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Membrane protein folding on the example of outer membrane protein A of *Escherichia coli*

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Abstract. The biophysical principles and mechanisms by which membrane proteins insert and fold into a biomembrane have mostly been studied with bacteriorhodopsin and outer membrane protein A (OmpA). This review describes the assembly process of the monomeric outer membrane proteins of Gram-negative bacteria, for which OmpA has served as an example. OmpA is a two-domain outer membrane protein composed of a 171-residue eight-stranded β-barrel transmembrane domain and a 154-residue periplasmic domain. OmpA is translocated in an unstructured form across the cytoplasmic membrane into the periplasm. In the periplasm, unfolded OmpA is kept in solution in complex with the molecular chaperone Skp. After binding of periplasmic lipopolysaccharide, OmpA insertion and folding occur spontaneously upon interaction of the complex with the phospholipid bilayer. Insertion and folding of the β-barrel transmembrane domain into the lipid bilayer are highly synchronized, i.e. the formation of large amounts of β-sheet secondary structure and β-barrel tertiary structure take place in parallel with the same rate constants, while OmpA inserts into the hydrophobic core of the membrane. In vitro, OmpA can successfully fold into a range of model membranes of very different phospholipid compositions, i.e. into bilayers of lipids of different headgroup structures and hydrophobic chain lengths. Three membrane-bound folding intermediates of OmpA were discovered in folding studies with dioleoylphosphatidylcholine bilayers. Their formation was monitored by time-resolved distance determinations by fluorescence quenching, and they were structurally distinguished by the relative positions of the five tryptophan residues of OmpA in projection to the membrane normal. Recent studies indicate a chaperone-assisted, highly synchronized mechanism of secondary and tertiary structure formation upon membrane insertion of β-barrel membrane proteins such as OmpA that involves at least three structurally distinct folding intermediates.

Key words. Membrane protein folding; outer membrane proteins; OmpA; membrane protein chaperones; lipid-protein interactions; lipopolysaccharide; protein insertion.

Introduction

A central question of the assembly of biological membranes is how the integral membrane proteins are folded and inserted into the lipid bilayer host matrices that separate the aqueous phases of the different compartments of the cell. The fatty core of a phospholipid bilayer requires hydrophobic amino acid residues at the interface of the integral membrane protein to the fatty acyl chains of the phospholipids. Also, amide hydrogens of transmembrane (TM) proteins that are located in the fatty region of the membrane must form hydrogen bonds with a carbonyl oxygene of a peptide bond in close vicinity to allow the stable assembly of a protein segment in the hydrophobic region of the lipid bilayer. Based on these requirements, which are reflected in the currently known structures of integral membrane proteins, two major classes of transmembrane proteins (TMPs) can be distinguished. The first class covers proteins that form very hydrophobic transmembrane α helices. A prominent example is bacteriorhodopsin (BR), a seven-α-helix bundle membrane protein (see e.g. [1–3]). In α-helix bundle proteins, multiple helices are aligned in the form of bundles and may contain polar residues at the interfaces between the helices that are not exposed to the lipid chains. Monomeric and oligomeric α-helix bundle membrane proteins are known. Folding and assembly of α-helix bundle membrane proteins such as BR have been reviewed recently

(cf. [4]). The second class comprises proteins with transmembrane β-sheet secondary structure. The geometry of the β strands excludes that individual β strands can exist in a lipid bilayer, and all known integral membrane proteins with transmembrane β strands form barrel structures in which at least eight neighboring β strands are connected by hydrogen bonds. An even number of transmembrane β strands ranging from 8 to 22 has been observed in β-barrel membrane proteins of known crystal structure. Examples are the bacterial outer membrane proteins (OMPs), such as outer membrane protein A (OmpA) with 8 β strands (β-s) [5, 6], OmpT (10 β-s) [7], OmPlA (12 β-s) [8], OmpF (16 β-s), maltose (LamB) [9, 10] or sucrose (ScrY) [11] porins (18 β-s), and the iron transporters FhuA [12, 13] or FepA [14] (22 β-s) (see fig. 1 for some examples). Predicted 14-stranded β-barrel membrane proteins are FomA [15] and OmpG [16, 17]. Monomers (for example OmpA, FhuA), dimers (OmPlA) and trimers (OmpF, PhoE) are known. In the transmembrane β strands, only every second amino acid faces the apolar lipid phase and must be a hydrophobic residue, while the others face the interior of the β barrel and are mostly polar. Therefore, the average hydrophobicity of transmembrane β barrels is low (–0.5 to –0.6 on the Kyte-Doolittle scale vs. > +0.5 for α-helix bundle transmembrane proteins). This review focuses on the insertion and folding of outer membrane protein A of *Escherichia coli*, which has most often been used as a representative model in folding studies of monomeric transmembrane β barrels.

In vitro requirements for membrane protein folding

Initial studies on membrane protein folding demonstrated that representatives of both classes of integral membrane proteins can be refolded in vitro from a completely denatured state into their native, functionally active state without any requirement of proteinaceous cofactors. Huang et al. first demonstrated this on the example of BR [18], which can be delipidated and completely denatured in 88% formic acid or anhydrous trifluoroacetic acid. BR regains α-helix secondary structure upon addition of ethanol. Neutralization of the acid with ammonia followed by dialysis against a solution of sodium dodecyl sulfate (SDS) and subsequent addition of the chromophore, phospholipids and cholate lead to complete recovery of BR activity. Schweizer et al. [19] showed that the eight-stranded β-barrel OmpA partially regains native structure in the presence of lipopolysaccharide and Triton-X-100 after dilution of the denaturants SDS or urea. Similarly, Dornmair et al. [20] demonstrated that after heat denaturation in SDS micelles, the eight-stranded β-barrel OmpA can refold into micelles of the detergent octylglucoside in the absence of lipopolysaccharide (LPS). These studies suggest that the information for formation of native structure in integral membrane proteins is contained in their amino acid sequence, as previously described by the Anfinsen paradigm for soluble proteins [21].

Surrey and Jähnig [22] showed first that OmpA spontaneously inserts and folds into phospholipid bilayers. The oriented insertion and folding in the absence of detergent

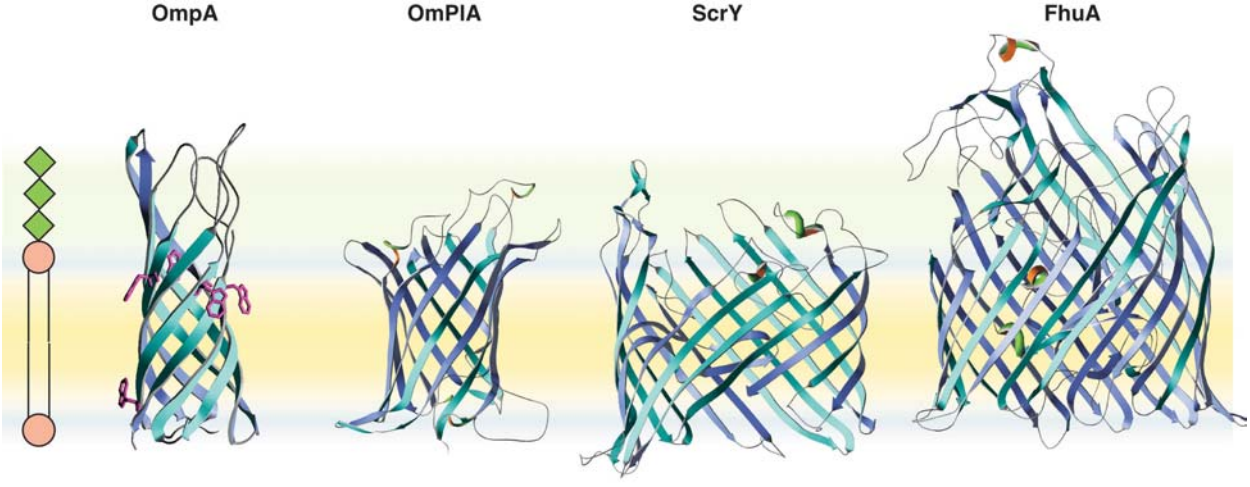


Figure 1. Some representative crystal structures of β-barrel membrane proteins of the outer membranes of bacteria. The different structures are characterized by TM β strands that span the lipid bilayer with an angle of ~45° and often extend into the LPS region of the outer membrane. TM β barrels have an even number of antiparallel TM strands, which is 8 for OmpA [69], 12 for OmPlA [8], 18 for ScrY [11] and 22 for FhuA [12]. OmpA is a small ion channel [29], ScrY is a sucrose-specific porin that forms trimers, FhuA is an active transporter for iron-uptake and OmPlA a phospholipase that forms a dimer. Another 8-stranded TM β barrel is OmpX of *E. coli* [70]. OMPs of mitochondria are predicted to form similar TM β barrels. Examples are the VDAC channels, out of which more than a dozen have been sequenced [71].

can be induced from a completely unfolded and solubilized state in 8 M urea upon strong dilution of the denaturant in presence of small unilamellar vesicles (SUVs) of dimyristoylphosphatidylcholine ($diC_{14}PC$). These bilayers have to be in the lamellar-disordered (liquid-crystalline) phase [22, 23], and vesicles have to be sonicated. In contrast, insertion and folding do not complete when the lipid bilayers are in the lamellar ordered (gel) phase or when refolding attempts are made into $diC_{14}PC$ bilayers of large unilamellar vesicles (LUVs) that are prepared by extrusion. Similarly, folding and trimerization of OmpF [24] take place after interaction of urea-unfolded OmpF with preformed lipid bilayers in the absence of detergent. Membrane-inserted dimers of OmpF are observed transiently. In vitro, the folding yields of OmpF are relatively modest (< ~30%) under optimized conditions [24]. In comparison, folding yields of OmpA approach 100% at pH 10, but are only ~70% at neutral pH [23], which is very likely a consequence of an increased negative surface charge of OmpA (pI = 5.9) at pH 10 that increases the solubility of OmpA, i.e. suppresses the aggregation side reaction.

To explore constraints for the folding and membrane insertion of OMPs, Kleinschmidt et al. [25] investigated the folding of OmpA into a wide range of different phospholipids and detergents at different concentrations. They reported the successful folding of OmpA into 64 different detergents and phospholipids that have very different compositions of the polar headgroup, do not carry a net charge and have a hydrophobic carbon chain length ranging from 7 to 14 carbon atoms. The concentrations of these detergents or phospholipids have to be above the critical micelle concentration (CMC) for successful OmpA folding, demonstrating that a supramolecular assembly, either a micelle or a lipid bilayer, is a minimal requirement for native structure formation in β-barrel integral membrane proteins. Folding of OmpA can be detected by circular dichroism (CD) spectroscopy and by electrophoretic mobility measurements. Both methods indicate 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, depending on the presence of supramolecular assemblies (micelles, bilayers). OmpA folding into micelles appears to be a thermodynamically controlled two-state process. The requirement of a supramolecular assembly for secondary and tertiary structure formation also indicates that structure does not form upon adsorption of detergent or lipid monomers to a newly formed hydrophobic surface of the protein. Instead, the hydrophobic core of the micelle or bilayer must first be present to allow folding of OmpA.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) has been a very valuable tool to monitor folding of OmpA because the electrophoretic mobilities of folded and denatured outer membrane proteins are different if the samples are not boiled prior to electrophoresis. This property was described by Henning et al. for OmpA [19] and has also been observed for other β barrels, such as FhuA [26] and OmpG [17]. For example, folded OmpA migrates at 30 kDa, whereas unfolded OmpA migrates at 35 kDa [19]. The 30-kDa form has been shown by Raman, FT-IR and CD spectroscopy [20, 22, 23, 25, 27, 28], by phage inactivation assays [19] and by single-channel conductivity measurements [29] to correspond to completely folded and functionally active OmpA.

The different electrophoretic mobilities of folded and unfolded OmpA have also been used to determine the kinetics of native structure formation with a simple kinetic gel-shift assay, taking advantage of the inhibition of OmpA folding by SDS [23, 30, 31]. In this kinetic assay, SDS is added to small volumes of the reaction mixture that are taken out at defined times after initiation of folding. In these samples, SDS binds quickly to folded and unfolded OmpA and stops OmpA folding. At the end of the kinetic experiment, the fractions of folded OmpA in each sample are determined by cold SDS-PAGE (i.e. without heat-denaturing the samples), and subsequent densitometry of the bands of folded and of unfolded OmpA. This method monitors the formation of tertiary structure in OmpA as a function of time (kinetics of tertiary structure formation by electrophoresis, KTSE).

In contrast to quantitative refolding into a wide range of neutral detergents, completely denatured OmpA does not refold into SDS micelles, as determined by electrophoretic mobility measurements and by CD spectroscopy (cf. [20]). However, the negative charge of SDS only contributes to this effect of the SDS headgroup and cannot be the only cause of inhibition of OmpA folding in the presence of SDS, since OmpA can fold partially into micelles of negatively charged LPS [32] and also into bilayers containing negatively charged phosphatidylglycerol, which are both components of the bacterial outer membrane [32, 33]. A range of detergents have also been used for refolding other β-barrel membrane proteins for subsequent membrane protein crystallization (for an overview, see e.g. [34]). While the presence of an aggregated state of detergents or lipids seems to be a minimal requirement for in vitro folding of β-barrel membrane proteins such as OmpA, in vivo there are proteinaceous and other cofactors that promote efficient folding.

In vivo requirements for outer membrane protein folding

Sequence constraints

Koebnik [35] investigated amino acid sequence constraints of the folding of OmpA in vivo and showed that efficient assembly of OMPs requires that in transmem-

brane β strands, at least four of five randomized residues with side chains pointing to the membrane lipids must be hydrophobic. In addition, none of the three central residues must be charged. Side chains pointing inward to the relatively small eight-stranded β barrel of OmpA should not be large, and proline residues are hardly tolerated in transmembrane β strands. Interestingly, two complementary OmpA fragments that are split at the second or third periplasmic turn can be coexpressed in *E. coli* and assemble efficiently, with all termini located in the periplasmic space [36]. When pairs of the transmembrane β strands are permuted on the DNA level, only the three possible circular permutations lead to correctly assembled OmpA variants, although their assembly is less efficient than the assembly of OmpA [37].

Role of periplasmic proteins (chaperones, isomerases)

OMPs of Gram-negative bacteria are translocated across the cytoplasmic membrane in a mostly unfolded form by the SecA/E/Y/G export system (for recent reviews, see e. g. [38, 39]). In the periplasm, a signal peptidase cleaves off the signal sequence. Genetic studies of possible periplasmic chaperones and biophysical assays with these chaperones and soluble proteins as their substrates suggest that, for example, SurA (45 kDa) [40–42] and FkpA (26 kDa) [43–45] assist in the assembly of OMPs. In these studies, periplasmic chaperones prevented the aggregation of soluble proteins. In vivo, concentrations of some OMPs in the outer membrane of *E. coli* are decreased when the genes of the periplasmic proteins Skp [46] and SurA [40, 41] are deleted. Representatives of three different families of peptidyl-prolyl cis/trans isomerases are found in the periplasm. Examples are the parvulin type SurA [42, 43], the FKBP type FkpA [43–45, 47] and the cyclophilin type PpiA (RotA, 18 kDa) [48]. SurA binds the 18-stranded LamB in vitro [42]. Recently, it was also reported that an outer membrane protein, *omp85* (86 kDa) of *Neisseria meningitidis* that has homologues in many organisms, is likely to play a role in outer membrane protein assembly [49] because deletion of the Omp85 gene strongly impairs the assembly of outer membrane proteins of these bacteria.

The periplasmic seventeen kDa protein (Skp) was identified as the major component of a mixture of proteins from the periplasm that binds to sepharose-linked OMPs on affinity columns [46]. An *E. coli* mutant lacking the *skp* gene also displays reduced levels of OmpA, OmpC, OmpF and LamB [46]. Mutants in which both *surA* and *skp* are deleted are not viable [42, 50]. Skp also binds to and inserts into monolayers of negatively charged lipids [51]. Skp binds to the NH_2-terminal part of OmpA and is required for the release of OmpA into the periplasm [52, 53]. Skp does not bind to folded OmpA [46], suggesting that Skp recognizes nonnative structures of OMPs.

Role of lipopolysaccharide

The *skp* gene maps at the 4-min region on the chromosome and is located only four bases upstream of genes that encode proteins involved in lipid A biosynthesis [54–56], which is an essential component of LPS. Since LPS is also a major component of the outer membrane of *E. coli*, several studies have addressed its role in the folding of OMPs.

Early studies suggested that LPS may be required for efficient assembly of OMPs, such as monomeric OmpA [19, 33], into outer membranes. DeCock and Tommassen [57] reported that LPS micelles and divalent cations promote the formation of folded monomers of the trimeric porin PhoE in presence of Triton-X-100 detergent. They further showed that LPS of deep rough mutant strains of *E. coli* is much less efficient than wild-type LPS, indicating that the core region of LPS plays an important role in the formation of folded PhoE monomers. In a subsequent study with a range of different types of lipopolysaccharides, de Cock et al. [58] demonstrated that the negative charges in the inner core region of LPS contribute to high folding efficiencies of PhoE into LPS/Triton-X-100 mixtures. LPS with a lipid A part with more flexible hydrophobic chains is more efficient in inducing the formation of folded monomers than LPS with more rigid chains. Extensive interactions of both the core region and the lipid A moiety of LPS with unfolded PhoE are apparently necessary contributions to high efficiency of PhoE folding. While these studies very nicely demonstrate the role of LPS in outer membrane protein folding, they were performed with small micelles of LPS and Triton-X-100, and the micelle/folded protein complexes were then fused with outer membranes. OMPs that are prefolded in micelles can quickly fuse with membranes without maintaining a preferred direction. However, in cells the long loops have to be oriented towards the extracellular space, while the short β-turns face the periplasmic space. Interestingly, specific binding motifs of LPS to folded outer membrane proteins were reported from X-ray crystal structures, e. g. for FhuA [59] and for OmpT [7]. Furthermore, LPS is also required for enzymatic activity of OmpT [60].

An assisted folding pathway of OmpA from a completely unfolded state

In vivo, the folding and assembly of β-barrel proteins into membranes do not require a basic pH as observed in studies in vitro [23]. Also, there must be a pathway for the successful folding of other OMPs, which exhibit poor

folding yields in vitro such as OmpF [24] and FhuA [C. L. Pocanschi and J. H. Kleinschmidt, unpublished results]. Poor in vitro refolding 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 OMPs soluble in the periplasm before these insert into the outer membrane. Obviously, these chaperones are more efficient in preventing OMP aggregation compared with the denaturant urea that has been used in folding studies in vitro and that must be diluted before OMPs can insert and fold.

Bulieris et al. [32] demonstrated a first chaperone-assisted pathway of OmpA folding that consists of three major stages. The periplasmic chaperone Skp is sufficient to keep OmpA soluble in vitro at pH 7 in an unfolded form even after strong dilution of the denaturant urea. As a molecular chaperone, Skp substitutes in vivo the denaturant urea that has been used to keep OmpA unfolded and soluble in vitro. In vitro, Skp prevents folding of OmpA into LPS micelles. Skp also inhibits folding of OmpA into phospholipid bilayers. However, when Skp complexes with unfolded OmpA are reacted with LPS in a second stage, a folding-competent form of OmpA is formed that inserts and folds after addition of phospholipid bilayers in the third stage. The folding kinetics are faster and yields of folded OmpA are higher in this Skp/LPS-assisted folding pathway compared with the folding kinetics and yields observed in the absence of Skp and LPS under otherwise identical conditions. The insertion and folding kinetics into lipid bilayers are inhibited when only one component, either Skp or LPS, is present (fig. 2). It is possible that higher yields in the Skp/LPS-assisted folding pathway (as compared with direct OmpA refolding into lipid bilayers upon urea dilution) simply reflect the fact that Skp binding to OmpA after urea dilution in vitro takes place more quickly than both aggregation of OmpA and productive folding of OmpA into lipid bilayers. Nevertheless, LPS is required for OmpA insertion from complexes with Skp.

Studying the binding of LPS and Skp to unfolded OmpA, Bulieris et al. found that about 25 molecules of LPS bind to unfolded OmpA with a binding constant of $K_{LPS} = 1.2 \pm 0.7\ \mu M^{-1}$ (i.e. with a free energy of binding $\Delta G = -34.7 \pm 1.5$ kJ/mol). In contrast, only 3 molecules of Skp bind to OmpA with a much larger binding constant of $K_{Skp} = 46 \pm 30\ \mu M^{-1}$ (i.e. with $\Delta G = -43 \pm 2$ kJ/mol) [32]. The 8–150-fold greater OmpA binding constant of Skp explains that Skp prevents the folding of OmpA upon addition of LPS micelles. However, LPS is absolutely necessary to promote efficient folding of OmpA into preformed phospholipid membranes at optimal stoichiometries of 0.5–1.7 mol LPS/mol Skp and 3 mol Skp/mol unfolded OmpA. Therefore, ~1.5–5 mol LPS bind to Skp-OmpA complexes (i.e. much lower amounts than observed in the absence of Skp) to catalyze membrane insertion and folding [32]. Binding of LPS to Skp-OmpA complexes is quite specific to promote subsequent insertion into membranes. 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 [32]. 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 a folding catalyst. The described assisted folding pathway [32] is shown in figure 3.

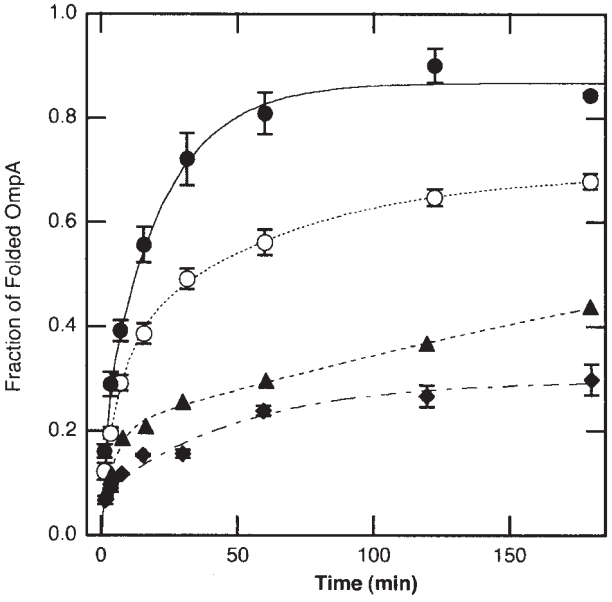


Figure 2. An assisted pathway of OmpA folding into lipid bilayers requires the simultaneous presence of Skp and LPS (data taken from Bulieris et al. [32]). Insertion and folding of OmpA were initiated by addition of preformed lipid bilayers 30 min after dilution of the denaturant urea in the absence of Skp and LPS (○); in the presence of Skp (◆); in the presence of LPS (▲) and in the presence of both Skp and LPS (●). The fractions of folded OmpA as a function of time were determined using the KTSE assay described in the text.

Folding of OmpA into bilayers of short-chain phospholipids

OmpA develops most of its structure upon interaction with lipid membranes, i.e. independent of whether insertion and folding are initiated directly from a urea-denatured state or from the unfolded form in a complex with the chaperone Skp and LPS. Therefore, the general mechanism of OmpA folding also appears to be largely independent of whether Skp and/or LPS are present. The simpler experimental design of folding OmpA from a urea-denatured state into lipid bilayers [22] has been used

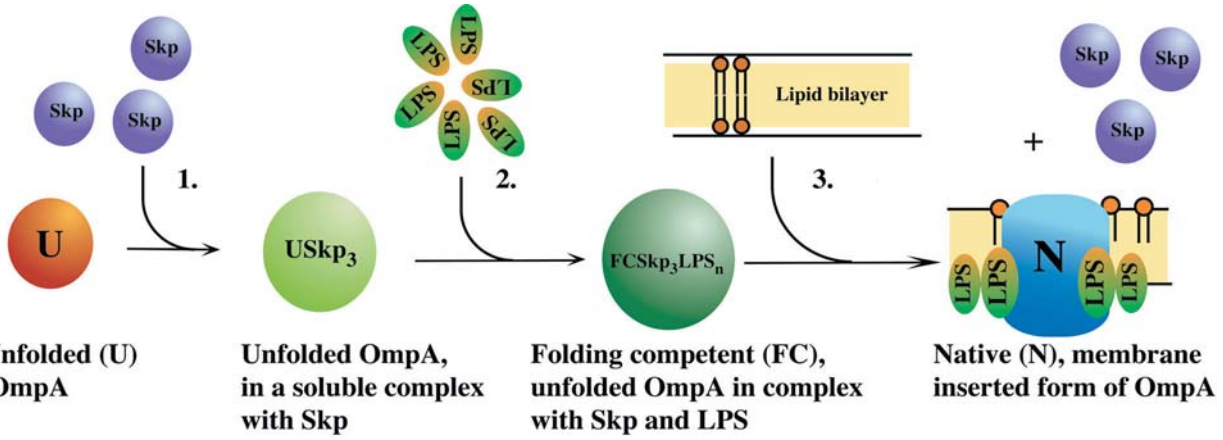


Figure 3. Scheme for an assisted folding pathway of a bacterial outer membrane protein. OmpA is translocated through the cytoplasmic membrane in an unfolded form (U) and binds to a small number of molecules of the periplasmic chaperone Skp, which solubilizes OmpA in the unfolded state ($USkp_3$). The complex of unfolded OmpA and Skp interacts with a small number of 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 (from [32]).

successfully in a series of membrane protein folding studies [22–25, 30, 31, 61, 62].

OmpA folding depends on the hydrophobic chain length of the phospholipid

Early folding experiments with urea-unfolded OmpA and membranes of $diC_{14}PC$ indicated that OmpA folds into lipid bilayers of SUVs prepared by sonication, but not into bilayers of LUVs with a diameter of 100 nm prepared by extrusion [22, 23]. More recent KTSE experiments in which OmpA folding into different phospholipid bilayers was investigated show that insertion and folding can take place into LUV bilayers of phospholipids if the acyl chain length is tridecanoyl or shorter [31]. When OmpA is reacted with LUV bilayers composed of longer-chain phospholipids, CD spectra indicate that in comparison to spectra of hydrophobically collapsed OmpA in the absence of lipids, no additional secondary structure is formed in the presence of these LUVs [31]. Lipids with longer chains such as $diC_{14}PC$ and dioleoylphosphatidylcholine ($diC_{18:1}PC$) require the preparation of SUVs by ultrasonication and temperatures greater than ~25–28 °C for successful OmpA insertion and folding. In spite of the use of sonicated vesicles and increased reaction temperature, insertion and folding of OmpA is still very slow, for example with $diC_{18:1}PC$ (fig. 4).

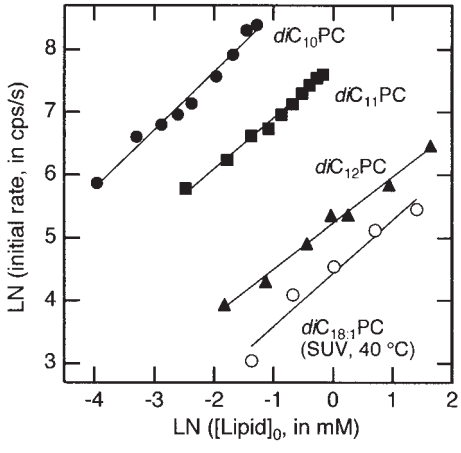


Figure 4. Double logarithmic plot of the initial rates of the OmpA folding kinetics as a function of the lipid concentrations ($T = 20$ °C) of the phospholipids $diC_{10}PC$, $diC_{11}PC$ and $diC_{12}PC$. The concentration dependence of the initial rates of OmpA folding into SUVs of $diC_{18:1}PC$ at 40 °C is shown for comparison. The data were taken from [31].

OmpA folding into bilayers of short-chain phospholipids follows a single-step second-order rate law

Investigation of the concentration dependence of OmpA folding into a range of different phospholipid bilayers revealed that the OmpA folding kinetics into LUVs of short-chain phospholipids and also into SUVs of $diC_{18:1}PC$ at 40 °C follow a single-step second-order rate law [31]. The folding kinetics can also be fit to a pseudo first-order rate law at high lipid concentrations (>90 mol lipid per mol protein) with a rate constant that is identical to the product of the second-order rate constant and the lipid concentration. The concentration-independent second-order rate constants strongly depend on the acyl chain length of the lipids. When monitored by fluorescence spectroscopy, the second-order rate constants of OmpA folding into bilayers of large unilamellar vesicles (100 nm diameter) are 0.4 l mol^{-1} s^{-1} into $diC_{12}PC$, 5.2 l mol^{-1} s^{-1} into $diC_{11}PC$, and 30 l mol^{-1} s^{-1} into $diC_{10}PC$ [31].

The kinetics of secondary and tertiary structure formation in OmpA have the same rate constant

When OmpA folding into bilayers of short-chain phospholipids (LUVs) is monitored by CD spectroscopy to determine the kinetics of secondary structure formation, a similar dependence of the rate constants on the length of the hydrophobic acyl chains of the lipids is observed, but the rate constants are always smaller than the corresponding rate constants of the fluorescence time courses [31]. Secondary structure formation is fastest with $diC_{10}PC$ and slowest with $diC_{12}PC$. No change in the circular dichroism signals is observed at 204 nm when OmpA is reacted with $diC_{14}PC$ or with $diC_{18:1}PC$.

Very similar observations are made when the folding kinetics are analyzed using KTSE assays to determine the rate constants of tertiary structure formation. The OmpA folding kinetics into $diC_{12}PC$ bilayers at different concentrations can be fitted to a second-order rate law. The calculated second-order rate constants are concentration independent and virtually identical to the concentration-independent rate constants of secondary structure formation. The OmpA folding kinetics of secondary and tertiary structure formation in $diC_{12}PC$ have a second-order rate constant of ~0.090 l mol^{-1} s^{-1}. When monitored by fluorescence spectroscopy, the folding kinetics into this lipid are about four- to fivefold faster ($k = 0.4$ l mol^{-1} s^{-1}), indicating that the adsorption and insertion of the fluorescent tryptophan residues of OmpA into the apolar regions of the lipid bilayer are faster than the formation of the fully folded form of OmpA. The tryptophans of OmpA are located at the front end of the β-barrel, which explains why the fluorescence kinetics are faster than the kinetics of secondary and tertiary structure formation. Together, the results of this study [31] indicate that formation of the β-strands and formation of the β-barrel of OmpA take place in parallel and are coupled to the insertion of the membrane protein.

The mechanism of OmpA folding into bilayers of lipids with 14 or more carbons in the fatty acyl chains

Slow-folding kinetics and multi-step membrane insertion and folding of OmpA

Kinetic folding studies with phospholipids of a hydrophobic chain length of 14 carbons or longer require the preparation of SUVs by ultrasonication [22, 23]. Lipid bilayers of SUVs have a relatively high surface curvature and are likely to temporarily contain local defects in the lipid surface. Therefore, more hydrophobic surface is exposed to OmpA than is adsorbed at the membrane water interface. Insertion of OmpA into SUVs is therefore facilitated compared with insertion into bilayers of LUVs. While OmpA insertion is successful when SUVs, for example of $diC_{14}PC$ or $diC_{18:1}PC$, are used as target membranes, the folding kinetics into such vesicles are strongly temperature dependent. At 40 °C, the fluorescence kinetics of folding can still be fit to a single-step pseudo first-order rate law [30, 31]. However, when a lower temperature is selected for the folding reaction, a single-step rate law is no longer sufficient [30]. Kinetic experiments at temperatures between 2 and 40 °C have shown that insertion and folding of OmpA into bilayers of $diC_{18:1}PC$ (SUVs) consist of at least three kinetic phases which are each described by pseudo first-order or first-order kinetics at lipid/protein ratios of 400. The first step is fast ($k_1 = 0.16$ min^{-1}, at 0.5 mM lipid) and not very dependent on temperature. The second step is up to two orders of magnitude slower at low temperatures, but approaches the rate of the first step at higher temperatures (0.048–0.063 min^{-1} at 0.5 mM lipid at 40 °C). The activation energy for this process is 46 ± 4 kJ/mol [30]. When KTSE assays are performed, an even slower phase of OmpA folding is observed, with a rate constant of $k_3 = 0.9 \times 10^{-2}$ min^{-1} (at 3.6 mM lipid and 40 °C) [30]. This reduced rate of tertiary structure formation in comparison to the fluorescence kinetics of OmpA folding is consistent with the faster fluorescence kinetics of OmpA folding into short-chain phospholipid bilayers compared with the kinetics of secondary and tertiary structure formation observed with these bilayers. The kinetic phases that are observed for OmpA folding into $diC_{18:1}PC$ bilayers (SUVs) suggest that at least two membrane-bound intermediates exist when OmpA folds and inserts into lipid bilayers with 14 or more carbons in the hydrophobic acyl chains. Both membrane-bound intermediates can be stabilized in $diC_{18:1}PC$ bilayers at low temperatures between 2 and 25 °C (the gel-to-fluid phase transition temperature of $diC_{18:1}PC$ is $T_c = -18$ °C). Temperature jump experiments demonstrate that low-temperature intermediates can be rapidly converted to fully inserted, native OmpA [30].

Identification and characterization of membrane-bound folding intermediates of OmpA

The membrane-bound folding intermediates were then characterized by determination of the positions of the fluorescent tryptophans of OmpA in these intermediates [61, 62]. A new experimental approach was developed by combining tryptophan fluorescence quenching at different depths in the lipid bilayer with the kinetics of the refolding process [62]. The position of fluorescent tryptophans with reference to the center of the phospholipid bilayer can be determined using a set of membrane-bound fluorescence quenchers that carry either two bromines or alternatively a doxyl group. Attached to the *sn*-2 acyl chain of a phospholipid, these groups quench the fluores-

cence of tryptophan residues of integral membrane proteins that are exposed to the acyl chains of the membrane lipids. The tryptophan fluorescence of OmpA is most efficiently quenched by a quencher that is closest to the average location of the tryptophan residues. For example, 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 [63, 64]. From the relative fluorescence intensities of tryptophan in a set of four different samples containing the same amounts of these four different brominated lipids, the vertical location of Trp in the membrane in projection to the bilayer normal has been determined using the parallax method [65, 66] or distribution analysis [67, 68].

To characterize folding intermediates in the folding of OmpA into lipid bilayers (SUVs of di$C_{18:1}$PC), the structural information obtained with this method was combined with kinetic refolding experiments [62]. Five kinetic experiments were carried out at a selected temperature with each of the four different quenchers and without a fluorescence quencher. The experiments were 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 have called this method time-resolved distance determination by tryptophan fluorescence quenching [62]. Using this method, previously unidentified folding intermediates on the pathway of OmpA insertion and folding into lipid bilayers can be detected, trapped and characterized. Three membrane-bound intermediates have been described, in which the average distances of the Trps from the bilayer center are 14–16 Å, 10–11 Å and 0–5 Å, respectively [62]. The first folding intermediate is stable at 2 °C for at least 1 h. A second intermediate can be isolated at temperatures between 7 and 20 °C. The Trps move 4–5 Å closer to the center of the bilayer at this stage. Subsequently, in an intermediate that is observable at 26–28 °C, the Trps move another 5–11 Å closer to the center of the bilayer. This intermediate appears to be less stable. The distribution parameter σ calculated from distribution analysis is largest for the Trp distribution of this intermediate. This can be caused by the fact that according to the structure of folded OmpA [5, 6, 69] and according to experiments with single Trp mutants of OmpA [61] (see below), Trp-7 has to remain in the first leaflet of the lipid bilayer, while the other Trps have to be translocated across the bilayer to the second leaflet. When folding is monitored by KTSE experiments at 28–30 °C, a 32-kDa band can be observed in the first few minutes of the OmpA folding reaction [30]. Since the folding conditions for this experiment are nearly identical to those of the fluorescence quenching experiments at 28–30 °C, this 32-kDa form is very likely identical to the third folding intermediate of OmpA, in which the average Trp location is 0–5 Å from the center of the lipid bilayer. The comparison indicates that in this intermediate, a significant part of the β-barrel must have formed which is resistant to treatment with SDS at room temperature. The final (native) structure is observed at higher temperatures of refolding. In this structure, all five Trps are located on average ~9–10 Å from the bilayer center, Trp-7 in the periplasmic leaflet and the other four Trps in the outer leaflet of the outer membrane.

OmpA folds and inserts into lipid membranes by a highly concerted folding mechanism

To further dissect the folding pathway of OmpA, the five different single-site mutants of OmpA were made by site-directed mutagenesis, each containing a single tryptophan and four phenylalanines in the five tryptophan positions of the wild-type protein. Successful in vitro refolding of all mutants into lipid bilayers allowed time-resolved distance determinations (TDFQ) on each of the tryptophan residues during the folding of the single Trp mutants of OmpA. More structural detail on the folding mechanism of OmpA was obtained when TDFQ experiments were performed for each single Trp mutant of OmpA at selected temperatures between 2 and 40 °C [61]. Below 30 °C, each Trp approaches a distance of 10 Å from the bilayer center. Trp-7 always exhibits this distance decrease with time, even at 40 °C, and has never been detected closer to the bilayer center than ~10 Å. In contrast, Trp-15, Trp-57, Trp-102 and Trp-143 are detected very close to the center of the lipid bilayer in the first minutes of refolding at 30, 32, 35 and 30 °C, respectively. When monitored at 40 °C, which resolves the last steps of OmpA refolding, these four Trps have to cross the center of the bilayer and approach distances of ~10 Å from the center after refolding is complete. The translocation process at 40 °C is biphasic, and the translocation rate constants of the first phase of fast distance changes at 40 °C are 0.55, 0.46, 0.26 and 0.43 min^{-1} for Trp-15, Trp-57, Trp-102 and Trp-143, respectively. The common distances of these four Trps from the membrane center that are observed in each of the membrane-bound folding intermediates demonstrate a synchronous translocation of those segments of OmpA that form the four β-hairpins in the native structure of OmpA.

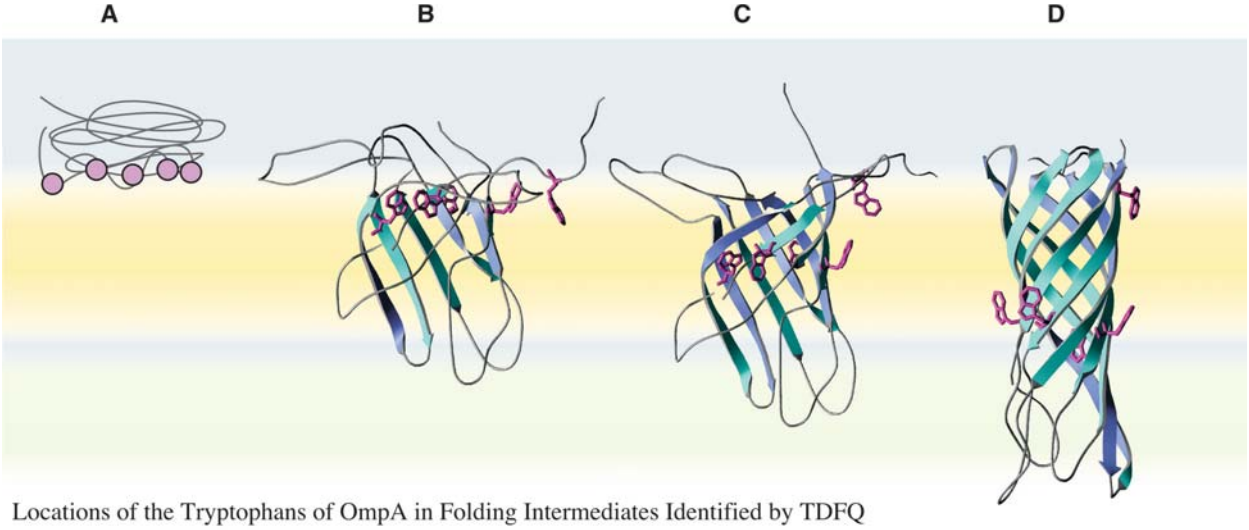
In summary, the folding mechanism of OmpA might be envisaged as is shown in figure 5: kinetic data on OmpA folding into short-chain phospholipid bilayers (LUVs) indicate that β-strand secondary and β-barrel tertiary structure formation are synchronized with the same rate constant [31], which is smaller than the rate constant of OmpA adsorption to the lipid bilayer determined from

fluorescence kinetics. When the much slower folding of OmpA into longer-chain phospholipid bilayers (SUVs) is investigated in detail, strongly temperature dependent kinetics are observed, and several kinetic phases can now be distinguished. In the first phase of OmpA folding into di$C_{18:1}$PC bilayers, OmpA adsorbs to the water-membrane interface (intermediate A). In this phase, the intrinsic fluorescence of OmpA increases strongly due to the translocation of the fluorescent Trps into a less polar environment at the membrane/water interface. The second, slower phase of the fluorescence kinetics indicates the migration of the Trps from the membrane/water interface into the more hydrophobic regions of the lipid bilayer. Once the environment of the fluorescent Trps is very hydrophobic, the subsequent translocation of the Trps across the bilayer can only be monitored with membrane-inserted fluorescence quenchers, i.e. by using the TDFQ method.

TDFQ experiments indicate an average location of the Trps at 14–16 Å from the bilayer center after adsorption to the membrane/water interface at low temperatures [62]. In a second slower phase, the Trps move into more hydrophobic regions to a distance of about 10 Å from the bilayer center. This intermediate (B) is quite stable and can be trapped at temperatures lower than 25–27 °C. At 27–29 °C, a third membrane-bound intermediate (C) can be detected. This intermediate corresponds to the 32-kDa form that can be detected by electrophoresis [30]. In this intermediate all Trps except Trp-7 are located close to the center of the lipid bilayer at a distance of 0–5 Å. Trp-7 remains at the same location as in intermediate B. In the last step, which is observed at temperatures above 28–30 °C, Trp-15, Trp-57, Trp-102 and Trp-143 move from the bilayer center outward to a location of 10 Å from the center. This preliminary folding model is based on the temperature-dependent observation of multiple kinetic phases, on TDFQ measurements of the five Trp residues at different temperatures, on the appearance of a semi-compact 32-kDa folding intermediate that correlates with the third folding intermediate characterized by TDFQ, and on the identical rate constants of secondary and tertiary structure formation.

Perspectives

While the working model depicted in figure 5 is compatible with existing experimental data, it will have to be improved when additional experimental data become available. For example, the locations of other amino acids will have to be determined in the intermediate states of OmpA insertion into the phospholipid bilayer. Also, it is unclear



Locations of the Tryptophans of OmpA in Folding Intermediates Identified by TDFQ

	Distance from the Center of the Lipid Bilayer			
Tryptophan	**A**	**B**	**C**	**N**
⑦	14-16 Å	10 Å	10 Å	10 Å
⑮,57,102,143		10 Å	0-5 Å	10 Å

Figure 5. Preliminary scheme describing how synchronized insertion folding of the TM domain of OmpA may be envisaged. From experiments, it is known that the kinetics of β-sheet secondary and β-barrel tertiary structure formation in OmpA have the same rate constants and are tightly coupled to the penetration of OmpA into the lipid bilayer [31]. Also, the positions of the five fluorescent Trps in three identified membrane-bound folding intermediates and in the completely refolded state of OmpA have been determined [61, 62] as indicated. Further details are not yet known. For example, the translocation of the long polar loops across the lipid bilayer must be investigated in detail, since a contact of these loops with the hydrophobic core of the bilayer would be thermodynamically very unfavorable.

how the polar loops of OmpA cross the hydrophobic interior of the lipid bilayer. OmpA insertion requires relocation of phospholipids to make space for formation of the β-barrel inside the lipid bilayer. Therefore, the thermodynamics of the insertion and folding process must be investigated in detail to determine the driving forces for bilayer insertion and folding. It will be interesting to compare the folding of OmpA to membrane insertion and folding of larger β-barrel membrane proteins to identify common principles of membrane protein folding of outer membrane proteins. In this context, the roles of molecular chaperones and membrane lipids such as LPS in outer membrane protein folding must be investigated extensively.

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