ASSEMBLY OF INTEGRAL MEMBRANE PROTEINS FROM THE PERIPLASM INTO THE OUTER MEMBRANE

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The outer membrane (OM) of gram-negative bacteria consists of a lipid bilayer, which is composed of phospholipids in the periplasmic leaflet and of lipopolysaccharide in the outer leaflet. Integral membrane proteins facilitate transport, for example, of nutrients, across this hydrophobic barrier. After their biosynthesis, outer membrane proteins (OMPs) are targeted to the cytoplasmic membrane in complex with SecB/SecA and they are then transported through the SecY/E/G translocon of the cytoplasmic membrane into the periplasm. In the periplasm, the signal sequences of the OMPs are cleaved by a leader peptidase and the proteins cross the periplasm in an unfolded form prior to their assembly into the OM. Misfolding of OMPs leads to their proteolysis and to the activation of extracytoplasmic stress response, which results in expression of periplasmic and outer membrane folding factors, such as chaperones, isomerases, and proteases. In the periplasm, the OMPs are kept soluble in largely unfolded form by periplasmic chaperones. A complex of several proteins in the OM is involved in targeting and assembly of the integral membrane proteins into the OM. OMPs insert and fold into lipid bilayers by a highly concerted mechanism, in which secondary and tertiary structure formation is synchronized and coupled to lipid bilayer insertion. This chapter summarizes the current knowledge about the transport of OMPs through the periplasm and about their assembly into membranes.

Biological membranes are needed for maintaining the integrity of cells and cell organelles as well as their shape. All membranes including the OM of bacteria are composed of a lipid bilayer that has a hydrophobic core and polar surfaces. The lipid bilayer prevents the arbitrary passage of solutes across the membrane. Integral membrane proteins, also called transmembrane proteins (TMPs), are embedded into the lipid bilayers to perform many different functions, including the transport of solutes and signals across the hydrophobic barrier. Therefore, TMPs are essential for the survival of the cell. The biogenesis of membranes is of fundamental interest to basic research from the perspectives of cell biologists, structural biologists, biochemists, and biophysicists. Important for the understanding of membrane biogenesis is how integral membrane proteins are inserted and folded into the lipid bilayer host matrix. For insertion and folding into the OM, TMPs cross the cytoplasmic membrane in an unfolded

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The Periplasm
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form. They must then traverse the periplasm. How insertion and folding of OMPs into the OM takes place is largely unknown, and this chapter aims to give an overview about our current knowledge on the insertion and folding of OMPs from the periplasm into the OM. To understand the insertion and folding process of integral membrane proteins, it is important to consider their transmembrane structure and biophysical properties. Therefore, some of the structures and properties of OMPs are described first in this chapter, followed by an overview of the currently known periplasmic folding factors of OMPs. Subsequently, recently discovered OMPs and lipoproteins that are associated with the OM and involved in OMP targeting, insertion, and/or folding are described. Finally, I summarize recently performed biochemical and biophysical investigations on OMP insertion and folding.

STRUCTURE OF OMPs

TMPs can be classified by their transmembrane secondary structure into the two categories α-helical and β-sheet membrane proteins. Within the hydrophobic core of the membrane, all polar amide hydrogen-bonding donors and carbonyl hydrogen-bonding acceptors of the polypeptide backbone form hydrogen bonds. Single helices can span the hydrophobic bilayer because, within the helix, each polar amide group of an amino acid forms a hydrogen bond with the carbonyl group of the fourth following amino acid in the helix. In a β-barrel, the hydrogen bonds between polar amide and carbonyl groups of the peptide bond are formed between neighboring strands. Single β-strands would expose the polar groups of the polypeptide chain toward the hydrophobic region of the membrane and therefore cannot cross the hydrophobic core of the lipid bilayer for energetic reasons. In TMPs, the nonpolar side chains face the hydrophobic acyl chains of the membrane lipids. Although the more abundant α-helical TMPs are found in the cytoplasmic (or inner) membranes, the TMPs with β-barrel structures are known from the OMs of bacteria, mitochondria, and chloroplasts.

In gram-negative bacteria, OMPs cross the periplasm before they assemble into the OM. All currently known OMPs from bacteria form transmembrane β-barrels. The β-barrel is characterized by the number of antiparallel β-strands and by the shear number, which is a measure for the inclination angle of the β-strands against the barrel axis. The OMPs of bacteria form transmembrane β-barrels with even numbers of β-strands ranging from 8 to 22 with shear numbers from 8 to 24 (Schulz, 2002). The strands are tilted by 36° to 44° relative to the barrel axis (Marsh and Páli, 2001; Schulz, 2002). Some examples for OMPs are given in Table 1, which also lists the molecular weight, the pI, the number of β-strands, the number of amino acid residues, the oligomeric state, and the function of the OMP. Structures of several OMPs are shown in Color Plate 2. β-Barrel membrane proteins of bacteria serve a wide range of different functions. Currently, they may be grouped into nine families: (i) general nonspecific diffusion pores (OmpC, OmpF, PhoE); (ii) passive, specific transporters, for example, for sugars (LamB, ScrY) or nucleosides (Tsx); (iii) active transporters for iron complexes (FhuA, FepA, FecA) or cobalamin (BtuB); (iv) enzymes such as proteases (OmpT), lipases (OmPlA), acyltransferases (PagP); (v) toxin binding defensive proteins (OmpX); (vi) structural proteins (OmpA, outer membrane protein A of *Escherichia coli*); (vii) adhesion proteins (NspA, OpcA); (viii) channels involved in solute efflux (TolC); and (ix) autotransporters (NalP, adhesin involved in diffuse adherence [AIDA]). Recent reports suggest that there may be OMPs with an even larger number of transmembrane β-strands than reported for the TonB-dependent active transporters. An example may be the OM usher PapC, for which a β-barrel with 26 TM β-strands has been proposed (Henderson et al., 2004; Thanassi et al., 2002). PapC is an OMP required for assembly and secretion of P pili by the chaperone/usher pathway in *E. coli* (Dodson et al., 1993; Norgren et al., 1987).

Recently developed screening algorithms for the genomic identification of β-barrel

TABLE 1 Examples of outer membrane proteins of known high-resolution structure

OMP	Organism	MW (kDa)	pI[a]	Residues	Residues in β-barrel domain	β-Strands in barrel domain	Oligomeric state	Function	PDB entry	Reference(s)
Outer membrane proteins with single-chain β–barrels										
OmpA	*E. coli*	35.2	5.6	325	171	8	Monomer	Structural	1QJP, 1BXW	Pautsch and Schulz, 1998, 2000
OmpA[b]	*E. coli*	35.2	5.6	325	171	8	Monomer	Structural	1G90	Arora et al., 2001
OmpX	*E. coli*	16.4	5.3	148	148	8	Monomer	Toxin binding	1QJ8	Vogt and Schulz, 1999
OmpX[b]	*E. coli*	16.4	5.3	148	148	8	Monomer	Toxin binding	1Q9F	Fernandez et al., 2004
NspA	*N. meningitidis*	16.6	9.5	153	153	8	Monomer	Cell adhesion	1P4T	Vandeputte-Rutten et al., 2003
PagP	*E. coli*	19.5	5.9	166	166	8	Monomer	Palmitoyltransferase	1HQT	Ahn et al., 2004
PagP[b]	*E. coli*	19.5	5.9	166	166	8	Monomer	Palmitoyltransferase	1MM4, 1MM5	Hwang et al., 2002
OmpT	*E. coli*	33.5	5.4	297	297	10	Monomer	Protease	1I78	Vandeputte-Rutten et al., 2001
OpcA	*N. meningitidis*	28.1	9.5	254	254	10	Monomer	Adhesion protein	1K24	Prince et al., 2002
Tsx	*E. coli*	31.4	4.9	272	272	12	Monomer	Nucleoside uptake	1TLW, 1TLY	Ye and van den Berg, 2004
NalP[c]	*N. meningitidis*	111.5	6.7	1,063	267	12	Monomer	Autotransporter	1UYN	Oomen et al., 2004
OmPlA	*E. coli*	30.8	5.1	269	269	12	Dimer	Phospholipase	1QD6	Snijder et al., 1999
FadL	*E. coli*	45.9	4.9	421	378	14	Monomer	Fatty acid transporter	1T16, 1T1L	van den Berg et al., 2004a
Omp32	*C. acidovorans*	34.8	8.8	332	332	16	Trimer	Porin	1E54	Zeth et al., 2000
Porin	*Rhodobacter capsulatus*	31.5	4.0	301	301	16	Trimer	Porin	2POR	Weiss et al., 1991; Weiss and Schulz, 1992
Porin	*R. blastica*	30.6	3.8	290	290	16	Trimer	Porin	1PRN	Kreusch and Schulz, 1994
OmpF	*E. coli*	37.1	4.6	340	340	16	Trimer	Porin	2OMF	Cowan et al., 1992
PhoE	*E. coli*	36.8	4.8	330	330	16	Trimer	Porin	1PHO	Cowan et al., 1992
OmpK36	*Klebsiella pneumoniae*	37.6	4.4	342	342	16	Trimer	Porin	1OSM	Dutzler et al., 1999
LamB	*E. coli*	47.4	4.7	420	420	18	Trimer	Maltose-specific porin	1MAL, 1AF6	Schirmer et al., 1995; Wang et al., 1997

Maltoporin	Serovar Typhimurium	48.0	4.7	427	427	18	Trimer	Maltose-specific porin	2MPR	Meyer et al., 1997
ScrY	Serovar Typhimurium	53.2	5.0	483	415	18	Trimer	Sucrose porin	1A0S, 1A0T	Forst et al., 1998
FhuA	*E. coli*	78.7	5.1	714	587	22	Monomer	Ferrichrome iron transporter	2FCP, 1BY3	Ferguson et al., 1998; Locher et al., 1998
FepA	*E. coli*	79.8	5.2	724	574	22	Monomer	Ferrienterobactin transporter	1FEP	Buchanan et al., 1999
FecA	*E. coli*	81.7	5.4	741	521	22	Monomer	Iron (III) dicitrate transporter	1KMO, 1PNZ	Ferguson et al., 2002; Yue et al., 2003
BtuB	*E. coli*	66.3	5.1	594	459	22	Monomer	Vitamin B_{12} transporter	1NQE, 1UJW	Chimento et al., 2003; Kurisu et al., 2003
FpvA	*P. aeruginosa*	86.5	5.1	772	538	22	Monomer	Ferripyoverdine transporter	1XKH	Cobessi et al., 2005

Organism	OMP	MW (kDa)	pI	Residues	Residues in β-barrel domain	β-Strands in barrel domain	Chains in the β-barrel	Function	PDB entry	References
Outer membrane proteins with multichain β-barrels										
TolC	*E. coli*	51.5	5.2	471	285 (95 × 3)	12 (4 × 3)	Trimer	Export channel	1EK9	Koronakis et al., 2000
MspA	*M. smegmatis*	17.6	4.4	168	432 (32 × 8)	16 (2 × 8)	Octamer	Porin	1UUN	Faller et al., 2004
α-Hemolysin	*S. aureus*	33.2	7.9	293	378 (54 × 7)	14 (2 × 7)	Heptamer	Toxin	7AHL	Song et al., 1996

[a]Calculated by Protparam/SWISS-PROT.
[b]NMR structure.
[c]Translocator domain.

membrane proteins indicate that many encoded OMPs are still not characterized, for example, in the genomes of *E. coli* and *Pseudomonas aeruginosa*, where their genes comprise about 2 to 3% of the entire genome (Wimley, 2002, 2003). Soluble bacterial toxins that can insert into membranes, such as α-hemolysine from *Staphylococcus aureus* (Song et al., 1996) and perfringolysine O from *Clostridium perfringens* (Heuck et al., 2000; Shepard et al., 1998), also form β-barrels, but these are oligomeric. Oligomeric β-barrels are also found in mycobacteria, such as MspA from *Mycobacterium smegmatis* (Faller et al., 2004; for a review, see Niederweis, 2003). In this chapter, I focus on membrane insertion and assembly of the porins that form single-chain transmembrane β-barrels.

PERIPLASMIC PROTEINS INVOLVED IN OMP ASSEMBLY

After their biosynthesis, OMPs bind to the chaperone SecB in the cytoplasm and are then targeted in concert with the ATPase SecA to the cytoplasmic membrane (Driessen et al., 2001; Müller et al., 2001). Some secreted proteins instead use the signal recognition particle (SRP) pathway for targeting; for example, lipoproteins (Froderberg et al., 2004) or certain autotransporters (Sijbrandi et al., 2003). The OMPs are then translocated in an unfolded form across the cytoplasmic (inner) membrane via the SecYEG translocon (Breyton et al., 2002; Van den Berg et al., 2004b), requiring ATP and electrochemical energy. The pathway of OMPs from the cytoplasm toward the OM is illustrated in Fig. 1. After their translocation, a signal peptidase (SPase), which is bound to the cytoplasmic membrane, cleaves the N-terminal signal sequence of the OMP in the periplasmic space, recognizing the Ala-X-Ala motif at the end of the OMP signal sequence (Tuteja, 2005), which typically comprises the first 15 to 30 residues of the unprocessed OMP. After signal sequence cleavage, the mature OMP traverses the periplasm toward the OM for integration. Overproduction of OMPs or accumulation of unfolded OMPs in the periplasm activates the alternative stress σ-factor, σ^E (RpoE) (Mecsas et al., 1993), in the cytoplasm, which then causes production of periplasmic proteases and folding factors. Eσ^E RNA polymerase transcribes, for example, the genes of the periplasmic proteins Skp, SurA, DegP, and FkpA, which act as chaperones and affect the assembly of OMPs (Chen and Henning, 1996; Lazar and Kolter, 1996; Missiakas et al., 1996; Rizzitello et al., 2001; Rouvière and Gross, 1996); the genes of periplasmic proteases such as DegP (HtrA); the genes of certain outer membrane lipoproteins, such as YfiO; genes of enzymes involved in the biosynthesis of lipopolysaccharide (LPS), such as HtrM (RfaD), LpxD, and LpxA (Dartigalongue et al., 2001; Rouvière et al., 1995); and the gene of the OMP Imp (OstA) (Dartigalongue et al., 2001). Stress in the periplasm, such as heat, leads to misfolding of periplasmic proteins and OMPs, which is sensed by the trimeric protease DegS (Alba et al., 2001; Wilken et al., 2004) (for reviews, see, e.g., Alba and Gross, 2004; Ehrmann and Clausen, 2004) that is anchored to the periplasmic side of the inner membrane and binds the carboxy terminus of misfolded OMPs, recognizing the C-terminal peptide motif Tyr-X-Phe-COOH (Walsh et al., 2003). This motif is frequently found in the C terminus of the β-barrel domain of OMPs (Struyve et al., 1991; Walsh et al., 2003). Upon binding of misfolded OMPs in a DegS PDZ-domain (a PDZ-domain is a protein domain involved in protein or peptide binding and named after three eukaryotic proteins in which it was observed: postsynaptic density protein, discs large protein, and zona occludens protein; see Sheng and Sala, 2001, for a recent review), DegS is activated and cleaves the anti-σ-factor RseA, which is in complex with RseB in the cytoplasmic membrane and with σ^E (RpoE) in the cytoplasm (Campbell et al., 2003; Collinet et al., 2000; De Las Peñas et al., 1997; Missiakas et al., 1997). Cleavage of RseA is then completed by the inner membrane metalloprotease YaeL (RseP) (Ades et al., 1999; Alba et al., 2002; Kanehara et al., 2003), leading to the release of σ^E into the cytoplasm (Ades et al., 2003). Under nor-

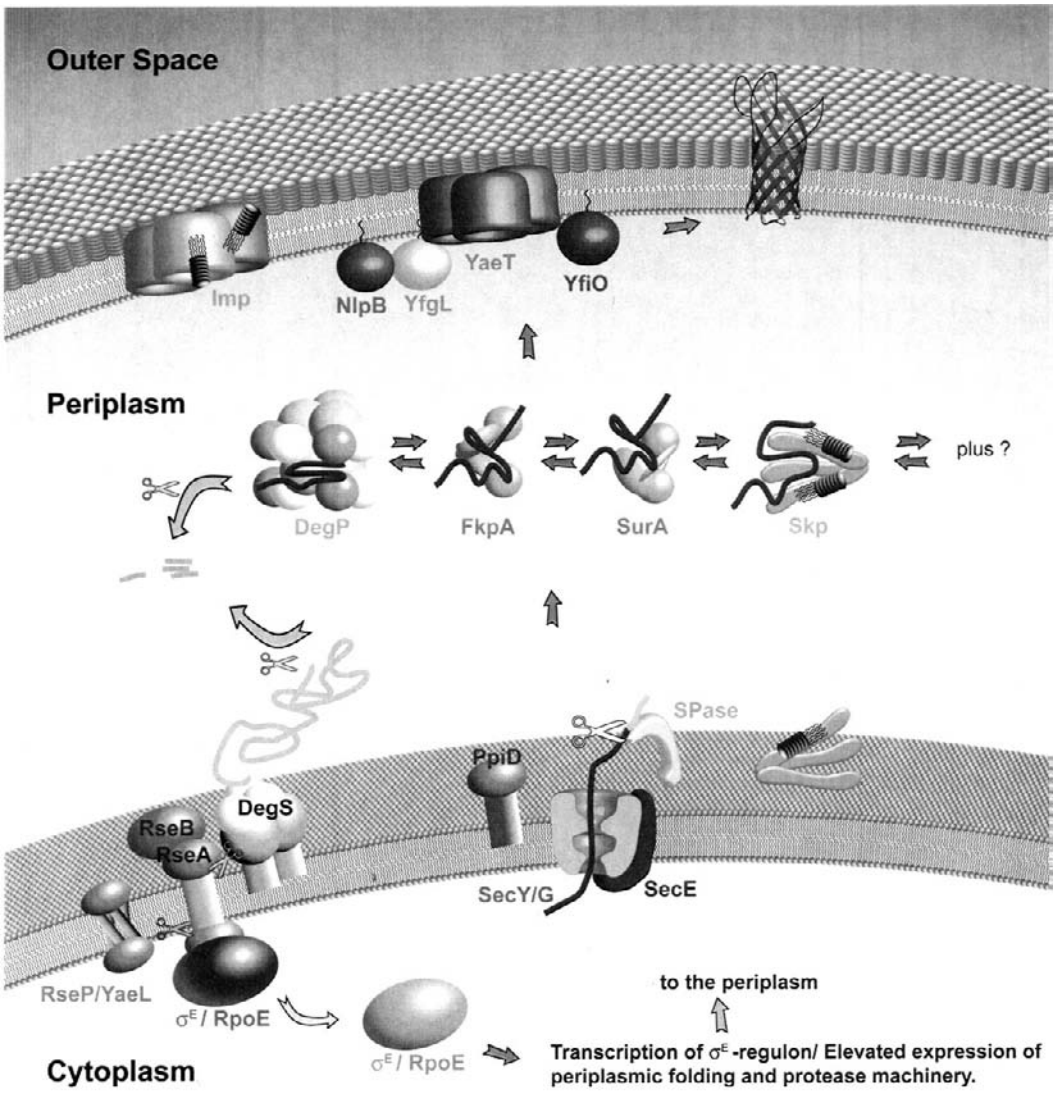

FIGURE 1 Export of integral membrane proteins through the periplasm to the OM. The OMP traverses the inner membrane through the SecY/G/E translocon in an unfolded form. A leader peptidase (SPase), anchored to the inner membrane, cleaves the signal sequence in the periplasm. The OMP then traverses the periplasm bound to a periplasmic chaperone. Among the periplasmic proteins that were either reported or likely to bind unfolded OMPs are Skp, SurA, DegP, and FkpA. All of the currently discovered soluble periplasmic folding factors are bifunctional. Skp binds unfolded OMPs and LPS; SurA and FkpA have chaperone function and—independent of the chaperone function—PPIase activity. DegP is a protease and a chaperone. From the periplasm, OMPs are targeted to or assembled into the outer membrane by membrane-bound proteins, namely YaeT (Omp85) and the lipoproteins YfiO, YfgL, and NlpB. This process has not been investigated yet. Misfolded proteins in the periplasm are degraded by proteases such as DegP and DegS. DegS is a sensor for misfolded OMPs and consequently cleaves the cytoplasmic RseA in the periplasm. In a second cleavage step, the cleaved RseA is degraded further by RseP (YaeL), leading to the release of σ^E (RpoE), which results in an elevated expression of periplasmic chaperones, isomerases, and proteases, and of OM-associated folding factors. See the text for further details.

mal conditions, the RseA-RseB complex binds about half of the σ^E content of the cell (Campbell et al., 2003; Collinet et al., 2000; De Las Peñas et al., 1997; Missiakas et al., 1997), and RseA degradation, initiated by DegS, upregulates the transcription of the heat shock genes by release of bound σ^E (Ades et al., 2003). The σ^E-dependent stress response is also activated in *rfa* mutants. The *htrM* (*rfaD*) gene product was shown to encode an ADP-L-glycero-D-mannoheptose-6-epimerase, an enzyme required for the biosynthesis of an LPS precursor (Pegues et al., 1990; Raina and Georgopoulos, 1991). Lack of the core heptose moiety in LPS was observed in such *htrM* (*rfaD*) mutants (Missiakas et al., 1996).

Searches for folding factors in the periplasm resulted in the discovery of several interesting proteins that function as chaperones or peptidyl-prolyl *cis/trans* isomerases (PPIases), which are discussed in the following sections. For example, the periplasmic seventeen kDa protein, Skp, was found to bind to unfolded OMPs on affinity columns, and the deletion of the *skp* gene led to reduced levels of OMPs in the *E. coli* OM (Chen and Henning, 1996). The concentrations of some OMPs in the OM of *E. coli* were also decreased, when one of the genes of the periplasmic PPIases, SurA (Lazar and Kolter, 1996; Rouvière and Gross, 1996) or PpiD (Dartigalongue and Raina, 1998), was deleted. There is no ATP in the periplasm (Wülfing and Plückthun, 1994) and therefore periplasmic chaperones are expected to function differently from cytoplasmic chaperones, which utilize ATP in their catalytic cycles (Craig, 1993).

Representatives of three different families of PPIases were found in the periplasm. These may assist the folding of OMPs, which traverse the periplasm in unfolded form. Examples are the parvulin type SurA (Behrens et al., 2001; Missiakas et al., 1996), the FKBP type FkpA (Bothmann and Plückthun, 2000; Missiakas et al., 1996; Ramm and Plückthun, 2000, 2001), and the cyclophilin type PpiA (RotA) (Liu and Walsh, 1990) (see Table 2).

Skp

The periplasmic seventeen-kilodalton protein, Skp (141 residues, 15.7 kDa), was identified as the major component of a mixture of periplasmic proteins that bound to sepharose-linked unfolded OMPs on affinity columns (Chen and Henning, 1996). *E. coli* cells lacking the *skp* gene display reduced levels of OmpA, OmpC, OmpF, and LamB in the OM (Chen and Henning, 1996; Missiakas et al., 1996), a phenotype which resembles that of *surA* mutants (Missiakas et al., 1996; Rouvière and Gross, 1996). Furthermore, Skp was found to improve the functional expression of a soluble fragment of the antibody 4-4-20 (Bedzyk et al., 1990; Whitlow et al., 1995) in the periplasm of *E. coli* (Bothmann and Plückthun, 1998). Skp almost completely prevents the aggregation of the soluble protein lysozyme at a molar ratio of 3:1 Skp/lysozyme (Walton and Sousa, 2004), consistent with previous observations on the 3:1 stoichiometry of Skp binding to OmpA (Bulieris et al., 2003).

Skp appears to be under control of both the σ^E (Alba and Gross, 2004; Ehrmann and Clausen, 2004) and the two-component CpxA/CpxR (Duguay and Silhavy, 2004) stress-response systems (Dartigalongue et al., 2001). Skp forms stable homotrimers in solution as determined by gel-filtration and cross-linking experiments (Schlapschy et al., 2004). The protein is highly basic with a calculated pI between 9.6 and 10.3 (depending on the algorithm used). The structure of the Skp trimer (Korndörfer et al., 2004; Walton and Sousa, 2004) (see Color Plate 3A) resembles a jellyfish with α-helical tentacles protruding about 60 Å from a β-barrel body and defining a central cavity. The entire Skp trimer is about 80 Å long and 50 Å wide. The Skp monomer has two domains. The small association domain (residues 1 to 21 and 113 to 141 of the mature sequence) is composed of three β-strands and two short α-helices, forms the limited hydrophobic core, and mediates the trimerization of Skp. The second, tentacle-shaped α-helical domain is formed by amino acids 22 to 112.

TABLE 2 Proteins suggested to be involved in assembly of outer membrane proteins

Protein	Organism	Residues[a]	Molecular mass (kDa)	Est. pI	Class	Oligomeric state	Essential	PDB entry	Reference(s)
Periplasmic proteins									
Skp[b]	*E. coli*	141	16	9.5	Chaperone	Trimer	No	1U2M, 1SG2	Korndörfer et al., 2004; Walton and Sousa, 2004
SurA[b]	*E. coli*	408	45	6.1	Chaperone/PPIase	Monomer	No	1M5Y	Bitto and McKay, 2002
DegP[b]	*E. coli*	448	47	7.9	Chaperone/Protease	Hexamer	No[c]	1KY9	Krojer et al., 2002
FkpA[b]	*E. coli*	245	26	6.7	Chaperone/PPIase	Dimer	No	1Q6U,1Q6H, 1Q6I	Saul et al., 2003, 2004
PpiA[b]	*E. coli*	166	18	8.2	PPIase	Monomer	No	1CLH, 1CSA, 1J2A	Clubb et al., 1994; Fejzo et al., 1994; Konno et al., 2004
PpiD[b]	*E. coli*	623	68	4.9	PPIase	Monomer	No		Dartigalongue and Raina, 1998
DegS	*E. coli*	327	35	5.0	Protease	Trimer	Yes	1SOT, 1SOZ, 1VCW, 1TE0	Wilken et al., 2004; Alba et al., 2001
Outer membrane proteins									
YfgL	*E. coli*	373	40	4.6	OM lipoprotein		No		Ruiz et al., 2005
YfiO	*E. coli*	226	26	5.5	OM lipoprotein		Yes		Wu et al., 2005
NlpB	*E. coli*	320	34	5.0	OM lipoprotein		No		Wu et al., 2005
YaeT	*E. coli*	790	88	4.9	Integral OMP		Yes		Doerrler and Raetz, 2005; Werner and Misra, 2005; Wu et al., 2005
Omp85	*N. meningitidis*	797	85	8.6	Integral OMP		Yes		Genevrois et al., 2003; Gentle et al., 2004; Voulhoux et al., 2003
HMW1B	*H. influenzae*	545	61	9.3	Integral OMP	Tetramer ?			Surana et al., 2004
Imp	*N. meningitidis*	802	89	8.8	Integral OMP		No		Bos et al., 2004
Imp	*E. coli*	760	87	4.9	Integral Omp		Yes		Braun and Silvahy, 2002

[a]With the exception of PpiD and the proteins from *N. meningitidis* and *H. influenzae,* parameters are given for the mature protein sequence. Number of residues, molecular mass, and pI were obtained from the SWISS-PROT database.

[b]While Skp, SurA, and DegP are individually not essential, double-null mutations in *skp* and *surA* (Rizzitello et al., 2001) and in *degP* and *surA* are (Rizzitello et al., 2001). Strains in which the four genes *surA, fkpA, ppiD,* and *ppiA* were deleted simultaneously were viable (Justice et al., 2005).

[c]DegP is essential for growth at elevated temperatures (Lipinska et al., 1990).

This domain is conformationally flexible. The charge distribution on the Skp surface gives the trimer an extreme dipole moment of ~3,700 Debye (770 eÅ) (Korndörfer et al., 2004), with positive charges all over the tentacle domain and, in particular, at the tips of the tentacle-like helices, while negative surface charge is found in the association domain. The surface of the tentacle-shaped domain contains hydrophobic patches inside the cavity formed by the tentacles. It may be that Skp binds its substrates in this central cavity (Korndörfer et al., 2004; Walton and Sousa, 2004). While the size of the cavity could be large enough to accommodate the transmembrane domain of OmpA in a folded form (Korndörfer et al., 2004), biochemical and spectroscopic data suggest that the OmpA barrel domain is largely unstructured when in complex with Skp (Bulieris et al., 2003). Also, the cavity would not be large enough for folded β-barrels of other OMPs to which Skp also binds, as shown for OmpF (Chen and Henning, 1996) and, in cross-linking experiments, for LamB and PhoE (Harms et al., 2001; Schäfer et al., 1999). As described later in this chapter, there is evidence for a connection between the function of Skp and LPS. Skp has a putative LPS binding site (Walton and Sousa, 2004) that was found by using a previously identified LPS binding motif (Ferguson et al., 2000). The binding site is formed on the surface of each Skp monomer by residues K77, R87, and R88, similar to the LPS binding motif in FhuA with residues K306, K351, and R382. Q99 in Skp may also form a hydrogen bond to an LPS phosphate, completing the four-residue LPS binding motif.

The structure of Skp resembles that of prefoldin (Pfd, GimC) of the archaeon *Methanobacterium thermotrophicum* (Siegert et al., 2000). However, in contrast to Skp, Pfd has a negative electrostatic surface potential and the pI is 4.6 (Devereux et al., 1984; Korndörfer et al., 2004). Pfd prevents aggregation of proteins in the cytosol and delivers them to class II cytosolic chaperonins (also called c-cpn, CCT, or TriC) independent of the presence of ATP or other nucleotides (Vainberg et al., 1998). Although prefoldin is a hexamer ($\alpha_2\beta_4$), it can be seen as a dimer of trimers (Walton and Sousa, 2004), with the trimers resembling the Skp trimer. Electron microscopy reconstructions of Pfd bound to unfolded substrates have shown that the substrates are located within the cavity formed by the α-helical tentacles, where they appear to bind to the tips of the helices (Lundin et al., 2004; Martin-Benito et al., 2002). Eukaryotic prefoldin only binds stably to nascent actin chains if they are at least 145 residues long, suggesting a synergistic action of multiple weaker binding sites (Siegert et al., 2000). Pfd efficiently prevented aggregation of denatured rhodanese at a 1:1 molar ratio of the Pfd hexamer to rhodanese (Siegert et al., 2000), similar to the substrate binding stoichiometry of trimeric Skp.

Skp was found to insert into monolayers of negatively charged lipids (de Cock et al., 1999b). Consistent with this observation, two forms of Skp could be distinguished based on their sensitivity to proteolysis with trypsin or proteinase K: a free periplasmic form that is degraded and a form that is protected against digestion by association with membrane phospholipids (de Cock et al., 1999b). The presence of LPS in digestion experiments reduced the relative amount of protease-resistant Skp (de Cock et al., 1999b). Skp binds to the NH_2-terminal transmembrane β-barrel of OmpA in its unfolded form and is required for the release of OmpA into the periplasm (Schäfer et al., 1999). Skp binds neither to folded OmpA nor to the periplasmic domain (Chen and Henning, 1996), suggesting that Skp recognizes nonnative structures of OMPs. The *skp* gene maps at the 4-min region on the chromosome and is located upstream of genes that encode proteins involved in lipid A biosynthesis (Dicker and Seetharam, 1991; Roy and Coleman, 1994; Thome et al., 1990), an essential component of LPS of the OM. The gene *firA*, which codes for UDP-3-*O*-[3-hydroxymyristoyl]-glucosamine-*N*-acyltransferase starts only 4 bases downstream of the *skp* stop codon (Bothmann and Plückthun, 1998). The presence of a putative binding site for LPS in Skp (Walton and Sousa, 2004) could be related to the location of *skp* close to *firA*.

SurA

A third periplasmic protein, the survival factor A, SurA, has been demonstrated to affect OMP assembly. SurA was first identified as necessary for stationary-phase survival (Tormo et al., 1990). *E. coli* mutants in which the *surA* gene is deleted have reduced concentrations of OmpA and LamB in the OM (Lazar and Kolter, 1996; Rouvière and Gross, 1996). SurA$^-$ strains are constitutively induced for the σ^E-dependent extracytoplasmic stress response (Missiakas et al., 1996; Rouvière and Gross, 1996), one of two signal transduction pathways known to communicate the folding state in the periplasm to the cytoplasm (Connolly et al., 1997; Danese and Silhavy, 1997; Mecsas et al., 1993).

The crystal structure of SurA is shown in Color Plate 3B. SurA consists of an N-terminal domain (N), which is composed of 148 amino acids and contains the α-helices H1 to H6. This domain is connected to the domain P1 (residues 149 to 260) and the domain P2 (residues 261 to 369). P2 connects the P1 domain to the C-terminal domain C (residues 370 to 428). Together, the N and C domains constitute a compact core with a broad deep crevice of about 50 Å in length. The P2 domain is tethered to this core by two extended peptide segments. The P1 and P2 domains have sequence similarity to parvulin, a cytoplasmic PPIase (Rahfeld et al., 1994).

The function of SurA in the transport and folding pathway to the OM was first assigned to its activity as a PPIase through its two parvulin-like domains, P1 and P2 (Color Plate 3B) (Lazar and Kolter, 1996; Missiakas et al., 1996; Rouvière and Gross, 1996). Only the P2 domain displayed PPIase activity in assays with reduced and carboxymethylated RNase T1 variant (RCM-T1), as demonstrated with SurA mutants lacking either the P1 or the P2 domain (Behrens et al., 2001). However, with a mutant form of SurA, from which the PPIase domains P1 and P2 were removed, it was shown that the N domain containing helices H1 to H6 functions as chaperone when linked with the C helix. A plasmid containing the gene for this mutant restored wt-SurA function and eliminated the extracytoplasmic stress response seen by activation of σ^E in *surA* deletion strains (Behrens et al., 2001). Since the two parvulin domains were obviously not necessary for SurA function in the maturation of OMPs, the SurA mutant lacking the parvulin domains was tested for function as a chaperone. In light-scattering assays on the aggregation of soluble citrate synthase during thermal stress, the presence of a 64-fold molar excess of SurA eliminated citrate synthase aggregation (Behrens et al., 2001).

The N-terminal amino acids 21 to 133 of SurA were able to bind peptides independent of the presence of proline (Webb et al., 2001). The SurA "core domain" has been proposed to bind the tripeptide motif aromatic-random-aromatic, which is prevalent in the aromatic girdles of β-barrel membrane proteins (Bitto and McKay, 2003). Isothermal titration calorimetry revealed that both the SurA and the SurA core domain bind a heptameric peptide with the sequence WEYIPNV with high affinity in the range of 1 to14 μM (Bitto and McKay, 2003). Both forms of SurA exhibited affinity to the peptide consensus motif aromatic-polar-aromatic-nonpolar-proline (Ar-Pol-Ar-NonPol-Pro). Ar-X-Ar tripeptide motifs, where X can be any amino acid residue, are found with high frequency in OMPs, in particular, in two aromatic girdles close to the polar-apolar interfaces of the lipid bilayer. On average, the Ar-X-Ar motif is found about twice as often in membrane proteins as in cytoplasmic or periplasmic proteins (Bitto and McKay, 2003). For example, the number of Ar-X-Ar motifs in the β-barrel domains of OMPs is 7 for OmpF, 10 for LamB, 3 for OmpA, and 1 for TolC. Consistent with this, SurA bound unfolded OmpF and OmpG one order of magnitude more tightly than the soluble protein reduced carboxymethylated lactalbumin (Bitto and McKay, 2004). DegS binds the Ar-X-Ar-COOH motif with affinities in a range of 0.6 to 15 μM (Walsh et al., 2003), which is similar to the affinity of SurA to the WEYIPNV peptide and stronger than the affinity of SurA to the Ar-X-Ar motif (Bitto and McKay, 2003). Binding to SurA required a minimum of five amino acids, and a proline at position 5 in-

creased the binding affinity (Bitto and McKay, 2003). A recent study indicated that, in addition to the presence of the Ar-X-Ar motif, the orientation of the amino acid side chains is important (Hennecke et al., 2005).

Although deletion of *surA* led to decreased levels of the OMPs LamB and OmpA in vivo (Lazar and Kolter, 1996; Rouvière and Gross, 1996), SurA had no effect on the assembly of TolC in vivo (Werner et al., 2003) or of AIDA in vitro (Mogensen et al., 2005), which contain only one and two Ar-X-Ar motifs, respectively, in their β-barrel domains. In preliminary experiments in the authors' laboratory, SurA facilitated the membrane insertion and folding of OmpA into preformed lipid bilayers (P. V. Bulieris, S. Behrens, and J. H. Kleinschmidt, manuscript in preparation). More recently, it was found that *surA* deletion severely diminished the expression of P and type 1 pili, which are adhesive cell surface organelles produced by uropathogenic strains of *E. coli* and assembled in the chaperone/usher pathway (Justice et al., 2005).

Genetic evidence suggests that SurA and Skp act as chaperones that are involved in parallel pathways of OMP targeting to the OM (Rizzitello et al., 2001). Null mutations in *skp* and *surA* as well as in *degP* and *surA* resulted in synthetic phenotypes. The *skp surA* null combination had a bacteriostatic effect and led to filamentation, while the *degP surA* null combination was bactericidal. It was suggested that the redundancy of Skp, SurA, and DegP is in the periplasmic chaperone activity, in which Skp and DegP are components of one pathway and SurA is a component of a parallel pathway. While the loss of either pathway was tolerated, the loss of both pathways was lethal (Rizzitello et al., 2001).

DegP

The widely conserved periplasmic DegP (also called HtrA, 448 residues, 47 kDa) is a heat shock protein and a member of the HtrA family of proteases (Lipinska et al., 1990; Spiess et al., 1999; Strauch et al., 1989), for which, in addition, a chaperone activity has been discovered (Spiess et al., 1999). Transcription of *degP* in response to periplasmic stress is under control of σ^E stress response system. Its double function as a protease and chaperone makes DegP an interesting quality control protein in the periplasm. In refolding experiments with soluble proteins as substrates, equimolar concentrations of DegP or $DegP_{S210A}$, which lacks protease activity due to substitution of serine by alanine in the active site, led to functional refolding of citrate synthase at 28°C (Spiess et al., 1999). Similarly, DegP facilitated refolding of periplasmic MalS, an α-amylase that hydrolyzes maltodextrins. Folding of MalS by $DegP_{S210A}$ was facilitated at moderate temperatures of 28°C or below, while experiments at 37°C or higher had only a very minor stimulating effect on the reactivation of MalS. By contrast, the proteolytic activity of wild-type DegP increased dramatically above 30°C (Spiess et al., 1999).

Two different conformational states of DegP, corresponding to the open and closed state of the homo-hexamer, were observed in the X-ray crystal structure of DegP (1KY9), which has been solved (Krojer et al., 2002). One DegP subunit is shown in Color Plate 4A. One DegP molecule consists of a protease domain and two protein binding domains, the PDZ domains. PDZ domains typically bind sequence specific a short C-terminal protein sequence, which folds into β-finger (Sheng and Sala, 2001). Six DegP molecules form a complex that consists of two loosely stacked rings of trimers (Krojer et al., 2002). The protease domains are located in a central cavity, while the 12 PDZ domains form the mobile side walls. These PDZ-domain side walls bind the protein substrates and mediate the opening and closing of the hexamer, allowing the exclusively lateral access of the substrate to the inner cavity formed by the two stacked rings of DegP trimers. The inner cavity is lined by hydrophobic residues, which are proposed to act as docking sites for unfolded proteins in the chaperone state of DegP. The protease domain is not active when DegP is in the chaperone conformation and further substrate binding

is not possible. The structural organization of the DegP hexamer differs from other known cage-forming proteins, where access is allowed through narrow axial or lateral pores. The PDZ domains of the neighboring subunits interact with each other and form gatekeepers for the inner chamber (Krojer et al., 2002).

There are currently no experimental studies on OMP assembly that demonstrate a direct chaperone effect of DegP on the concentrations of TMPs in the OM. However, it was recently observed that DegP tightly binds to OmpC (M. Ehrmann, personal communication). Also, the chaperone function of OmpC was important to maintain viability of cells producing assembly-deficient mutants of OmpF, in which the conserved carboxy-terminal phenylalanine was nonconservatively replaced by dissimilar amino acids, leading to accumulation of monomeric, unfolded OmpF in the periplasm (Misra et al., 2000). In this study, the mutant $DegP_{S210A}$ (Spiess et al., 1999) was used. Overproduction of $DegP_{S210A}$ led to mislocalization of OmpF to the inner membrane. No effect of DegP or $DegP_{S210A}$ could be observed in folding studies with the autotransporter AIDA, which forms a transmembrane β-barrel domain in the OM. Instead, the presence of DegP resulted in proteolytic cleavage of AIDA (Mogensen et al., 2005). In contrast to observations with OmpA (Bulieris et al., 2003), the chaperone Skp also did not bind to AIDA and it did not affect the folding of the transmembrane β-barrel domain of AIDA (Mogensen et al., 2005). Therefore, further experiments are needed to clarify whether DegP directly affects the folding of OMPs into membranes.

FkpA

The structure of FkpA (245 residues, 26 kDa) has been determined in three different forms (Saul et al., 2004): for wild-type FkpA, for a truncated mutant lacking the last 21 residues, FkpAΔCT, and for this mutant (see Color Plate 4B) in complex with the macrolide FK506, which is an immunosuppressant. FkpA forms V-shaped dimers and the 245-residue subunit is divided into two domains. The N-terminal domain (residues 15 to 114) includes three α-helices that are interlaced with those of the other subunit. The C-terminal domains (residues 115 to 224) are located at the two ends of the V. The C domain, which is structurally similar to human FKBP12, belongs to the FKBP-type of PPIases, well-characterized PPIases that are inhibited by FK506. The two FKBP type C domains are held apart by the long α-helix 3 of the N domains, with their FK506 binding sites facing toward each other. In the FK506-bound FkpAΔCT crystal structure the centers of the ligands are about 49 Å apart from each other.

FkpA isomerase activity was measured in a protein-folding assay with Rnase T1 and determined to k_{cat}/K_m = 4,000 $mM^{-1}\cdot s^{-1}$ (Ramm and Plückthun, 2000). This underlines the efficiency of FkpA compared with other FKBPs, such as FKBP12 with k_{cat}/K_m = 800 $mM^{-1}\cdot s^{-1}$ (Dolinski et al., 1997) and trigger factor with k_{cat}/K_m = 740 $mM^{-1}\cdot s^{-1}$ (Stoller et al., 1995). In comparison, activities of the cyclophilin PpiA were determined to k_{cat}/K_m = 6,000 $mM^{-1}\cdot s^{-1}$ (Compton et al., 1992) and to 10,000 $mM^{-1}\cdot s^{-1}$ (Pogliano et al., 1997). The activity of the parvulin type SurA was k_{cat}/K_m = 30 to 40 $mM^{-1}\cdot s^{-1}$ (Behrens et al., 2001) and that of PpiD was k_{cat}/K_m = 400 to 3,400 $mM^{-1}\cdot s^{-1}$ (Dartigalongue and Raina, 1998).

A selection system for periplasmic chaperones (Bothmann and Plückthun, 1998), using a genomic library from an *skp* deletion strain (Bothmann and Plückthun, 2000), led to the discovery of FkpA as a folding promoter for the single-chain antibody fragment (scFv) of the antibody 4-4-20 (Bedzyk et al., 1990; Whitlow et al., 1995). Subsequently, coexpression of FkpA was also shown to improve functional expression of scFv (Bothmann and Plückthun, 2000), even for those antibody fragments that did not contain any *cis*-prolines, suggesting that the effect of FkpA as a folding facilitator is independent of its PPIase activity (Bothmann and Plückthun, 2000). In contrast, SurA or PpiA showed no increase in the expression levels of functional scFv, neither upon coexpression in the periplasm nor by the phage

display method. The chaperone function of FkpA was also observed with the periplasmic protein MalE31, a maltose binding protein with a high tendency to aggregation (Arie et al., 2001), and could be assigned to the N-terminal domain, which exists in solution as a mixture of monomers and dimers (Saul et al., 2004), while the C-terminal FKBP domain contained the PPIase function (Saul et al., 2004). Light-scattering assays indicated that an 8-fold molar excess of FkpA reduced citrate synthase aggregation to a high extent and delayed it considerably (Ramm and Plückthun, 2001).

Homologues of FkpA are found in many pathogenic bacteria such as *Legionella pneumophila* (Horne and Young, 1995). FkpA is not essential, but *fkpA*$^-$ strains display an increased permeability of the OM to certain detergents and to antibiotics (Missiakas et al., 1996).

Periplasmic DegP is upregulated in *fkpA*$^-$ strains, suggesting that FkpA is involved in folding of proteins in the periplasm. Transcription of *fkpA* is under control of the alternative σ-factor σ^E (Danese and Silhavy, 1997; Dartigalongue et al., 2001; Missiakas et al., 1996). The elevated expression of the σ^E regulon that is induced in *htrM (rfaD)* deletion mutants, i.e., in strains producing a modified form of LPS, is turned off by overexpression of FkpA or SurA. This was monitored using *htrM* mutants, in which the gene for β-galactosidase, *lacZ*, was fused to σ^E-transcribed promoters (Missiakas et al., 1996; Raina et al., 1995). Such *htrM* mutants display increased activitity of β-galactosidase because of increased σ^E stress response. The elevated activity of β-galactosidase was reduced upon overexpression of FkpA or SurA in these *htrM* mutants. A similar observation was made for *htrA dsbC* double-null mutants (Missiakas et al., 1996).

To date, a direct effect of FkpA on the assembly of OMPs has not been demonstrated, and FkpA will be an interesting target for future studies.

PpiA (Rotamase A, RotA)

PpiA (16 kDa, 166 residues), which is sometimes also called RotA, is a member of the cyclophilin class of PPIases (Hayano et al., 1991; Liu and Walsh, 1990). Both the NMR solution structure of PpiA (Clubb et al., 1994; Fejzo et al., 1994) and the X-ray crystal structure (Konno et al., 2004) have been solved. PpiA is expressed under control of the two-component CpxR-CpxA stress response system (Pogliano et al., 1997). It is homologue to human cyclophilin (34% sequence identity) and has similar PPIase activity. The catalytic activity is close to the upper diffusional limit with $k_{cat}/K_m \sim 1.0 \times 10^7\ M^{-1}s^{-1}$ at 10°C, but unlike human cyclophilin, PpiA is not inhibited by cyclosporin A (Pogliano et al., 1997), which binds to cyclophilin with high affinity (K_d = 17 nM), but to PpiD with low affinity (K_d = 3.4 μM) (Fejzo et al., 1994). PpiA is not essential (Kleerebezem et al., 1995) and deletion of *ppiA* did neither affect the levels of integral proteins in the OM nor the levels of soluble periplasmic proteins. Also, rates of OMP integration into the OM in vivo were not affected according to pulse-chase experiments, suggesting that PpiA does not play an important role in protein folding (Kleerebezem et al., 1995).

PpiD

PpiD belongs to the parvulin class of PPIases (Dartigalongue and Raina, 1998). Transcription is controlled by the CpxAR stress response system (Danese et al., 1995; Danese and Silhavy, 1997; Dartigalongue and Raina, 1998). PpiD (68 kDa, 623 residues) is anchored to the inner membrane by a transmembrane helix. Recently it was reported that inactivation of all four genes *ppiD*, *surA*, *ppiA*, and *fkpA* encoding the known periplasmic PPIases resulted in a viable strain. This strain had reduced levels of the OMPs LamB and OmpA, with OMP levels similar to that of the *surA* null mutant (Justice et al., 2005). This result suggests that neither PpiD, nor FkpA, nor PpiA has a direct role in OMP folding.

Other Periplasmic Chaperones

In addition to the chaperones discussed above, other classes of chaperones are in the periplasm. For example, the PapD-like superfam-

ily of chaperones, such as PapD and FimC, are important in the chaperone/usher pathways for the assembly of bacterial surface organelles such as fimbria/pili, which are involved in cell-surface attachment and cell-cell contacts. The PapD-like chaperones act very specifically with nascent pilus (FGS chaperones) or nonpilus (FGL chaperones) subunits as they emerge from inner membrane translocon, and they subsequently deliver the subunits to the corresponding usher pore in the OM, at which the subunits are assembled into linear fibers before their secretion through the pore. A detailed discussion of these specialized chaperones is beyond the scope of this chapter. For a review, see Thanassi (2002). Another class of periplasmic chaperones is involved in transport of outer membrane lipoproteins across the periplasm to the OM. An example is the lipoprotein chaperone LolA (Taniguchi et al., 2005). Most of the 90 different species of lipoproteins are known to be associated with the periplasmic side of the OM, to which they are anchored by a lipoid linked to the N-terminal cysteine of the lipoprotein. For a recent review on the transport of outer membrane lipoproteins to the OM, see Tokuda and Matsuyama (2004).

OMPs INVOLVED IN OMP ASSEMBLY

Omp85, YaeT, and HMW1B

It was discovered recently that Omp85 of the OM of *Neisseria meningitidis* is essential for cell growth (Voulhoux et al., 2003). Omp85 was necessary for the integration of several OMPs, such as the trimeric porins PorA and PorB, the heterooligomeric complex of the siderophore receptor FrpB/RmpM, or the outer membrane phospholipase A (OmpLA). In strains depleted of Omp85, the passenger domain of the immunoglobulin A1 protease autotransporter (IgA1) was difficult to detect, while the full-length autotransporter accumulated, indicating that the passenger domain of IgA1 was not cleaved, possibly because the β-barrel domain of IgA1 did not insert into the OM to allow the translocation of the passenger domain. Oligomers of the secretin PilQ, which plays a role in type IV pili formation, were strongly reduced in the OM, while monomeric, likely unfolded PilQ was accumulated. Immunofluorescence microscopy with antibodies directed against PorB and PilQ and Alexa fluorochrome-conjugated secondary antibodies indicated reduced surface exposure of the OMPs PorB and PilQ in Omp85 deletion strains. A direct interaction of immobilized Omp85 on nitrocellulose membranes with denatured PorA was demonstrated with PorA antibodies. All these observations indicated that OMPs were not correctly inserted into the OM in *omp85*-deletion strains (Voulhoux et al., 2003). Omp85 is a highly conserved protein in gram-negative bacteria with homologues also in eukaryotic cells, such as Tob55 in mitochondria (Gentle et al., 2004; Paschen et al., 2003) and Toc75 in chloroplasts (Voulhoux et al., 2003).

Similar to depletion of Omp85 of *N. meningitidis*, depletion of homologue YaeT in *E. coli* also led to severe defects in the biogenesis of OMPs (Werner and Misra, 2005). OMPs were found to accumulate in the periplasm, suggesting that YaeT facilitates the insertion of soluble periplasmic intermediates into the OM, supporting the role of Omp85 and its homologues in other organisms in the targeting or assembly of TMPs into OMs (Werner and Misra, 2005).

Another homologue to Omp85 of *N. meningitidis* and YaeT of *E. coli* is HMW1B of *Haemophilus influenzae* (Surana et al., 2004). HMW1B has a pore size of about 2.7 nm and was found to form multimers, possibly tetramers. HMW1B is critical for secretion of the *H. influenzae* adhesin HMW1 and interacts with the N terminus of the adhesin.

It was also suggested that Omp85 may be involved in the transport of LPS and phospholipids to the OM (Genevrois et al., 2003) and that the effect of Omp85 on OMP assembly may be indirect. Omp85 is located in a cluster of genes involved in lipid A, fatty acid, and phospholipid biosynthesis, which would support a role in transport of LPS or phospholipid. However, the gene encoding for the periplasmic chaperone Skp (Bothmann and Plückthun, 1998; Chen and Henning, 1996) is located in the same cluster, and the Skp trimer

(Korndörfer et al., 2004; Walton and Sousa, 2004) has been shown to bind unfolded OMPs (Bulieris et al., 2003; Chen and Henning, 1996). It has been proposed that Skp also contains an LPS binding site (Walton and Sousa, 2004). Deletion of *omp85* in *N. meningitidis* led to a significant portion of cells showing signs of lysis in electron microscopy (Genevrois et al., 2003). These cells also showed accumulation of vesicular lipid structures and of electron-dense, proteinaceous material in the periplasm, suggesting that these components were no longer incorporated into the OM in the absence of Omp85. Electron microscopy also indicated some LPS accumulation at the outside of the OM, verified by a cytochemical reaction for polysaccharide, while a modified form of LPS was found to cofractionate with the inner membrane fraction in isopycnic sucrose gradient centrifugation. Radioactive labeling of phospholipids indicated that the phospholipid distribution between inner and outer membrane fractions was shifted toward the inner membrane in Omp85-depleted strains, suggesting a role of Omp85 in phospholipid transport toward the OM (Genevrois et al., 2003).

While a role of Omp85 of *N. meningitidis* in the transport of phospholipids and LPS toward the OM was reported, another study with a temperature-sensitive mutant of *E. coli*, carrying 9 amino acid substitutions in YaeT, displayed no alterations in the phospholipid and LPS compositions under permissive and nonpermissive temperatures for bacterial growth (Doerrler and Raetz, 2005). In this study, levels of OMPs in the OM of the mutant were low. Moderately overexpressed SecA, a cytoplasmic ATPase, was found to function as a multicopy suppressor of the temperature-sensitive phenotype, partially restoring the level of OMPs in the OM. One likely explanation for this was that SecA may be compensating for a decrease in secretion levels of mutant YaeT at elevated temperatures in the temperature-sensitive mutant. OMPs accumulated neither in the periplasm nor in the inner membrane, and it was suggested that most likely they were degraded (Doerrler and Raetz, 2005).

Imp

Besides *N. meningitidis* Omp85, another integral OMP, the increased membrane permeability protein (Imp), also called the organic solvent tolerance protein A (OstA), was shown to play an important role in the assembly of the OM in *E. coli* (Braun and Silhavy, 2002) and later also in *N. meningitidis* (Bos et al., 2004). While Imp is essential in *E. coli*, it is not in *N. meningitidis*, which, in contrast to most other gram-negative bacteria, is viable even when LPS is not synthesized (Steeghs et al., 2001). Deletion of *imp* in *E. coli* resulted in the appearance of a novel high-density membrane fraction that contained properly folded OMPs in sodium dodecyl sulfate (SDS)-stable conformation (Braun and Silhavy, 2002). *E. coli* Imp cofractionated with OMPs such as LamB, but Imp was expressed at much lower levels. The *imp* gene encodes an 87-kDa protein composed of 784 amino acids and sequence analysis predicted that Imp has a high content of β-sheet secondary structure and is disulfide bonded in a high-molecular-weight complex (Braun and Silhavy, 2002). The *imp* gene is located upstream of the gene *surA*, which encodes a periplasmic PPIase with chaperone activity (Behrens et al., 2001), and both genes are cotranscribed. Imp depletion in cells led to filamentation, followed by membrane rupture and cell lysis (Braun and Silhavy, 2002). *Imp* deletion strains of *N. meningitidis* produced much lower amounts of LPS (Bos et al., 2004). Accessibility assays with LPS-modifying enzymes, neuraminidase and PagL, demonstrated that the residual LPS was not exposed to the cell surface, suggesting that Imp facilitates the integration of LPS into the outer leaflet of the OM. However, since *imp* deletion strains of neisseriae are viable (Bos et al., 2004), it is unlikely that Imp is involved in the transport of phospholipids, a role previously assigned to Omp85 (Genevrois et al., 2003).

YfgL, YfiO, and NlpB Outer Membrane Lipoproteins

YaeT of *E. coli* was found to be part of a larger complex, which contained the outer mem-

brane lipoproteins YfgL, YfiO, and NlpB (Wu et al., 2005), suggesting that these lipoproteins are required for YaeT function. YfgL was identified by using chemical conditionality as a new genetic method to probe OM assembly (Ruiz et al., 2005). In this method, antibiotics were used in selection studies on strains with defects in OM permeability to create chemical conditions that demanded specific suppressor mutations to partially restore membrane impermeability against these antibiotics. The mutations were not in the target of the toxic molecule, but in thegenes, which after mutation allowed survival of the cell by changing the composition and properties of the membrane to prevent the import of the antibiotics across the OM and therefore their action on the target molecules in the cell. The method was tested with an *imp* deletion strain of *E. coli* (Braun and Silhavy, 2002), with an increased permeability of the OM. These cells were sensitive to detergents and antibiotics (Sampson et al., 1989). Selections for resistance against the antibiotics chlorobiphenyl vancomycin and moenomycin, which both inhibit peptidoglycan biosynthesis, revealed only loss-of-function mutations in the gene *yfgL* (Eggert et al., 2001), which encodes a putative outer membrane lipoprotein. Lack of YfgL leads to changes in the OM that prevent the import of the antibiotics into the cell. YfgL deletion strains showed reduced levels of OMPs such as OmpA and LamB, suggesting a role of YfgL in OMP biogenesis (Ruiz et al., 2005). This effect is not as strong as the effect caused by depletion of the periplasmic chaperone and PPIase SurA. The reduction in LamB concentration is additive, because double mutants with deletions of *surA* and *yfgL* showed an even stronger reduction of LamB levels in the OM (Ruiz et al., 2005).

Coimmunoprecipitation experiments indicated that YfgL is present in a complex with YaeT and two other lipoproteins, NlpB and YfiO, which were identified by mass spectrometry. Essential for *E. coli* growth were YaeT (Werner and Misra, 2005; Wu et al., 2005) and YfiO (Onufryk et al., 2005), while YfgL and NlpB were not (Bouvier et al., 1991; Eggert et al., 2001; Ruiz et al., 2005).

IN VITRO STUDIES ON THE FOLDING OF OMPs

Folding of OMPs into Detergent Micelles

Many, but not all, outer membrane proteins that form transmembrane β-barrels can be successfully refolded in vitro from a urea-denatured state. Upon denaturation in urea at slightly elevated temperature, OMPs lose their quarternary, tertiary, and secondary structure, which was shown, for example, by circular dichroism (CD) spectroscopy. First, Henning and coworkers performed in vitro refolding studies of TMPs in 1978 and demonstrated that the 8-stranded β-barrel OmpA develops native structure when incubated with LPS and Triton X-100 after dilution of the denaturants SDS or urea (Schweizer et al., 1978). Similarly, it was later shown that after heat-induced unfolding in SDS micelles, OmpA refolds into micelles of the detergent octylglucoside even in absence of LPS (Dornmair et al., 1990). These results on the β-barrel OmpA, and the successful refolding of bacteriorhodopsin that consists of a bundle of seven transmembrane α-helices and was first refolded by Khorana and coworkers in 1981 (Huang et al., 1981), suggest that the information for the formation of native structure in TMPs is contained in their amino acid sequence, as previously described by the Anfinsen paradigm for soluble proteins (Anfinsen, 1973).

However, a difference in refolding experiments of α-helical and β-barrel membrane proteins is that the α-helical membrane proteins were first taken up in SDS-detergent micelles before they were refolded into mixed micelles of phosphatidylcholine lipid and the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) or into lipid bilayers of phosphatidylcholine. Completely unfolded bacteriorhodopsin in organic solvent can be transferred into the denaturing detergent SDS, in which it develops a large degree of α-helical structure. It is not clear

whether such an intermediate is an off-pathway product or a possible folding intermediate, since SDS is not found in cells.

Poor in vitro refolding of some OMPs upon denaturant dilution in the presence of preformed phospholipid bilayers appears to be a consequence of the fast aggregation of OMPs, which competes with bilayer insertion and folding. In vivo, molecular chaperones keep the OMPs soluble in the periplasm before they become part of the OM. The chaperones are likely more efficient at preventing OMP aggregation than the denaturant urea that has been used in folding studies in vitro and that must be diluted before OMPs can insert and fold into model membranes. In vivo, there must also be a targeting mechanism that prevents the insertion of OMPs from the periplasm into the cytoplasmic membrane and specifically directs them to the OM.

Folding of β-Barrel Membrane Proteins into Phospholipid Bilayers Is Oriented

Surrey and Jähnig (1992) showed that OmpA spontaneously inserts and folds into phospholipid bilayers upon denaturant dilution. Oriented insertion and folding of OmpA into lipid bilayers in the absence of detergent was observed when unfolded OmpA in 8 M urea was reacted with small unilamellar vesicles (SUVs) of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine ($di$$C_{14:0}$PC) under concurrent strong dilution of the urea. The insertion of OmpA into vesicles was oriented, because trypsin digestion was complete (100%) and resulted in a 24-kDa fragment, while the full-length OmpA (35 kDa) was no longer observed. Translocation of the periplasmic domain of OmpA across the lipid bilayer into the inside of the vesicle would have led to a full protection of OmpA from trypsin digestion. The 24-kDa fragment corresponded to the membrane-inserted β-barrel domain (19 kDa) and a smaller part of the periplasmic domain, which was largely digested by trypsin. By contrast, only 50% of detergent-refolded OmpA, which was reconstituted into $di$$C_{14:0}$PC vesicles after refolding into micelles, could be digested with trypsin, indicating random orientation of the periplasmic domain inside and outside of the phospholipid vesicles (Surrey and Jähnig, 1992).

For direct oriented insertion of OmpA into the bilayers, the preformed lipid vesicles had to be in the lamellar-disordered (liquid-crystalline) phase and the vesicles had to be sonicated (Rodionova et al., 1995; Surrey and Jähnig, 1995). By contrast, insertion and folding did not complete when the lipid bilayers were in the lamellar-ordered (gel) phase or when refolding attempts were made with $di$$C_{14:0}$PC bilayers of large unilamellar vesicles (LUVs) that were prepared by extrusion through membranes with 100-nm pore size (Kleinschmidt and Tamm, 2002). Similarly, folding and trimerization of OmpF (Surrey et al., 1996) was observed after interaction of urea-unfolded OmpF with preformed lipid bilayers in the absence of detergent. Membrane-inserted dimers of OmpF were detected transiently. In vitro, the yields of folded OmpF in lipid bilayers are small (≲30 %), even under optimized conditions (Surrey et al., 1996) and when compared with OmpA, which quantitatively folds at pH 10. The pH dependence observed for the folding of OMPs in vitro is typically linked to an increased solubility of the OMPs, when acidic or basic amino acid side chains are present in their charged form. Refolding experiments in vitro therefore result in better folding yields when carried out at a pH that is sufficiently different from the pI of the protein. The pI of OmpA and of other OMPs of *E. coli* or *Salmonella enterica* serovar Typhimurium is in between 5 and 6, while OMPs of *N. meningitidis or Comamonas acidovorans* have a pI in the basic pH region (see Table 1). In vivo, solubility of OMPs is conferred by binding to a periplasmic chaperone.

β-Barrel Structure Formation Requires a Supramolecular Assembly of Amphiphiles

To determine basic principles for the folding of β-barrel TMPs, folding of OmpA was examined with a large set of different phospholipids

and detergents at different concentrations (Kleinschmidt et al., 1999b). Folding of OmpA was successful with 64 different detergents and phospholipids that had very different compositions of the polar headgroup, did not carry a net charge, and had a hydrophobic carbon chain length ranging from 7 to 14 carbon atoms. In all cases, folding yields were near 100% at pH 10 (Kleinschmidt et al., 1999b), but folding kinetics of OmpA were different for the different detergents (unpublished results). For OmpA folding, the concentrations of these detergents or phospholipids must be above the critical micelle concentration (CMC), demonstrating that a supramolecular assembly (micelles or lipid bilayers) with a hydrophobic interior is the minimal requirement for the formation of a β-barrel transmembrane domain (Kleinschmidt et al., 1999b). In these experiments, OmpA folding was monitored by CD spectroscopy and by electrophoretic mobility measurements. Both methods indicated that after exposure to amphiphiles with short hydrophobic chains (with 14 or fewer carbons), OmpA assumes either both, secondary and tertiary structure (i.e., the native state) or no structure at all, dependent on the presence of supramolecular assemblies (micelles, bilayers). Thermodynamically, OmpA folding into micelles is a two-state process (Kleinschmidt et al., 1999b).

The necessary presence of amphiphiles (lipids, detergents) above the critical concentration for assembly (CCA) to induce the formation of native secondary and tertiary structure in OmpA also indicated that β-barrel structure does not develop while detergent or lipid monomers are adsorbed to a newly formed hydrophobic surface of the protein, i.e., absorption of single amphiphiles one after another will not help to form natively structured parts in the protein. To the contrary, a hydrophobic core of a micelle or bilayer must be present to allow folding of OmpA. (The term CCA is defined here to describe the amphiphile concentration at which a geometrically unique, water-soluble supramolecular assembly is formed, which can be a micelle, a lipid vesicle, or even an inverted or cubic lipid phase. The CCA is identical with the CMC in the special case of micelle-forming detergents. The CCA does not refer to the formation of random aggregates [for instance, of misfolded membrane proteins].) Conlan and Bayley (2003) reported later that another OMP, OmpG, folds into a range of detergents such as Genapol X-080, Triton X-100, *n*-dodecyl-β-D-maltoside, Tween 20, and octylglucoside. However, OmpG did neither fold into *n*-dodecylphosphocholine nor into the negatively charged detergents SDS and sodium cholate. Similar to OmpA, the detergent concentrations had to be above the CMC for OmpG folding (Conlan and Bayley, 2003). Different detergents have also been used for refolding of other β-barrel membrane proteins for subsequent membrane protein crystallization (for an overview, see, for example, Buchanan [1999]).

Electrophoresis as a Tool To Monitor Insertion and Folding of β-Barrel Membrane Proteins

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) has been very useful in monitoring the folding of OmpA into detergent micelles or lipid bilayers, provided that the samples are not boiled prior to electrophoresis (Bulieris et al., 2003; Dornmair et al., 1990; Kleinschmidt and Tamm, 1996, 2002; Kleinschmidt et al., 1999b; Kleinschmidt, 2003; Schweizer et al., 1978; Surrey and Jähnig, 1992, 1995). If samples are not heat denatured prior to electrophoresis, the folded and denatured OMPs migrate differently. For OmpA, Henning and coworkers described this property as heat modifiability (Schweizer et al., 1978). This has also been reported for other OMPs of bacteria such as FhuA (Locher and Rosenbusch, 1997), OmpG (Behlau et al., 2001; Conlan et al., 2000; Conlan and Bayley, 2003), and FomA (Kleivdal et al., 1995; Pocanschi et al., 2006; Puntervoll et al., 2002; C . L. Pocanschi, T. Dahmane, Y. Gohon, F. Rappaport, H.-J. Apell, J. H. Kleinschmidt, and J.-L. Popot, submitted for publication). Native OmpA, for example, migrates at 30 kDa, whereas unfolded

OmpA migrates at 35 kDa (Schweizer et al., 1978). See also Kleinschmidt (2006) for further examples.

Until now, all structural and functional experiments have shown a strict correlation between the 30-kDa form and structurally intact and fully functional OmpA. These previous studies included analysis of the OmpA structure by Raman, Fourier transform infrared (FT-IR), and CD spectroscopy (Dornmair et al., 1990; Kleinschmidt et al., 1999b; Rodionova et al., 1995; Surrey and Jähnig, 1992, 1995; Vogel and Jähnig, 1986), biochemical digestion experiments (Kleinschmidt and Tamm, 1996; Surrey and Jähnig, 1992), and functional assays such as phage inactivation (Schweizer et al., 1978) and single-channel conductivity measurements (Arora et al., 2000). A similar strict correlation was observed recently also for the major OMP of *Fusobacterium nucleatum*, FomA (Pocanschi et al., 2006; Pocanschi et al., submitted). There are, however, some β-barrel membrane proteins that do not exhibit a different migration on SDS-polyacrylamide gels. Examples are the nucleoside transporter Tsx and the mitochondrial hVDAC1, which unfolded in SDS even at room temperature, as can be shown by CD spectroscopy (B. Shanmugavadivu, H.-J. Apell, K. Zeth, and J. H. Kleinschmidt, submitted for publication).

It is possible to determine the kinetics of native structure formation in OmpA (Kleinschmidt and Tamm, 1996, 2002; Surrey and Jähnig, 1995), FomA (Pocanschi et al., 2006), OmpG (Conlan and Bayley, 2003), and probably also in other OMPs using the different electrophoretic mobility of folded and unfolded OMPs. Although SDS inhibits folding of OMPs, it often does not unfold them unless samples are boiled.

In an assay to determine, for example, the OmpA-folding kinetics, OmpA that was denatured in 8 M urea was reacted with preformed lipid bilayers (vesicles) under concurrent strong dilution of the denaturant (Kleinschmidt and Tamm, 1996, 2002). At defined times after initiation of folding, small volumes of the reaction mixture were taken out and an equal volume of SDS-treatment buffer, typically used in SDS-PAGE, was added. In these samples, SDS bound quickly to folded and unfolded OmpA and stopped further OmpA folding (Kleinschmidt and Tamm, 1996, 2002), while already folded OmpA was not unfolded at room temperature.

In the end of the kinetic refolding experiment, the fractions of folded OmpA in all samples were determined by cold SDS-PAGE (i.e., without heat denaturing the samples). The fractions of folded OmpA at each time were estimated by densitometric analyses of the bands of folded and of unfolded OmpA, thus monitoring the formation of tertiary structure in OmpA as a function of time (Kinetics of Tertiary Structure Formation by Electrophoresis, KTSE). This method was also applied successfully to studying the folding kinetics of FomA (Pocanschi et al., 2006) and OmpG (Conlan and Bayley, 2003).

Role of LPS

The OM contains mostly LPS in the outer leaflet. LPS has relatively short hydrocarbon chains, which are partially hydroxylated close to the glucosamine backbone at carbon 3, lowering the hydrophobic thickness of the OM. Several periplasmic proteins and LPS have been demonstrated to interact with OMPs in the periplasm, and initial studies suggested that LPS is required for efficient assembly of OMPs such as monomeric OmpA (Freudl et al., 1986; Schweizer et al., 1978) and trimeric PhoE (de Cock and Tommassen, 1996; de Cock et al., 1999a) into OMs.

Further evidence for a role of LPS came from genetic studies. In *rfa* mutants, the σ^E-dependent stress response was activated. The *htrM* (*rfaD*) gene product was shown to encode an ADP-L-glycero-D-mannoheptose-6-epimerase, an enzyme required for the biosynthesis of an LPS precursor (Pegues et al., 1990; Raina and Georgopoulos, 1991). Lack of the core heptose moiety in *htrM* mutants led to an altered LPS (Missiakas et al., 1996). In such mutants, the assembly of certain OMPs was affected because of the absence of proper LPS (Nikaido and Vaara, 1985; Schnaitman and Klena, 1993).

Also, the rate of OMP synthesis was decreased (Ried et al., 1990).

Together with divalent cations, LPS was reported to facilitate trimerization of PhoE in mixed micelles of Triton X-100 detergent in vitro (de Cock and Tommassen, 1996; de Cock et al., 1999a). However, in these studies, experiments were performed with micelles of LPS and Triton X-100 instead of phospholipid bilayers. It was later found that monomeric OmpA folds relatively fast into micelles but with rather slow kinetics into phospholipid bilayers (Surrey and Jähnig, 1995; Surrey et al., 1996). Proteins that are already folded in micelles may be easily inserted into OMs, especially when the membranes are present in large excess, but would end up with a random orientation in the bilayer, i.e., loops could be exposed to the periplasmic space instead of their normal orientation to the outer space when OMP-micelle complexes fuse with membranes. Since OmpA assumed a random orientation after micelle-bilayer fusion (Surrey and Jähnig, 1992), it is unlikely that OmpA would first fold into LPS micelles in the periplasm, which then fuse with the OM as first proposed for PhoE based on the appearance of a folded monomer in mixed micelles of LPS and Triton X-100 in vitro (de Cock and Tommassen, 1996). However, a PhoE mutant was later shown to fold in vivo and also in vitro into *N*-lauryl-*N*,*N*-dimethylamine-*N*-oxide (LDAO) micelles but not into mixed micelles of Triton X-100 and LPS, also leading to doubts about the existence of a folded monomeric intermediate of PhoE in LPS in vivo (Jansen et al., 2000).

Folding of the β-Barrel OmpA into Lipid Bilayers Is Assisted by Skp and LPS

Direct biochemical evidence for a chaperone-assisted three-step delivery pathway of an OMP to a model membrane was first given for OmpA (Bulieris et al., 2003). It was demonstrated that the periplasmic chaperone Skp keeps OmpA soluble in vitro at pH 7 in an unfolded form even when the denaturant urea was diluted out and not present at concentrations needed to keep OmpA soluble at pH 7. Skp was also shown to prevent folding of OmpA into LPS micelles and to inhibit the folding of OmpA into phospholipid bilayers composed of phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine (Bulieris et al., 2003). Only when Skp complexes with unfolded OmpA were reacted with LPS in a second stage, a folding-competent form of OmpA was formed that efficiently inserted and folded into phospholipid bilayers in a third stage. In this Skp/LPS-assisted folding pathway, faster folding kinetics and higher yields of folded OmpA were observed than with the direct folding of OmpA into the same lipid bilayers upon urea dilution in the absence of Skp and LPS (Bulieris et al., 2003). In the sole presence of either Skp or LPS, the kinetics of insertion and folding were inhibited (Fig. 2).

The higher folding yields of OmpA from the complex with Skp and LPS (in comparison with OmpA folding from the urea-denatured state) may be a consequence of faster Skp binding to unfolded OmpA in solution in comparison with the folding of OmpA into lipid bilayers. Faster rates of Skp binding in solution would result in relatively lower amounts of aggregated OmpA, thus increasing the amounts of OmpA available for folding. However, it was also shown that LPS is required for the efficient OmpA insertion from complexes with Skp into lipid bilayers (Bulieris et al., 2003). In this study, unfolded OmpA bound LPS or Skp or both. The binding stoichiometries were 25 molecules LPS with a binding constant of $K_{\mathrm{LPS}} \sim 1.2 \pm 0.7\ \mathrm{mM}^{-1}$ (i.e., with a free energy of binding $\Delta G = -8.3 \pm 0.3$ kcal/mol) and three molecules of Skp with a much larger binding constant of $K_{\mathrm{Skp}} \sim 46 \pm 30\ \mathrm{mM}^{-1}$ (i.e., with $\Delta G = -10.3 \pm 0.5$ kcal/mol) (Bulieris et al., 2003). The 8- to 150-fold greater OmpA binding constant of Skp explains that Skp prevents the folding of OmpA upon addition of LPS micelles. However, LPS was necessary to promote efficient folding of OmpA into preformed phospholipid membranes at optimal stoichiometries of 0.5 to 1.7 mol of LPS/mol of Skp and 3 mol of Skp/mol of unfolded OmpA. For fast kinetics and high yields

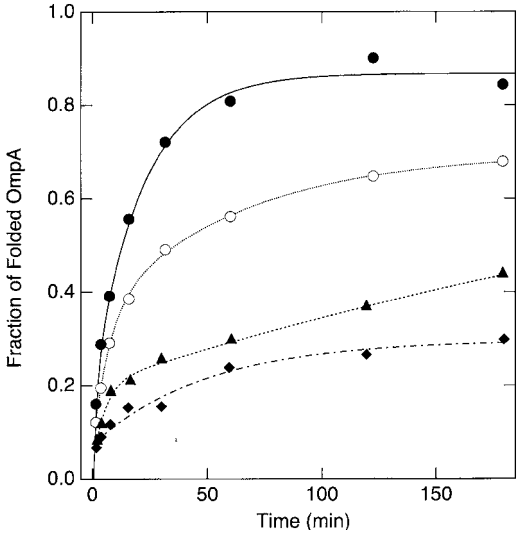

FIGURE 2 Folding of OmpA into lipid bilayers requires both Skp and LPS (adapted from Bulieris et al., 2003). Data shown correspond to folding experiments of urea-denatured OmpA into lipid bilayers, which were added 30 min after dilution of the denaturant urea in the absence of Skp and LPS (○), in the presence of Skp (◆), in the presence of LPS (▲), and in the presence of both Skp and LPS (●). The folding kinetics was fastest and folding yields were highest when both Skp and LPS were present. Folding was inhibited when either Skp or LPS was absent. The folding kinetics in the presence of Skp and LPS also compares favorably with the folding kinetics from the urea-denatured state in the absence of Skp and LPS, indicating that OmpA is insertion competent in vivo, in the absence of urea, when in complex with Skp and LPS. The data shown in Bulieris et al. (2003) also indicated that OmpA did not develop native structure in complex with Skp and LPS, but only in the presence of lipid bilayers.

of membrane insertion and folding of OmpA, about 1.5 to 5 mol of LPS bound to Skp-OmpA complexes (i.e., much lower amounts than observed in absence of Skp) (Bulieris et al., 2003). CD spectroscopy and KTSE assays indicated that large amounts of secondary and tertiary structure in OmpA only form in the third stage of the assembly pathway, upon addition of phospholipid bilayers (Bulieris et al., 2003), suggesting that Skp and LPS deliver OmpA to the membrane, which is absolutely necessary for the formation of complete secondary and tertiary structure in OmpA.

The role of LPS in this stage of membrane insertion and folding was questioned in a recent review (Tamm et al., 2004), arguing that the stimulation of folding and insertion of OmpA was only 19%. This number is the absolute, not the relative change (28%) in folding yield of OmpA, when comparing the results of two OmpA-folding experiments, which are performed either with denatured OmpA in 8 M urea or with OmpA in complex with Skp and LPS. This complex was formed after strong dilution of urea (see also Fig. 2). This comparison unfortunately is not very meaningful, because there is no urea in the periplasm. Instead, it is better to compare OmpA folding experiments into lipid bilayers with either only LPS or only Skp present and the same experiments with both Skp and LPS present (Fig. 2). Therefore, as indicated in Fig. 2, the effect of LPS on the folding of OmpA bound in complex with Skp is between 96% and 291%. The effect on the folding rate is even greater. Furthermore, in the same review Tamm et al. (2004) speculated that the role of LPS may just be to displace the basic Skp from OmpA. If that were the case, a part of OmpA would aggregate instead of leading to higher folding yields into lipid bilayers. Furthermore, binding of Skp to unfolded OmpA is stronger than binding of LPS (see above) as demonstrated in the reviewed paper, indicating that LPS is unable to completely displace Skp from OmpA. LPS is at least transiently associated with the periplasmic side of the inner membrane (Wang et al., 2004), a location also reported for Skp (de Cock et al., 1999b; Schäfer et al., 1999), suggesting an early interaction of Skp with LPS or of Skp/OmpA complexes with LPS.

The interaction of the OmpA/Skp/LPS complex with the lipid bilayer is apparently the most important event to initiate folding of OmpA in the presence of chaperones and LPS as folding catalysts. The described assisted folding pathway and discovered 3:1 stoichiometry for Skp binding to OmpA (Bulieris et al., 2003) was later supported by the observation that Skp is trimeric in solution (Schlapschy et al., 2004) and by the description of the crystal structure of Skp and a putative LPS bind-

ing site in Skp (Korndörfer et al., 2004; Walton and Sousa, 2004) (Color Plate 3A). One LPS binding site per Skp monomer is consistent with the observation of optimal folding kinetics of OmpA from an OmpA/Skp/LPS complex at 0.5 to 1.7 mol of LPS/Skp (Bulieris et al., 2003). In this case, a 1:1 mol/mol stoichiometry indicates that LPS only binds to the LPS binding site of Skp and OmpA is completely shielded from interactions with LPS, probably by binding in between the tentacles of the Skp trimer, while LPS is bound to the outer surface of Skp. A current folding model for this assisted OmpA-folding pathway is shown in Fig. 3.

MECHANISM OF INSERTION AND FOLDING OF β-BARREL MEMBRANE PROTEINS INTO LIPID BILAYERS

Lipid Acyl Chain Length Dependence and Rate Law for β-Barrel Membrane Protein Folding

The rate law of OmpA folding into a range of different phospholipid bilayers was determined using the method of initial rates (Kleinschmidt and Tamm, 2002). The folding kinetics of OmpA into LUVs of short-chain phospholipids, such as 1,2-lauroyl-*sn*-glycero-3-phosphocholine (*di*$C_{12:0}$PC), at 30°C, and also into SUVs of phospholipids with longer chains, such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (*di*$C_{18:1}$PC), at 40°C followed a single-step second-order rate law. The folding kinetics of OmpA into these phospholipids could also be approximated with a pseudo-first-order rate law, when the lipid concentration was high compared with the protein concentration (>90 mol of lipid per mol protein). With this approximation, a rate constant was observed that was identical with the product of the second-order rate constant and the lipid concentration. When fitted with a second-order rate law, the kinetic rate constants depended neither on the lipid nor on the protein concentration, if the lipid/protein ratio was above ~90 mol/mol, while the first-order rate constant depended on the lipid concentration. However, the second-order rate constants strongly depended on the acyl chain lengths of the lipids. When OmpA folding into bilayers of *di*$C_{12:0}$PC was monitored by fluorescence spectroscopy, this rate constant was $k_{2.ord} \sim 0.4$ liter $mol^{-1}\ s^{-1}$, while it was $k_{2.ord} \sim 5.2$ liters $mol^{-1}\ s^{-1}$ for OmpA folding into bilayers of 1,2-diundecanoyl-*sn*-glycero-3-phosphocholine (*di*$C_{11:0}$PC) and $k_{2.ord} \sim 30$ liters $mol^{-1}\ s^{-1}$ for OmpA folding into 1,2-dicapryl-*sn*-glycero-3-phosphocholine (*di*$C_{10:0}$PC) bilayers (Klein-

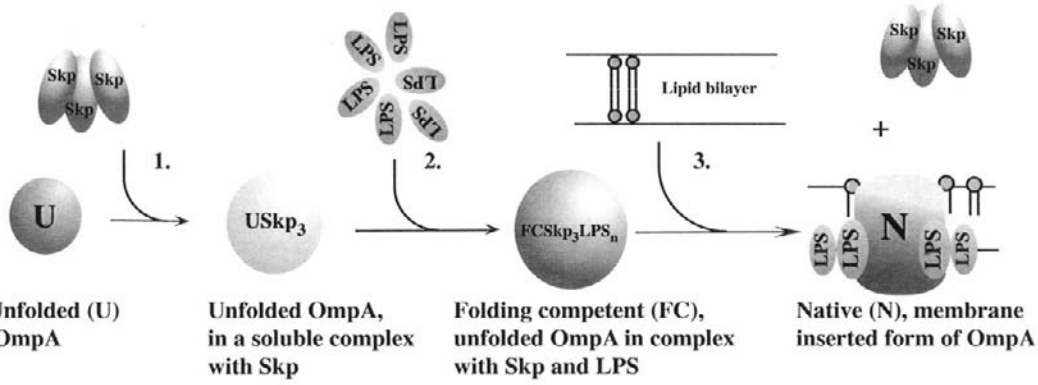

FIGURE 3 A model of the Skp/LPS-assisted folding pathway of the β-barrel protein OmpA of the OM of *E. coli* is depicted. After translocation across the cytoplasmic membrane by the SecY/E/G system in unfolded form (U), OmpA binds three molecules of the trimeric Skp, which is a periplasmic chaperone and keeps OmpA soluble in an unfolded state ($USkp_3$). The complex of unfolded OmpA and Skp interacts with LPS molecules to form a folding-competent intermediate of OmpA ($FCSkp_3LPS_n$). In the final step, folding-competent OmpA inserts and folds into the lipid bilayer. (Adapted from Kleinschmidt, 2003.)

schmidt and Tamm, 2002). Faster folding kinetics into thinner lipid bilayers were recently explained with an increased flexibility of the thinner bilayers (Marsh et al., 2006).

The Kinetics of Secondary and Tertiary Structure Formation in the β-Barrel Domain of OmpA Are Synchronized

The kinetics of membrane insertion and structure formation of OmpA initiated by denaturant dilution in the presence of preformed lipid bilayers may also be monitored by CD spectroscopy or by KTSE. When the kinetics of secondary structure formation were measured for OmpA insertion and folding into LUVs of saturated short-chain phospholipids, a similar dependence of the rate constants on the length of the hydrophobic acyl chains of the lipids was observed as by fluorescence spectroscopy. However, in general, the second-order rate constants were smaller than the corresponding rate constants of the fluorescence time courses (Kleinschmidt and Tamm, 2002). Secondary structure formation was fastest with diC$_{10:0}$PC and slowest with diC$_{12:0}$PC as determined from the CD kinetics at 204 nm. When OmpA was reacted with preformed lipid bilayers (LUVs) of diC$_{14:0}$PC or diC$_{18:1}$PC, the CD signals did not change with time, indicating no changes in the secondary structure of OmpA upon incubation with these lipids.

Interaction of OmpA with the Lipid Bilayer Precedes Folding

When folding kinetic values were analyzed by using KTSE assays to determine the rate constants of tertiary structure formation, observations corresponded to those made by CD spectroscopy. The folding kinetics of OmpA depended on the length of the hydrophobic chains, but OmpA did not fold when the experiments were performed with diC$_{14:0}$PC or diC$_{18:1}$PC. The OmpA folding kinetics into diC$_{12:0}$PC bilayers at different concentrations were fitted to a second-order rate law, and second-order rate constants were determined. Over a range of different lipid concentrations, the second-order rate constants obtained by KTSE were practically indistinguishable from the second-order rate constants of secondary structure formation. The rate constants of the secondary and tertiary structure formation of OmpA in diC$_{12:0}$PC were both $^{s/t}k_{2.ord} \sim 0.090$ liters mol^{-1} s^{-1}. By contrast, the second-order rate constant obtained from the fluorescence time courses of the OmpA folding kinetics into this lipid was about 4- to 5-fold higher ($^{pla}k_{2.ord} \sim 0.4$ liters mol^{-1} s^{-1}), indicating that the adsorption and insertion of the fluorescent tryptophan residues of OmpA into the hydrophobic core of the lipid bilayer were faster than the formation of the fully folded form of OmpA.

Four of the five tryptophans of OmpA are at the front end of the β-barrel and presumably interacted first with the hydrophobic core of the membrane, leading to fast fluorescence kinetics compared with the CD kinetics and kinetics of tertiary structure formation by electrophoresis. Together, these results indicated that the formation of the β-strands and the formation of the β-barrel of OmpA take place in parallel and are a consequence of the insertion of the membrane protein into the lipid bilayer. The previous observation that a preformed supramolecular amphiphile assembly is necessary for structure formation in OmpA was therefore further detailed by a kinetic characterization of the faster rates of interaction of OmpA with the lipid bilayer and by the slower rates of secondary and tertiary structure formation in OmpA.

Temperature Dependence and Multistep Folding Kinetics of the β-Barrel of OmpA into DOPC Bilayers

Early folding experiments with urea-unfolded OmpA and membranes of dimyristoyl-phosphatidylcholine (diC$_{14:0}$PC) indicated that OmpA folds into lipid bilayers of small unilamellar vesicles (SUVs) prepared by sonication, but not into bilayers of LUVs with a diameter of 100 nm prepared by extrusion (Surrey and Jähnig, 1992, 1995). Lipids with longer chains such as diC$_{14:0}$PC and dioleoyl-phosphatidyl-

choline (diC$_{18:1}$PC) required the preparation of SUVs by ultrasonication and temperatures greater than ~25 to 28°C for successful OmpA insertion and folding (Kleinschmidt and Tamm, 1996; Surrey and Jähnig, 1992).

Lipid bilayers of SUVs have a high surface curvature and intrinsic curvature stress. OMP insertion increases the diameter of the vesicles and therefore reduces the curvature stress. The high curvature is also linked to an increased exposure of the hydrophobic surface to OmpA after it is adsorbed at the membrane-water interface, facilitating insertion of OmpA into SUVs compared with insertion of OmpA into bilayers of LUVs, where curvature stress is much lower and no insertion was observed.

The folding kinetics of OmpA into SUVs of diC$_{14:0}$PC or diC$_{18:1}$PC were slower than the folding kinetics into LUV short-chain phospholipids and strongly temperature dependent (Kleinschmidt and Tamm, 2002). The fluorescence kinetics of OmpA folding that could still be fitted to a single-step pseudo-first-order rate law at 40°C (Kleinschmidt and Tamm, 1996, 2002) were more complex when the temperature for folding was 30°C or less. A single-step rate law was not sufficient to describe the kinetics (Kleinschmidt and Tamm, 1996).

Insertion and folding of OmpA into bilayers of diC$_{18:1}$PC (SUVs) were characterized by at least three kinetic phases, when experiments were performed at temperatures between 2 and 40°C. These phases could be approximated by pseudo-first-order kinetics at a lipid/protein ratio of 400. Two folding steps could be distinguished by monitoring the fluorescence time courses at 30°C. The first (faster) step was only weakly temperature dependent ($k_1 = 0.16$ min^{-1}, at 0.5 mM lipid). The second step was up to two orders of magnitude slower at low temperatures, but the rate constant approached the rate constant of the first step at higher temperatures (~0.0058 min^{-1} at 2°C and ~0.048 to 0.14 min^{-1} at 40°C, in the presence of 0.5 mM lipid). The activation energy for the slower process was 46 ± 4 kJ/mol (Kleinschmidt and Tamm, 1996). An even slower phase of OmpA folding was observed by KTSE assays, indicating that tertiary structure formation was slowest with a rate constant of $k_3 = 0.9 \times 10^{-2}$ min^{-1} (at 3.6 mM lipid and at 40°C) (Kleinschmidt and Tamm, 1996). This is consistent with the smaller rate constants of secondary and tertiary structure formation in comparison with the rate constants of protein association with the lipid bilayer, which were later observed for OmpA folding into LUVs of short-chain phospholipids (Kleinschmidt and Tamm, 2002) (see "Interaction of OmpA with the Lipid Bilayer Precedes Folding," above).

The kinetic phases that were observed for OmpA folding into diC$_{18:1}$PC bilayers (SUVs) suggest that at least two membrane-bound OmpA-folding intermediates exist when OmpA folds and inserts into lipid bilayers with 14 or more carbons in the hydrophobic acyl chains. These membrane-bound intermediates could be stabilized in fluid diC$_{18:1}$PC bilayers at low temperatures between 2 and 25°C (the temperature for the phase transition of diC$_{18:1}$PC from the lamellar-ordered to the lamellar-disordered, liquid crystalline phase is $T_c = -18$°C). The low-temperature intermediates could be rapidly converted to fully inserted, native OmpA, as demonstrated by temperature-jump experiments (Kleinschmidt and Tamm, 1996).

Characterization of Folding Intermediates by Fluorescence Quenching

Tryptophan fluorescence quenching by brominated phospholipids (see, e.g., Alvis et al., 2003; Bolen and Holloway, 1990; Everett et al., 1986; Ladokhin and Holloway, 1995; Ladokhin, 1999a, b; Markello et al., 1985; Williamson et al., 2002) or by lipid spin labels (see, e.g., Abrams and London, 1992, 1993; Cruz et al., 1998; Fastenberg et al., 2003; Piknova et al., 1997; Prieto et al., 1994) traditionally has been very valuable to determine characteristic elements of the transmembrane topology and lipid-protein interactions of TMPs.

The positions of fluorescent tryptophans with reference to the center of the phospho-

lipid bilayer can be determined by using a set of membrane-integrated fluorescence quenchers that carry either two vicinal bromines or, alternatively, a doxyl group at the *sn*-2 acyl chain of the phospholipid. When in proximity to the fluorescent tryptophan residues of TMPs, these groups quench the tryptophan fluorescence. The positions of the bromines in 1-palmitoyl-2-(4,5-dibromo-)stearoyl-*sn*-glycero-3-phosphocholine (4,5-DiBrPC), in 6,7-DiBrPC, in 9,10-DiBrPC, and in 11,12-DiBrPC are known from X-ray diffraction to be 12.8, 11.0, 8.3, and 6.5 Å from the center of the lipid bilayer (McIntosh and Holloway, 1987; Wiener and White, 1991). From the extent of Trp fluorescence quenching by each of these membrane-inserted quenchers, the location of the Trp can be obtained by interpolation or extrapolation of the distance-dependent fluorescence-quenching profiles.

To further characterize the folding process of OmpA, we combined this method with the study of the folding kinetics of OmpA into bilayers (SUVs) of *di*$C_{18:1}$PC (Kleinschmidt et al., 1999a; Kleinschmidt and Tamm, 1999). The average positions of the five fluorescent tryptophans of OmpA relative to the center of the lipid bilayer were measured as a function of time and therefore determined for the membrane-bound folding intermediates that were previously implicated by the discovery of multistep folding kinetics (Kleinschmidt and Tamm, 1996). A new method was developed by studying the kinetics of the refolding process in combination with the tryptophan fluorescence quenching at different depths in the lipid bilayer (Kleinschmidt and Tamm, 1999) using membrane-embedded quenchers.

The fluorescence intensity of the tryptophans of OmpA was measured as a function of time after initiation of OmpA folding by dilution of the denaturant in the presence of preformed lipid bilayers containing one of the brominated lipids as a fluorescence quencher. In a set of four equivalent folding experiments, bilayers were used that contained 30 mol% of one of the four brominated lipids and 70% *di*$C_{18:1}$PC. The fluorescence intensities in the four different time courses of OmpA folding in the presence of each of the four brominated lipids were subsequently normalized by division with fluorescence intensities obtained upon OmpA folding into bilayers of 100% *di*$C_{18:1}$PC (i.e., in the absence of any quencher). Thus, depth-dependent quenching profiles were obtained at each time after initiation of OmpA folding. From these profiles, the vertical location of Trp in the membrane in projection to the bilayer normal was then determined by using the parallax method (Abrams and London, 1992; Chattopadhyay and London, 1987) or the distribution analysis (Ladokhin and Holloway, 1995; Ladokhin, 1999b).

A large set of experiments was performed in the temperature range between 2 and 40°C. At each selected temperature, the average distances of the tryptophans to the center of the lipid bilayer were determined as a function of time. Therefore, we called this method time-resolved distance determinations by tryptophan fluorescence quenching (TDFQ) (Kleinschmidt and Tamm, 1999). Previously unidentified folding intermediates on the pathway of OmpA insertion and folding into lipid bilayers were detected, trapped, and characterized. Three membrane-bound intermediates were described, in which the average distances of the Trps from the bilayer center were 14 to 16 Å, 10 to 11 Å, and 0 to 5 Å, respectively (Kleinschmidt and Tamm, 1999).

The first folding intermediate was stable at 2°C for at least 1 h. A second intermediate was characterized at temperatures between 7 and 20°C. The Trps moved 4 to 5 Å closer to the center of the bilayer at this stage. Subsequently, in an intermediate that was observed at 26 to 28°C, the Trps moved another 5 to 11 Å closer to the center of the bilayer. This intermediate appeared to be less stable. The distribution parameter, calculated from distribution analysis, was largest for the Trp distribution of this intermediate. This was a consequence of the mechanism of folding and of the structure of folded OmpA (Arora et al., 2001; Pautsch and Schulz, 1998, 2000). The large distribution parameter observed for this intermediate was

consistent with experiments on single Trp mutants of OmpA (Kleinschmidt et al., 1999a) (see below). Trp-7 has to remain in the first leaflet of the lipid bilayer, while the other Trps must be translocated across the bilayer to the second leaflet. Therefore, with symmetrically incorporated brominated lipids as fluorescence quenchers, the largest distribution parameter was observed when the four translocating Trps were in the center of the lipid bilayer. Formation of the native structure of OmpA was observed at temperatures $\gtrsim$28°C. In the end of these kinetic experiments, all 5 Trps were finally located on average about 9 to 10 Å from the bilayer center, Trp-7 in the periplasmic leaflet and the other 4 Trps in the outer leaflet of the OM.

When KTSE experiments were performed to monitor OmpA folding at 30°C, a band at 32 kDa was observed in the first few minutes of OmpA folding (Kleinschmidt and Tamm, 1996). The folding conditions for this experiment were nearly identical with those of the fluorescence-quenching experiments at 28 to 30°C. Therefore, this 32-kDa form is very likely identical with the third folding intermediate of OmpA, in which the average Trp location is 0 to 5 Å from the center of the lipid bilayer. The comparison indicated that in this intermediate, a significant part of the β-barrel had formed, which is resistant to treatment with SDS at room temperature.

The β-Barrel Domain of OmpA Folds and Inserts by a Concerted Mechanism

TDFQ experiments were subsequently performed with the five different single tryptophan mutants of OmpA. These mutants were prepared by site-directed mutagenesis (Kleinschmidt et al., 1999a), and each contained a single tryptophan and 4 phenylalanines in the 5 tryptophan positions of the wild-type protein. All mutants were isolated from the *E. coli* OM and refolded in vitro into lipid bilayers. TDFQ for each of the single Trp mutants of OmpA gave more structural detail on the folding mechanism of OmpA. These TDFQ experiments were carried out at selected temperatures between 2 and 40°C (Kleinschmidt et al., 1999a).

When kinetic experiments were performed below 30°C, each of the 5 tryptophans approached a distance of 10 to 11 Å from the bilayer center in the end of the fluorescence time course of OmpA folding. A distance decrease with time was observed even at 40°C for Trp-7 (Fig. 4A). The TDFQ results showed that Trp-7 did not migrate any closer to the bilayer center than ~10 Å independent of the experimental conditions. However, Trp-15, Trp-57, Trp-102, and Trp-143 were detected very close to the center of the lipid bilayer in the first minutes of refolding at temperatures of 30°C, 32°C, 35°C, and 40°C, respectively. This is shown for Trp-143 in Fig. 4B. TDFQ experiments performed at 40°C resolved the last two steps of OmpA refolding, and the translocation rate constants of the first phase of fast distance change were 0.55, 0.46, 0.26, and 0.43 min^{-1} for Trp-15, Trp-57, Trp-102, and Trp-143, respectively. The four Trps crossed the center of the bilayer and approached distances of ~10 Å from the bilayer center in the final folding step of OmpA. These experiments demonstrated that Trp-15, Trp-57, Trp-102, and Trp-143 are similarly located in three folding intermediates that were also observed previously for wild-type OmpA. The similar distances of these Trps from the membrane center in each of the membrane-bound folding intermediates indicate a simultaneous translocation of the transmembrane segments of OmpA, coupled to the formation of the β-barrel structure upon insertion.

The results of these kinetic studies on the folding mechanism of OmpA may be used to develop a tentative model of OmpA folding (Color Plate 5). The time courses of OmpA folding into phospholipid bilayers (LUVs) of $di C_{12:0}PC$ indicated that β-strand secondary and β-barrel tertiary structure formation are synchronized with the same rate constant (Kleinschmidt and Tamm, 2002), which is lower than the rate constant of the fluorescence time course of OmpA adsorption to the lipid bilayer.

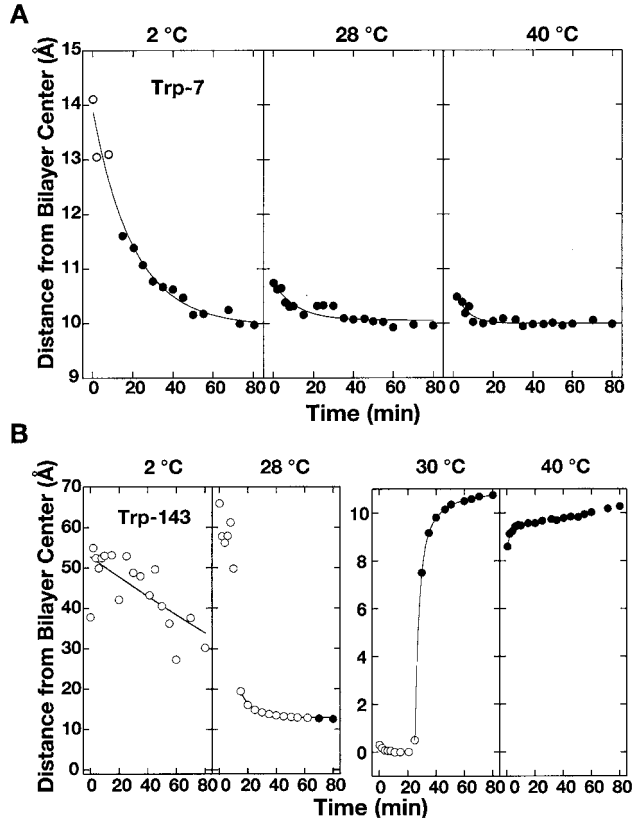

FIGURE 4 (A) Time courses of the movement of Trp-7 toward the bilayer center at 2, 28, and 40°C. Distances were obtained from curve fits to fluorescence-quenching profiles as described in the text. Data points represented by filled circles were the fitted quenching-profile minima, open circles denote extrapolated distances from the observed quenching profiles. The solid lines are fits of single- or double-exponential functions to the data. (B) Time courses of the movement of Trp-143 toward the bilayer center at 2 and 28°C and from the bilayer center at 30 and 40°C. At 2°C, the distances of Trp-143 could only be obtained by extrapolation (open circles). The solid lines are fits of the data to single- or double-exponential functions. (Adapted from Kleinschmidt et al., 1999a.)

Strongly temperature-dependent kinetic values were observed and several kinetic phases were distinguished when folding of OmpA was investigated with lipid bilayers of *di*$C_{18:1}$PC, which is a phospholipid with comparably long hydrophobic chains. OmpA first adsorbs to the water-membrane interface (intermediate A) and the intrinsic fluorescence of OmpA increases strongly due to the partitioning of the fluorescent Trps into the less polar environment at the membrane/water interface. Subsequently, the slower phase of the fluorescence changes reflects the migration of the Trps from the membrane/water interface into the hydrophobic core of the lipid bilayer.

The translocation of the Trps across the bilayer is best monitored with membrane-inserted fluorescence quenchers, because the intrinsic Trp fluorescence does not change much during Trp migration through the 30-Å hydrophobic core of *di*$C_{18:1}$PC. The average location of the Trps of 14 to 16 Å from the bilayer center after adsorption to the membrane-water interface was determined by TDFQ experiments at 2°C (Kleinschmidt and Tamm, 1999). At temperatures of 5 to 25°C, this initial phase of folding was fast and followed by a second, slower phase, in which the Trps move into more hydrophobic regions at a distance of about 10 Å from the bilayer center. The observed folding intermediate (B) is quite stable. A third membrane-bound intermediate (C) was identified at 27 to 29°C. In this intermediate, all Trps, except Trp-7, are detected a distance of 0 to 5 Å from the bilayer center in the first minutes of OmpA folding. Trp-7 remains at the same location as in intermediate B. Very likely, this intermediate is identical with the

32-kDa form of OmpA that was previously observed by KTSE experiments (Kleinschmidt and Tamm, 1996). Finally, at temperatures above 28 to 30°C, Trp-15, Trp-57, Trp-102, and Trp-143 move away from the center of the bilayer to a distance of about 10 Å. This distance of the Trp residues of OmpA compares well with the X-ray and NMR structures of OmpA (Arora et al., 2001; Pautsch and Schulz, 2000).

The basic elements of the model in Color Plate 5 are the synchronized kinetics of secondary and tertiary structure formation, the simultaneous migration of the tryptophans that cross the bilayer center, and the migration of Trp-7, which does not translocate. However, more structural information is needed to improve this preliminary model. For example, it is not known how the residues of the polar loops of OmpA cross the hydrophobic core of the lipid bilayer.

PERSPECTIVES

Although considerable progress has been made in recent years to understand the pathways and principles of targeting and assembly of OMPs into the OM, numerous unanswered questions remain. It is now clear that periplasmic chaperones, such as Skp and SurA, help to keep OMPs unfolded in the periplasm and prevent their aggregation without requiring ATP as an energy source. The identification of the integral OMP YaeT (Omp85 in *N. meningitidis*) and outer membrane lipoproteins as factors involved in targeting and/or insertion of OMPs into the OM on one side and the spontaneous assembly of OMPs into lipid bilayers in vitro on the other side raises several interesting questions. Obviously, there is no absolute requirement of proteinaceous machinery to refold OMPs to their functionally active state. Yet in cells, OMPs must be specifically sorted to the OM. Furthermore, the accumulation of misfolded OMPs in the periplasm upon deletion of *yaeT* (*omp85*) suggests that the properties of the OM lipid bilayer differ from the properties of the phospholipid bilayers into which OMPs successfully fold in vitro. In vitro, folding is successful with thin lipid bilayers (Kleinschmidt and Tamm, 2002; Pocanschi et al., 2006; Marsh et al., 2006) that are flexible. Flexibility is required, because the lipids must make space for the OMP during insertion, a process that is much slower with thicker bilayers formed, for example, by dioleoyl phospholipids. In the OM, spontaneous protein insertion may not be a favored process for the cell, which must maintain its integrity. Therefore, some mechanism appears to be necessary in vivo to specifically recognize OMPs and to facilitate OM insertion only for them. It is likely that proteins mediate this process and that their role is to locally alter the properties of the membrane for the insertion of OMPs into the OM. Insertion, if not spontaneous, would require energy and it is unclear what the source for this energy may be. Future studies must address this problem. One possibility may be a mechanism of energy transduction across the periplasm, similar to the mechanism proposed for the active import of iron siderophores or cobalamin.

The basic physical mechanism of β-barrel formation in the OM is likely the same as the mechanism of β-barrel formation in lipid bilayers (Kleinschmidt et al., 1999a; Kleinschmidt and Tamm, 2002), but it will be interesting to examine how the complex of YaeT, YfiO, YfgL, and NlpB facilitates targeting and membrane insertion of OMPs. The recently discovered, highly concerted mechanism of secondary and tertiary structure formation in OMPs, which is coupled to the bilayer insertion process, must be examined in more detail in the absence and in the presence of proteinaceous folding factors. A number of biophysically interesting questions must be addressed in this context. For instance, how do the polar outer loops traverse the hydrophobic core of the OM? What are the topologies of complexes of OMPs and their chaperones? How are OMPs recognized by the chaperones and by OM-folding facilitators? How is YaeT inserted into the OM? Is this an autocatalytic process? Are the lipoproteins required? What exactly is the role of the lipoproteins in OMP insertion? It will be interesting to shed more light into these and additional problems of OM assembly in future studies.

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