at the subunit interface to span the outer membrane, and therefore, such a folded monomer could not be accommodated in the lipidic environment of a membrane (Schulz, 1993). Although, the β-strands of the *E. coli* porins are long enough at the subunit interface to span the membrane, it seems likely that the assembly pathways of the porins of these two bacterial species are similar, which would be consistent with the notion that trimerization of the *E. coli* porins precedes insertion into the membrane. It should be noted, however, that oligomerization does not necessarily precede membrane insertion in the case of all oligomeric OMPs. The outer membrane phospholipase A (OMPLA) of *E. coli* is active as a dimer (Dekker *et al.*, 1997). Normally, the protein is present in the outer membrane in an inactive state. The protein could be activated, for example by expression of the bacteriocin release protein, and, concomitantly, dimerization could be detected by cross-linking (Dekker *et al.*, 1999). Hence, in the case of OMPLA, oligomerization can apparently occur after membrane insertion.

The exact role of the oxidoreductase DsbG was not yet completely resolved. It has been proposed that it could either act as an oxidant or that it displays isomerization activity. Our results showed that DsbG is capable of catalyzing the formation of disulfide bonds between already folded proteins. Andersen *et al.* (1997) reported that DsbG maintains the proper redox balance between the DsbA/DsbB and DsbC systems. However, since we did not observe an effect of the absence of either DsbA or DsbC on the formation of the artificial intersubunit disulfide bond in PhoE, it seems unlikely that DsbG exerts its effect in this case via DsbA or DsbC. Additionally, Andersen *et al.* (1997) reported that a *dsbG* mutant strain showed an overall defect in the maintenance of proteins in the oxidized state in the periplasm. Our results are in accordance with this observation. However, Bessette *et al.* (1999) reported that DsbG functions as a DsbC homologue, although with a narrower substrate

specificity. DsbC displays isomerization activity, *i.e.* it is capable of reshuffling incorrect disulfide bonds in a protein into the correct ones. Therefore, the active site cysteines of DsbC are in the reduced state, and the authors observed that the active site cysteines of DsbG are in a reduced state as well. However, it would be difficult to envisage how such a reduced enzyme could introduce disulfide bonds in a protein, as observed in our study (Chapter 3). Therefore, we favor the idea that DsbG is required for the maintenance of proteins in the oxidized state (Andersen *et al.,* 1997).

Role of the periplasmic SurA protein in the biogenesis OMPs

Recently, a role for the periplasmic peptidyl-prolyl *cis/trans* isomerase (PPIase) SurA in the folding of the OMPs OmpF, OmpA and LamB has been reported (Lazar and Kolter, 1996; Rouvière and Gross, 1996). Similarly, we observed that SurA is required for the efficient assembly of PhoE (Chapter 4). In a *surA* mutant strain, the conversion of the unfolded monomer of LamB to the folded monomer was found to be affected (Rouvière and Gross, 1996), a result which is consistent with the notion that periplasmic folding precedes the insertion into the outer membrane. Remarkable was the severe effect of the *surA* mutation on OMP biogenesis, since many other PPIases reside in the periplasm, which could potentially take over the function of SurA. Moreover, in a *rotA* mutant strain, hardly any residual PPIase activity was detected in the periplasm (Kleerebezem *et al.,* 1995) and such a mutation did not result in any other phenotype than the lack of periplasmic PPIase activity. This raised the possibility that SurA is not only functioning as a PPIase, but that it exerts additional functions in the periplasm. To address this possibility, we constructed a proline-less mutant PhoE protein, which should therefore be independent of the PPIase activity of SurA. However, when expressed in a *surA* mutant strain, the kinetics of the assembly of this mutant PhoE protein still appeared to be affected. This result implies that SurA has an additional role besides its PPIase activity, and it probably exerts a chaperone-like function.

Dual functions for proteins involved in folding processes have been revealed over the past few years, including chaperones with additional enzyme functions, proteases that could also act as chaperones, and folding catalysts that display chaperone-like functions as well. For example, the eukaryotic PPIases cyclophilin and a cyclophilin-related protein, NK-TR, were reported to display a chaperone-like activity (Freskgård *et al.,* 1992; Rinfret *et al.,* 1994). However, Kern *et al*. (1994) reported that the chaperone activity of

cyclophilin could solely be explained by its isomerase activity. In an attempt to determine whether the *cis-trans* isomerization of the two *cis* Pro residues of cyclophilin affects the kinetics of refolding, these proline residues were substituted (Fransson *et al.*, 1992). However, these mutations lead to such a destabilization of the protein structure that kinetic studies of the refolding process were not feasible. Several oxidoreductases, including the periplasmic DsbA and DsbE proteins and the eukaryotic PDI protein (Yao *et al.*, 1997; Zheng *et al.*, 1997; Fabianek *et al.*, 1998), have been reported to display additional chaperone-like activity. The chaperone activity of DsbA has been reported by several authors. The secretion of a mutant form of pullulanase devoid of cysteines still required DsbA for efficient secretion (Sauvonnet and Pugsley, 1998). This DsbA-dependence could be explained by a chaperone-like function of DsbA. Alternatively, DsbA is required to create a disulfide bond in another, hypothetical chaperone involved in pullulanase folding. Direct evidence for a chaperone-like activity of DsbA was obtained, when the refolding of denatured D-glyceraldehyde-3-phosphate dehydrogenase and of rhodanese was studied (Zheng *et al.*, 1997). Although both proteins are devoid of disulfide bonds, DsbA increased reactivation and decreased aggregation of both proteins. This effect was suppressed by the presence of a 21 amino acid long oligopeptide in the assay, indicating that the peptide-binding activity of DsbA is responsible for the chaperone-like activity. It was shown that DsbA binds preferentially to unfolded proteins, not only by covalent disulfide bonds, but also by non-covalent interactions with other regions of the polypeptide chain (Martin *et al.*, 1993).

The combination of a chaperone and a folding catalyst in a single protein might provide this protein with a high efficiency for folding, since their substrates are unfolded proteins, which require the formation of disulfide bonds and/or the *cis/trans* isomerization of peptidyl-prolyl bonds for obtaining the native folded structure. The simultaneously present chaperone activity would maintain the protein in a folding-competent state by preventing that the target cysteines and/or proline residues are buried within a native-like or aggregated structure before the catalytic activity is exerted. Characteristic of an enzyme with a chaperone-like function is the organization of the enzymatic and chaperone activities in distinct domains. The most explicit example is the heteroligomeric complex ClpAP, in which the protease (ClpP) and chaperone (ClpA) reside in separate subunits (Gottesman *et al.*, 1997). SurA is characterized by the presence of two active site domains, whereas the

cytoplasmic member of this family of PPIases, Parvulin, which displays a higher PPIase activity than SurA, only has one such domain (Rouvière and Gross, 1996). Further dissection of SurA will be required to determine whether both domains contribute to SurA activity or specificity. In contrast, it has been suggested that in the periplasmic DegP protein, which switches between chaperone- and protease-activity depending upon the incubation temperature, the protease domain itself has chaperone activity (Spiess *et al.*, 1999). The authors suggested that the active site serine is buried away from other residues of the catalytic triad at low temperatures.

The finding that a *surA* mutant strain did not affect the assembly of periplasmic proteins (Lazar and Kolter, 1996) suggests that the chaperone-like function of SurA is specific for OMPs. This supposition is supported by the fact that overexpression of SurA can suppress the induced extracytoplasmic stress response, σ^E, caused by production of defective LPS (Missiakas *et al.*, 1996), which has already been described to lead to decreased levels of OMPs in the outer membrane. Also the Skp protein appears to interact specifically with OMPs (Chen and Henning, 1996; de Cock *et al.*, 1999). What feature of the OMPs is recognized by these chaperones is not clear, but it might be β-structure.

Model for the passage of PhoE from the inner to the outer membrane

The following model for the biogenesis of porins can be proposed. After translocation and cleavage of the signal sequence, the mature protein is released from the inner membrane, and it might be transported to a limited number of transit sites in the inner membrane. This process might require the activity of the periplasmic Skp protein, which has affinity for both unfolded OMPs and for phospholipids (de Cock *et al.*, 1999). At these transit sites, the formation of the folded monomer might be initiated by interaction with nascent LPS. This proposal is supported by the observations that the assembly of porins is affected *in vivo* when lipid synthesis is blocked (Bolla *et al.*, 1988) and that *in vitro* synthesized PhoE could be folded into a folded monomer in the presence of LPS and Triton X-100 (de Cock and Tommassen, 1996). The requirement of Triton X-100 in these *in vitro* studies might mimic the role of a periplasmic chaperone *in vivo*. It seems that SurA might function as this chaperone. The formation of this folded monomer might either be completed at the inner membrane or freely in the periplasm. Subsequently, this folded monomer should be targeted to the outer membrane, since the

pores in the peptidoglycan are too narrow to allow for the passage of completely assembled porin trimers (Dijkstra and Keck, 1996). Targeting to the outer membrane might require additional periplasmic components. The folded monomers are probably concentrated at specific sites at the periplasmic side of the outer membrane. After the release of the LPS molecules, which are probably bound at the subunit interface of the folded monomers (de Cock and Tommassen, 1996), dimerization is the next step in the biogenesis (Reid *et al.*, 1988) followed by trimerization. After trimerization, the protein inserts into the membrane. The driving force for this insertion might be the hydrophobicity of the trimer. The trimers that are initially formed are not yet completely mature, since they are less stable than the final trimers (Vos-Scheperkeuter and Witholt, 1984). The slow transition from the metastable to the stable trimer is the final step in the biogenesis process and occurs in the outer membrane, when the stable interactions between the subunits and between loops and LPS are made.

The porin PhoE

Mechanism of voltage gating of the porin pores

Porins appear as much more plastic and dynamic entities than has been suggested for a long time. Opening and closing of the channels can be modulated by conditions and compounds surrounding the channels. This dynamic behaviour must be highly significant for the survival of the bacteria, which can encounter changing and severe environmental conditions. It allows the cell to control the outer membrane permeability. Recently, patch-clamp studies revealed that cells maintain a large population of their porins in a closed state (Delcour, 1997). One of most intriguing unresolved questions with respect to the functioning of the porins in the outer membrane is the mechanism of channel closure. Studies with purified porins reconstituted in lipid bilayers revealed that the porins can exist in an open or a closed state, depending on the transmembrane potential. The channels close at high voltages, and closing transitions are often grouped in three steps, probably representing the successive closures of each monomer within a trimer.

Two mechanisms for the transition between open and closed conformation of the pores have been proposed: (i) a large conformational change of the L3 loop, and (ii) subtle local electrostatic changes in the eyelet region. Loop L3 seems to be a good candidate to gate the pore by swinging into the pore

lumen and, thus, physically block the channel. However, the position of loop L3 in the pore is fixed by a hydrogen bond network between the barrel wall and the tip of loop L3, by salt bridges at the root of loop L3 and by the electrical field across the pore. Nevertheless, molecular dynamic simulation studies suggested that L3 could actually sweep into the channel lumen (Watanabe *et al.*, 1997). To investigate whether physical movement of L3 into the pore lumen is the mechanism of pore closing, we studied a number of mutant PhoE proteins in which loop L3 is covalently tethered to the barrel wall via disulfide bonds (Chapter 5). We reasoned that these mutant pores should not be able to close when a gross movement of loop L3 is the basic mechanism of pore closure. However, since the mutant pores with disulfide bonds could still be closed, we concluded that a gross movement of the constriction loop within the channel is not implicated in voltage gating (Chapter 5). In a similar manner, loop L3 of porin OmpF has been tethered to the barrel wall by a disulfide bond (Phale *et al.*, 1997; Bainbridge *et al.*, 1998a). In accordance with our observation, voltage-dependent closure of the mutant porins was still observed. Moreover, in the study of Bainbridge *et al.* (1998a), disulfide bonds were created between L3 and the barrel wall, not only at the tip of L3, but also at other positions. In addition, internal disulfide bonds within L3 were constructed. All these mutant pores could be closed at a high transmembrane potential (Bainbridge *et al.*, 1998a). Therefore, neither a gross movement of L3, nor a more restricted movement of L3 into the channel lumen forms the mechanistic basis for channel closure. Consistent with this view, Tieleman and Berendsen (1998) recently performed molecular dynamic simulation studies, and, in contrast to similar studies by Watanabe *et al.* (1997), they did not observe a large scale motion of loop L3. They concluded that the absence of solvent in the study of Watanabe *et al.* (1997) is an oversimplification.

What might than be the mechanistic basis for channel closure? Bainbridge *et al.* (1998b) concluded that voltage gating is a general property of the β-barrel structure rather than of any particular substructure, since voltage gating occurs in all large β-structured pores, which lack any sequence homology. The β-strands are extremely symmetrical within the membrane. Moreover, the porins display symmetrical voltage gating, that is, they close in response to applied voltages of either polarity. Based on this notion, Bainbridge *et al.* (1998b) proposed that the physical basis of voltage gating lies within the transmembrane β-barrel. However, the mutant PhoE porin with a P109A

substitution could not be closed upon applying a transmembrane potential (Chapter 4). Moreover, the wild-type *P. denitrificans* porin was not sensitive for a transmembrane potential (Saxena *et al.*, 1999). The fact that a mutation in the L3 loop made this porin voltage sensitive supports the importance of amino acid residues located on the L3 loop for voltage gating. Therefore, closure of the channels solely caused by the movement of the surface-exposed loops, as was recently proposed on the basis of atomic force microscopy (Müller and Engel, 1999) seems unlikely. Mutations affecting the charged residues within the constriction zone clearly affected voltage-dependent gating. In the anion-selective porin PhoE, the substitution of positively charged residues within the transverse electrical field in the constriction zone decreased the voltage sensitivity (Van Gelder *et al.*, 1997b), suggesting that these residues act as voltage sensors. In contrast, the substitution of the same residues in the cation-selective porin OmpF increased the voltage sensitivity, whereas in this case, the substitution of negatively charged residues decreased voltage sensitivity (Saint *et al.*, 1996; Van Gelder *et al.*, 1997b). These results demonstrate that the charged residues that contribute to the transverse electrical field in the constriction zone play an important role in voltage gating. These charges are screened by water molecules and possibly by counter ions present in the water-filled pores. This reduces the attractive electrostatic forces between the oppositely charged clusters present on the barrel wall and loop L3. Redistribution of the water molecules and counter ions caused by a transmembrane potential and by ions passing through the pores might result in unscreening of the charges in the channel, thereby increasing the strength of the electrostatic field between loop L3 and the barrel wall. The increased electrical field may prevent the passage of ions, which, in contrast to dipolar solutes, cannot be oriented within the electrical field (Schulz, 1995). Thus, the pores would appear to be closed in the planar lipid bilayer experiments, where ion flow is measured, while at the same time, the pores remain open for passing water molecules (Steiert, 1993). In addition, numerous local structural rearrangements might be induced by the increased electrical field, which could result in increased gating.

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Samenvatting

Samenvatting

De Gram-negatieve bacterie *Escherichia coli* wordt omgeven door twee membranen, namelijk een binnenmembraan en een buitenmembraan, die van elkaar gescheiden zijn door het periplasma (Fig. 1, Hoofdstuk 1). Beide membranen zijn opgebouwd uit fosfolipiden en eiwitten, terwijl de buitenmembraan tevens lipopolysacchariden (LPS) bevat. De aanwezigheid van LPS in de buitenste monolaag van de buitenmembraan dient ter bescherming van de cel tegen schadelijke stoffen uit de directe omgeving, zoals galzouten en fosfolipases. Zowel hydrofiele als hydrofobe stoffen kunnen deze membraan nauwelijks passeren. Maar om de cel toch van zijn groeibehoeftes te voorzien, zijn diverse eiwitten aanwezig die poriën vormen in de buitenmembraan. Eén van deze porie-eiwitten is het PhoE eiwit, waarvan de synthese wordt geïnduceerd gedurende fosfaat-limiterende groeiomstandigheden. De 3D-structuur van PhoE is opgehelderd. PhoE vormt een trimeer in de buitenmembraan, opgebouwd uit drie identieke monomeren. Iedere monomeer bestaat uit 16 β-strengen, die een cilinder vormen en verbonden zijn door korte lussen die aan de periplasmatische zijde en door langere lussen aan de buitenkant van de cel. Eén van deze langere lussen, L3,

vouwt naar binnen in het kanaal en vormt daarbij een vernauwing, de zogenaamde constrictiezone (Fig. 2, Hoofdstuk 1). Door deze ligging bepaalt lus L3 een aantal porie eigenschappen van PhoE, zoals de voorkeur voor opname van negatief geladen deeltjes en de grootte van de deeltjes die opgenomen kunnen worden. Lus L3 is tevens betrokken bij het sluiten van de porie. Sluiting van de porie zou de cel de mogelijkheid bieden de opname van stoffen te reguleren. De monomeren zijn onderling stevig met elkaar verbonden, waarbij onder andere diverse aminozuren in lus L2 een rol spelen. Deze lus vouwt naar de naast gelegen monomeer toe en vormt daarbij diverse verbindingen met zowel de wand van het kanaal als met lus L3 met die monomeer (Fig. 1, Hoofdstuk 3).

Het onderzoek naar het transport van buitenmembraaneiwitten van de plaats van synthese in het cytoplasma naar de buitenmembraan heeft zich de afgelopen jaren met name toegespitst op het transport naar en over de binnenmembraan. PhoE wordt in ons laboratorium gebruikt als modeleiwit om de processen die ten grondslag liggen aan de biogenese te bestuderen. PhoE wordt in het cytoplasma gesynthetiseerd met een extensie aan de N-terminus, de zogenaamde signaalsequentie. Na het passeren van de binnenmembraan en het verwijderen van de signaalsequentie, moet PhoE naar de buitenmembraan gedirigeerd worden. Twee modellen zijn gepostuleerd voor dit transport (Fig. 4, Hoofdstuk 1). Het eerste model stelt dat transport verloopt op plaatsen, waar de binnen- en buitenmembraan contact met elkaar maken. Buitenmembraaneiwitten zouden daarbij niet vrij komen in het periplasma. Het tweede model postuleert dat buitenmembraaneiwitten na transport over de binnenmembraan in het periplasma worden losgelaten, waarna een periplasmatisch intermediair naar de buitenmembraan wordt gedirigeerd. De conformatie van deze intermediair is onbekend. Enerzijds kan een ongevouwen intermediair naar de buitenmembraan worden gedirigeerd, anderzijds kan vouwing van de monomeer en zelfs vorming van de trimeer plaatsvinden in het periplasma voor insertie in de buitenmembraan. Hoewel men in het algemeen de voorkeur geeft aan het tweede model ontbreekt direct bewijs voor deze route. Kenmerkend voor het periplasma is de aanwezigheid van diverse chaperonnes en vouwingsenzymen. Chaperonnes voorkomen aggregatie van eiwitten, terwijl vouwingsenzymen een specifieke snelheidsbeperkende stap in het vouwingsproces stimuleren. Twee typen enzymen kunnen worden onderscheiden, namelijk de zogenaamde Dsb eiwitten, die de vorming van disulfidebruggen tussen twee cysteines katalyseren,

en de peptidyl-prolyl *cis/trans* isomerases (PPIases), die de overgang tussen de *cis* en de *trans* configuratie van de pepide-binding tussen 2 aminozuren, waarvan het tweede een proline is, katalyseren.

De studies beschreven in dit proefschrift zijn uitgevoerd om inzicht te krijgen (i) in de processen direct na het transport van buitenmembraaneiwitten over de binnenmembraan en met name in de plaats in de cel waar vouwing van deze eiwitten plaatsvindt, (ii) de rol van het periplasmatische eiwit SurA in de biogenese van PhoE en (iii) het mechanisme van sluiten van porie-eiwitten.

In de studie beschreven in Hoofdstuk 2 werd onderzocht via welke route PhoE de buitenmembraan bereikt. De gekozen strategie was als volgt: wanneer we zouden kunnen aantonen dat één van de periplasmatische enzymen betrokken is bij de vouwing van intermediairen in de biogenese dan zou dit een sterke aanwijzing zijn dat PhoE via de periplasma naar de buitenmembraaan wordt getransporteerd. Bovendien zou dit betekenen dat vouwing in ieder geval wordt geïnitieerd vóór insertie in de buitenmembraan plaatsvindt. Het vouwingsenzym DsbA katalyseert de vorming van disulfidebruggen in ongevouwen eiwitten. PhoE bevat echter geen cysteines in zijn eiwitsequentie en derhalve geen disulfidebruggen in de gevouwen conformatie. Op basis van de 3D-structuur waren wij in staat om cysteines in de PhoE sequentie in te bouwen op plaatsen, die in de gevouwen monomeer zo dicht bij elkaar komen dat ze een disulfidebrug zouden kunnen vormen (Fig. 1, Hoofdstuk 5). De beoogde disulfidebruggen bleken inderdaad gevormd te worden in de mutant eiwitten. Door de mutant PhoE eiwitten tot expressie te brengen in een bacteriestam die geen DsbA produceert, kon bovendien worden aangetoond, dat de vorming van deze bruggen afhankelijk was van DsbA. De betrokkenheid van een periplasmatische vouwingsenzym toont aan dat er een periplasmatische intermediair in de biogenese van buitenmembraaneiwitten bestaat en dat vouwing van de monomeer in ieder geval gedeeltelijk in het periplasma plaatsvindt.

Een logisch vervolg op deze resultaten was de studie beschreven in Hoofdstuk 3, waarin we de plaats van de vorming van trimeren onderzochten. Enerzijds zou dit proces kunnen plaatsvinden na insertie van de monomeren in de buitenmembraan, anderzijds zouden de monomeren al in het periplasma kunnen assembleren tot trimeren. *In vitro* experimenten hadden al aangetoond dat geassembleerde PhoE trimeren in buitenmembranen kunnen inserteren, maar de benodigde condities waren zeer artificieel. Om te bepalen in hoe-

verre deze *in vitro* resultaten de werkelijke situatie nabootsen, werden *in vivo* experimenten uitgevoerd, waarbij gebruik werd gemaakt van dezelfde strategie als beschreven in Hoofdstuk 2. Op basis van de 3D-structuur werden twee aminozuren in PhoE vervangen voor cysteines. Wanneer de trimeer in zijn goede conformatie vouwt, komen deze cysteines, gelegen op de tip van lus 2 van de ene monomeer en aan de basis van lus 3 van de andere monomeer, zo dicht bij elkaar dat ze een disulfidebrug kunnen vormen (Fig. 1, Hoofdstuk 3). Inderdaad konden wij aantonen dat een disulfidebrug tussen de monomeren in de trimeer gevormd werd, en bovendien bleek de vorming van de disulfidebrug afhankelijk te zijn van het periplasmatische DsbG eiwit. Dit bewijst dat vorming van de trimeer plaatsvindt in het periplasma, en dat PhoE als gevouwen trimeer inserteert in de buitenmembraan.

Intussen werd gepubliceerd dat het periplasmatische PPIase SurA betrokken is bij de biogenese van diverse buitenmembraaneiwitten. In de buitenmembraan van een bacteriestam die geen SurA produceert, bleken aanmerkelijk minder buitenmembraaneiwitten aanwezig te zijn dan in een wild-type stam. Nader onderzoek wees uit dat SurA betrokken is bij de vorming van de gevouwen monomeer. Opmerkelijk was het feit dat de afwezigheid van een ander periplasmatisch PPIase, namelijk RotA, dat een veel hogere PPIase activiteit heeft dan SurA, niet tot een vermindering in de hoeveelheid buitenmembraaneiwitten leidt. Dit suggereerde dat SurA, naast zijn enzymatische functie, wellicht nog een additionele rol als chaperonne in de biogenese van buitenmembraaneiwitten zou kunnen vervullen. Deze mogelijkheid werd onderzocht in de experimenten die beschreven zijn in Hoofdstuk 4. In dit hoofdstuk zijn eerst pulse-chase experimenten beschreven, waaruit bleek dat SurA ook bij de biogenese van PhoE betrokken is. In een stam die geen SurA produceert, bleken stabiele trimeren van PhoE aanmerkelijk langzamer gevormd te worden dan in een wild-type stam. Dit toonde aan dat SurA nodig is voor de efficiënte biogenese van PhoE. Om te bepalen of deze langzamere biogenese werd veroorzaakt door het ontbreken van de PPIase activiteit van SurA of van een eventuele andere functie die dit eiwit zou kunnen vervullen, werden alle drie de proline residuen in PhoE, die het mogelijke aangrijpingspunt voor de PPIase activiteit van SurA zijn, vervangen voor alanines. In pulse-chase experimenten bleek de biogenese van dit mutante PhoE nog steeds verstoord te zijn in een bacteriestam die geen SurA produceert. Daaruit concluderen we dat SurA betrokken is bij de biogenese van PhoE, maar niet door zijn PPIase activiteit, maar waarschijnlijk door een

chaperonne-achtige activiteit van dit eiwit.

Gezuiverde buitenmembraan porie-eiwitten kunnen na insertie in een artificiële lipiden bilaag, gesloten worden door een hoge potentiaal over de membraan aan te leggen. De fysiologische rol van dit fenomeen staat nog ter discussie, omdat de natuurlijk voorkomende Donnan potentiaal over de buitenmembraan nooit deze hoge waarden bereikt. Het mechanisme van sluiten is onbekend en werd onderzocht in de studies beschreven in Hoofdstuk 5. In diverse studies is gesuggereerd dat lus L3 betrokken is bij dit proces. De vraag op welke wijze deze lus de porie activiteit bepaalt, blijft echter een onderwerp van discussie. Lus L3 wordt op zijn plaats gehouden door diverse, niet-covalente interacties met de wand van het kanaal. Computer simulaties suggereerden echter dat L3 flexibel is en door een grote beweging het kanaal fysisch kan blokkeren. De PhoE constructen, die we beschreven hebben in Hoofdstuk 2 bleken ideaal om dit proces nauwkeuriger te bestuderen. Immers, in deze mutant PhoE eiwitten is lus L3 covalent verbonden met de wand van het kanaal via disulfidebruggen (Fig. 1, Hoofdstuk 5). Daardoor kan L3 geen grote bewegingen in het kanaal meer maken. De gezuiverde mutant trimeren bleken in de artificiële bilagen nog steeds te sluiten, wanneer een hoge potentiaal over de membraan werd aangelegd. Hieruit concluderen we dat een grote beweging van lus L3 in het kanaal niet ten grondslag kan liggen aan het mechanisme van porie-sluiting. Waarschijnlijk kunnen kleine veranderingen in de conformatie van de verschillende aminozuren in de constrictiezone het kanaal afsluiten.

Nawoord

Gedurende de afgelopen vijf jaar waren er soms wel eens momenten waarop ik twijfelde of het OIO-schap ergens toe zou leiden. Maar uiteindelijk blijken de stukjes perfect in elkaar te passen, hetgeen resulteerde in dit boekje. Het bereiken van het moment waarop je het manuscript naar de drukker brengt, is slechts mogelijk gebleken door de inbreng van velen, zowel binnen als buiten het lab.

Allereerst mijn promotor Wiel Hoekstra. Wiel, ondanks je drukke werkzaamheden als decaan (Wie(l) is toch die man die man ….) was je altijd aanwezig bij mijn praatjes voor het secretieplatform en de AIO-dag en betrokken bij het wel en wee van de studenten die ik begeleidde. Mijn copromotor Jan Tommassen. Jan, jij bent een onuitputtelijke bron van ideeën en suggesties en jouw corrigeerwerk van mijn schrijfwerk is essentieel gebleken bij het tot standkomen van dit proefschrift. Alleen betwijfel ik of het langzamer praten tijdens voordrachten, bijdraagt aan een beter begrip bij de "aandachtige" luisteraar (zeker op de maandagmorgen).

De W428-ers Ria (nooit meer *in vivo* metingen?) en Nico (door jouw begeleiding gedurende de eerste twee jaar weet ik dat er altijd nog mooiere gelen bestaan) voor de prettige (soms zwijgzame) samenwerking. Natuurlijk de studenten Nienke Westerink (ook al wilde je liever bij Patrick stage lopen!!), Arjen Kramer (vuurgevaarlijk), Floris Bikker (biotinylering van cysteines is echt mogelijk) en Ralph Bax (zingt eer ge begint) voor alle geslaagde en vooral minder geslaagde inspanningen (vissen???) tijdens jullie doctoraalstage maar vooral voor de gezelligheid op W428.

De oud- en collegae van West- en Oost-4, onder andere Patrick (altijd bereid om een pakketje uit Utrecht te ontvangen), en Peter (er gaat dus echt niks boven Groningen), Mohammed (altijd in voor nog een biertje), Hendrik (een geslaagde reis met z'n vieren naar dat kitsch kasteel in Tegernsee), Sally (ooit Carnegie Hall?), Corrine (ik ben nu echt weg), Thorsten (computeren blijkt een vak apart), en Hans (PhoE-besprekingen waren altijd onnavolgbaar in jouw aanwezigheid??).

De bacteriologische keuken, de afdelingen fotografie en vormgeving voor de bereidheid om snel nog een "klusje" tussendoor te verrichten.

Mijn ouders voor de interesse die jullie hebben getoond in mijn (onbegrijpelijke?) bezigheden als OIO en belangrijker nog jullie relativeringsvermogen,

mijn zusjes Eleonora (uiteindelijk ben ik toch dankzij jou in de microbiologie terecht gekomen) en Pauline (altijd in voor een goed gesprek) en mijn broertje Alexander (het drukken maar vooral het drukwerk ontwerpen is een verdomd leuke bezigheid; gelukkig komt er binnenkort nog eentje) voor de gezelligheid en het vele lachen.

En tenslotte Frans. Jouw steun, begrip en geduld gedurende de afgelopen drie jaar zijn van onschatbare waarden geweest bij het slagen van mijn OIO-periode (en de perfectionering van mijn cricketspel?).

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Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 27 mei 1970 in Groningen. In 1989 werd het V.W.O. diploma behaald aan de RSG "Noord-Kennemerland" te Alkmaar. In hetzelfde jaar werd begonnen met de studie Biomedische Wetenschappen aan de Universiteit Leiden. In juni 1994 werd het doctoraal examen afgelegd met als hoofdvak farmacologie waarbij transport over bloed-hersen barrière werd bestudeerd onder supervisie van Prof. dr. D.D. Breimer, Dr. A.G. de Boer en Dra. H.E. de Vries. Tijdens de bijvakken werd onderzoek gedaan bij de vakgroep Infectieziekten met als onderwerp 'intracellulair doden van microörganismen door humane monocyten na stimulatie van complement receptoren I en II' onder supervisie van Prof. dr. R. van Furth, Dr. P.H. Nibbering en Drs. L. Zheng, bij de vakgroep Farmacologie met als onderwerp 'karakterisatie van runder hersenendotheel d.m.v. de von Willebrand factor' onder supervisie van Prof. dr. D.D. Breimer, Dr. A.G. de Boer en Dra. H.E. de Vries, bij de vakgroep Anesthesiologie met als onderwerp 'effecten van propofol en thiopenton op centraal en totaal bloed volume' onder supervisie van Prof. dr. J.G. Bovill, Drs. F. Boer en Dra. F. Teng-van der Zande en tenslotte als extra bijvak bij de vakgroep Microbiologie, Vrije Universiteit Amsterdam met als onderwerp 'GTPase activiteit van de *E. coli* SRP-receptor FtsY' onder supervisie van Prof. dr. F.K. de Graaf, Dr. J. Luirink, Dr. R. Kusters. Van september 1994 tot september 1998 was zij als onderzoekster-in-opleiding, in dienst van de stichting Aard- en Levenswetenschappen, werkzaam bij de vakgroep Moleculaire Microbiologie van de Universiteit Utrecht. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht. Vanaf september 1999 is zij de komende drie jaar als post-doctoraal medewerkster werkzaam bij de vakgroep Maag-, darm-, en leverziekten (Prof. dr. G.N.J. Tytgat en Dr. R.P.M. Oude Elferink) van het AMC-UvA, Amsterdam.