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Review

Folding and assembly of β-barrel membrane proteins

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Abstract

Beta-barrel membrane proteins occur in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts. The membrane-spanning sequences of β-barrel membrane proteins are less hydrophobic than those of α-helical membrane proteins, which is probably the main reason why completely different folding and membrane assembly pathways have evolved for these two classes of membrane proteins. Some β-barrel membrane proteins can be spontaneously refolded into lipid bilayer model membranes in vitro. They may also have this ability in vivo although lipid and protein chaperones likely assist with their assembly in appropriate target membranes. This review summarizes recent work on the thermodynamic stability and the mechanism of membrane insertion of β-barrel membrane proteins in lipid model and biological membranes. How lipid compositions affect folding and assembly of β-barrel membrane proteins is also reviewed. The stability of these proteins in membranes is not as large as previously thought (<10 kcal/mol) and is modulated by elastic forces of the lipid bilayer. Detailed kinetic studies indicate that β-barrel membrane proteins fold in distinct steps with several intermediates that can be characterized in vitro. Formation of the barrel is synchronized with membrane insertion and all β-hairpins insert simultaneously in a concerted pathway.

Keywords: Lipid–protein interaction; Hydrophobic mismatch; Membrane curvature; OmpA; Equilibrium folding; Folding intermediate; *E. coli* lipid

Contents

Abbreviations: CD, circular dichroism; CL, cardiolipin; DLPC, dilauroyl-*sn*-phosphatidylcholine; DMPC, dimyristoyl-*sn*-phosphatidylcholine; DOPC, dioleoyl-*sn*-phosphatidylcholine; GdnHCl, guanidine hydrochloride; LPS, lipopolysaccharide; Omp, outer membrane protein; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylglycerol; SDS, sodium dodecyl sulfate; TM, transmembrane

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1. Introduction

Gram-negative bacteria are surrounded by two membranes, an inner cytoplasmic membrane and an outer membrane that faces the environment. The inner membrane forms the major permeability barrier between the inside and outside of the cell and carries out most membrane-associated metabolic functions. It consists of a normal lipid bilayer composed of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Proteins consisting of single or multiple membrane-spanning α-helices are inserted into the inner membrane. In marked contrast, the outer membrane of Gram-negative bacteria is highly asymmetric. Its outer leaflet is composed of lipopolysaccharides (LPS), but the inner leaflet is composed of the same phospholipid classes as the inner membrane. The general architecture of the outer membrane proteins is also different: these proteins form membrane-spanning β-barrels, which are self-closed β-sheets. Not surprisingly, the biogenesis of β-barrel outer membrane proteins (Omps) is also very different from the biogenesis of α-helical inner membrane proteins. Both classes of proteins are usually synthesized as precursors with N-terminal signal sequences, which target them to the translocon, an inner membrane protein channel consisting of the *secY*, *secE* and *secG* gene products. The atomic structure of the SecY/E/G complex has recently been solved by X-ray crystallography [1]. The transmembrane (TM) helices of inner membrane proteins are thought to be released laterally from the lumen of the translocon into the lipid bilayer by a mechanism that is not yet understood. Outer membrane proteins on the other hand are secreted into the periplasm between the inner and outer membrane, where their signal sequence is cleaved by the signal peptidase, which is also an inner membrane protein. Periplasmic chaperones are thought to bind unfolded Omps in the periplasmic space in order to prevent their aggregation. Many Omps have been observed to spontaneously fold into lipid bilayers in vitro. Therefore, it is often assumed that insertion into the outer membrane is also a spontaneous process in vivo that does not require metabolic energy. Another school of thought postulates that Omps bind and fold with LPS in the periplasmic space and that only a prefolded complex with LPS can insert into the outer membrane. In this review article, we summarize available evidence for these pathways. Since the spontaneous folding of Omps into lipid bilayers has been studied by biophysical methods in quite some detail, the emphasis of this review is mostly directed towards summarizing the energetics and mechanisms of folding of this class of membrane proteins in vitro. It is our belief that some general principles, especially those regarding the thermodynamic stability and lipid interactions (but not necessarily those regarding mechanisms of insertion) of outer membrane proteins also apply to helical membrane proteins, which are much harder to study by the same methods because of their lower solubility in chemical denaturants. Therefore, some of the results that have emerged from this work on Omps may be of more general significance and may apply to all membrane proteins. Beta-barrel membrane proteins are not restricted to Gram-negative prokaryotic organisms. In eukaryotic cells, several mitochondrial and chloroplast outer membrane proteins are also believed to adopt β-barrel structures. Since these plastids and their membranes are thought to be evolutionarily derived from endosymbiontic bacteria, it is likely that folding and biogenetic mechanisms have also been conserved between these membranes during evolution.

2. Structures and functions of β-barrel membrane proteins

The atomic structures of about two dozen β-barrel membrane proteins have been solved to date (Table 1). These structures range from 8-stranded to 22-stranded β-barrels. Many are monomers, one is a homo-dimer, and the porins are homo-trimers. A somewhat unusual case is MspA from *Mycobacterium smegmatis*, which forms a single barrel composed of eight identical subunits [2]. Therefore, the architecture of MspA resembles that of the staphylococcal toxin α-hemolysin, which forms a homo-heptameric single barrel when bound to membranes [3]. Some representative structures are shown in Fig. 1. Generally, these structures feature tight turns on the periplasmic side and large, often quite flexible loops on the extracellular side of the membrane. The lipid bilayer-facing surfaces of the barrels are composed of hydrophobic residues and the residues facing the interior of the barrel are mostly polar residues. Therefore, the general distribution of residues in β-barrel membrane proteins is inverted compared to that of most soluble proteins. The TM β-strands of β-barrel membrane proteins are rich in glycines and aromatic Trp and Tyr residues are frequently found in two rings that contact the lipid bilayer interfaces at both ends of the barrels.

The residues inside the smallest eight-stranded β-barrels are quite tightly packed so that much of the lumen inside the barrel is filled with polar side chains, which interact with each other through a network of hydrogen bond and electrostatic interactions. Pockets of ordered and unordered water molecules are also found within these proteins. An example is the structural outer membrane protein A (OmpA) whose structure is shown in Fig. 1. The lumen of the 12-stranded NalP protein from *Neisseria meningitidis* is large enough to accommodate a single polar TM α-helix that is stabilized through salt bridges and hydrogen bonds with the interior of the barrel wall (Fig. 1). The 16- and 18-stranded porins have large water-filled pores. In some cases, one or two outer loops fold back into the pore in order to provide moderate substrate specificity. The β-barrels of the coupled transporter proteins are 22-stranded and in addition contain a globular "plug" or "cork" domain that fills most of the lumen of these large β-barrels (Fig. 1). The 16-stranded

Table 1
Representative outer membrane proteins with known crystal or NMR structures

Protein	β-Strands	Oligomeric state	Organism	Residues	PDB code	Proposed function	Reference
OmpX	8	monomer	*E. coli*	148	1QJ8	toxin binding	[17]
OmpX[a]	8	monomer	*E. coli*	148	1Q9F		[63]
OmpA	8	monomer	*E. coli*	171	1QJP	structural	[18]
OmpA[a]	8	monomer	*E. coli*	176	1G90		[22]
PagP[a]	8	monomer	*E. coli*	170	1MM4, 1MM5	palmitoyl transferase	[64]
NspA	8	monomer	*N. meningitides*	155	1P4T	cell adhesion	[65]
OmpT	10	monomer	*E. coli*	297	1I78	protease	[16]
OpcA	10	monomer	*N. meningitides*	253	1K24	adhesion protein	[66]
NalP	12	monomer	*N. meningitides*	308	1UYN	autotransporter	[67]
OmpLA	12	dimer	*E. coli*	269	1QD6	phospholipase	[15]
TolC	3×4	trimer	*E. coli*	428	1EK9	export channel	[68]
FadL	14	monomer	*E. coli*	427	1T16, 1T1L	fatty acid transporter	[9]
OmpF	16	trimer	*E. coli*	340	2OMF	porin	[5]
PhoE	16	trimer	*E. coli*	330	1PHO	porin	[5]
Porin	16	trimer	*Rh. capsulatus*	301	2POR	porin	[69]
Porin	16	timer	*Rh. blastica*	289	1PRN	porin	[70]
OmpK36	16	trimer	*K. pneumoniae*	342	1OSM	porin	[71]
Omp32	16	trimer	*C. acidovorans*	340	1E54	porin	[72]
MspA	8×2	octamer	*M. smegmatis*	184	1UUN	porin	[2]
LamB	18	trimer	*E. coli*	421	1MAL, 1AF6	maltose porin	[7,73]
Maltoporin	18	trimer	*S. typhimurium*	421	2MPR	maltose porin	[74]
ScrY	18	trimer	*S. typhimurium*	413	1A0S	sucrose porin	[8]
BtuB	22	monomer	*E. coli*	594	1NQE	cobalamin transporter	[14]
FhuA	22	monomer	*E. coli*	723/714	2FCP, 1BY5	iron transporter	[10,11]
FepA	22	monomer	*E. coli*	724	1FEP	iron transporter	[12]
FecA	22	monomer	*E. coli*	741	1KMO	iron transporter	[75]
Non-constitutive β-barrel membrane proteins							
α-Hemolysin	7×2	heptamer	*S. aureus*	293	7AHL	toxin	[3]
LukF	7×2	heptamer	*S. aureus*	299	1LKF	toxin	[76]

[a] Determined by NMR.

structure of the mycobacterial porin MspA is unusual because it contains two consecutive β-barrels of different diameter (Fig. 1). The narrower barrel, which is hydrophobic on the outer surface, has a hydrophobic length of 37 Å, which is longer than the hydrophobic length of ~26 Å found in the β-barrels of Gram-negative bacteria. Mycobacteria, members of which cause tuberculosis, have outer membranes that do not contain LPS, but exceptionally long mycolic fatty acids that are covalently attached to the peptidoglycan on the inside and smaller extractable lipids on the outside. It is likely that the 37 Å match the otherwise unknown hydrophobic thickness of the mycobacterial outer membrane. The average length of the TM β-strands is 11 amino acid residues in trimeric porins and 13–14 residues in monomeric β-barrels. Since the strands are usually inclined at about 40° from the membrane normal, they span about 27–35 Å of the outer membrane, respectively.

The outer membrane proteins of Gram-negative bacteria may be grouped into six families according to their functions [4]: (i) general porins such as OmpC, OmpF, and PhoE [5,6], (ii) passive transporters such as LamB, ScrY, and FadL [7–9], (iii) active transporters of siderophores such as FepA, FecA, and Fhu A [10–13] and of vitamin B_{12} such as BtuB [14], (iv) enzymes such as the phospholipase OmpLA [15] or the protease OmpT [16], (v) defensive proteins such as OmpX [17], and (vi) structural proteins such as OmpA [18]. The loops exhibit the largest sequence variability and thus contain most of the functional characteristics of each protein within these families [4]. The general porins OmpC and OmpF are regulated by osmotic pressure and the phosphoporin PhoE is synthesized under limiting phosphate conditions. Although these porins are not selective for particular substrates, they only allow for the passage of molecules smaller than ~600 Da. The maltoporin LamB is selective for the permeation of maltodextrins. The specificity and size selectivity is conferred by one of the loops that folds back into the lumen of the porins and thereby controls the passive permeation of the various substrates. FhuA, FepA, FecA, and BtuB catalyze the active uptake of iron-siderophore complexes and vitamin B_{12}, respectively. These proteins are coupled to the periplasmic protein TonB, which acts as a transducer that is powered by inner membrane protein active transporters. The outer membrane phospholipase OmpLA exists in two forms, an active dimer and an inactive monomer. The active site lies at the outer edge of the barrel and at the interface between the two subunits of the dimer. Dimerization occurs through "knob-in-the-hole" van der Waals' interactions of several apolar residues and stacking interactions of some aromatic residues, and two inter-subunit hydrogen bonds within the

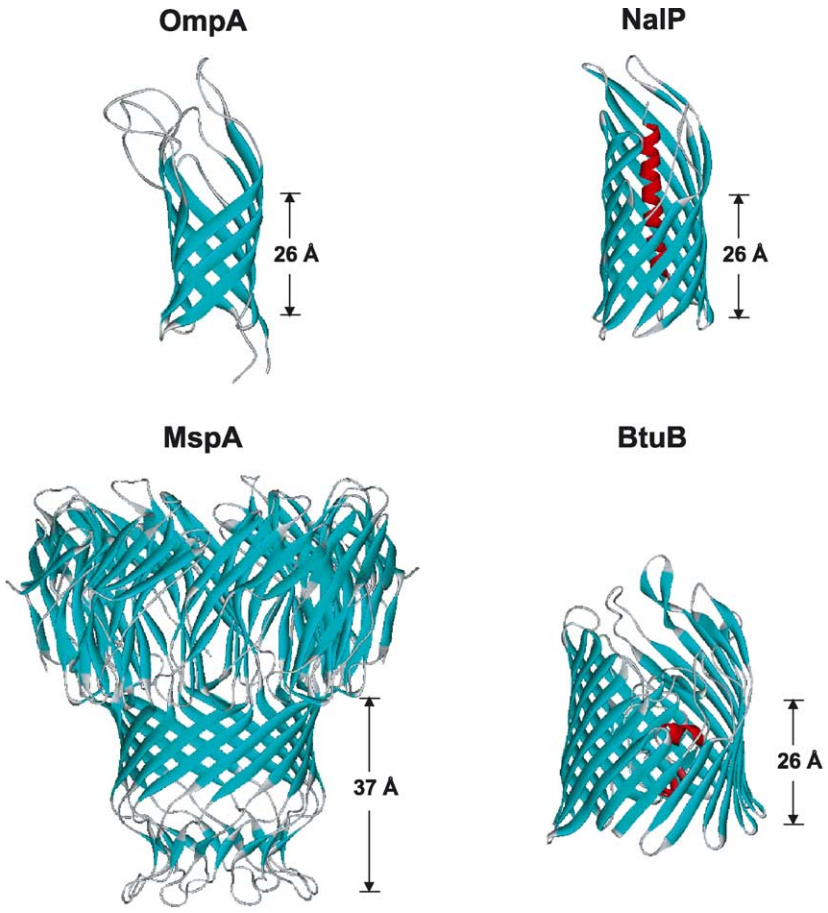

Fig. 1. Representative structures of β-barrel membrane proteins. OmpA, transmembrane domain of OmpA of *E. coli* (PDB entry 1G90; [22]); NalP, translocator domain of autotransporter of *N. meningitidis* (1UYN; [67]); MspA, porin of *M. smegmatis* (1UUN; [2]); BtuB, cobalamin transporter of *E. coli* (1NQE; [14]). The approximate location of the lipid bilayer is indicated in each structure. Note the much wider hydrophobic thickness of MspA.

hydrophobic membrane domain. These interactions are of the same type as the interactions that hold together pairs of TM α-helices [19]. Therefore, pairing of TM domains in membranes appears to follow the same rules, independent of whether these domains are TM α-helices or β-barrels. Dimer formation of OmpLA is promoted by membrane perturbation, e.g. by the presence of phospholipids in the outer leaflet of the outer membrane. Another outer membrane enzyme is OmpT, which is a protease that protrudes far from the lipid bilayer and has a unique catalytic site at the extracellular top. OmpT requires LPS for activity and preferentially cuts between two consecutive basic residues to defend the organism against antimicrobial peptides. PagP is an outer membrane enzyme of the LPS biosynthetic pathway that transfers the *sn*-1 palmitate chain from phospholipid to lipid A of LPS. OmpX binds, neutralizes, and thus protects Gram-negative bacteria from toxic proteins. Obviously this function is conferred by the outer loops. OmpA and its *Pseudomonas* ortholog OprF are structural proteins. They link the outer membrane to the periplasmic peptidoglycan via a globular C-terminal domain on the periplasmic side of the outer membrane. The β-barrel TM domain thus provides primarily a membrane anchor, but its outer loops also function as receptors for various phages and colicins. OmpA has also been shown to form an ion channel in planar lipid bilayers [20,21]. Although this function provides a convenient assay for measuring the recovery of native structure in folding experiments, its physiological significance is uncertain.

3. Thermodynamic stability of β-barrel membrane proteins

The thermodynamic stability of β-barrel membrane proteins has been investigated by solvent denaturation and by differential scanning calorimetry (DSC). In the following, we discuss the thermodynamic stability of the simple monomeric β-barrel protein OmpA, which has been investigated mostly by denaturation with urea. We then proceed to a discussion of complexities that arise by the presence of the central plug domain of FepA and FhuA, whose stability has been studied by solvent denaturation and DSC. Finally, we review experiments that address the contribution of trimer formation to the stability of porins. The most extensive thermodynamic stability studies have

been carried out with the OmpA of *Escherichia coli*. OmpA is a two-domain protein whose N-terminal 171 residues constitute the β-barrel membrane-anchoring domain and whose C-terminal 154 residues form a globular periplasmic domain that interacts with the peptidoglycan. The 3D structure of the TM domain has been solved by X-ray crystallography [18] and, more recently, by solution NMR spectroscopy [22]. Extensive mutagenesis studies show that OmpA is quite robust against many mutations especially in the loop, turn, and lipid bilayer facing regions of the barrel [23]. The TM domain of OmpA can even be circularly permutated without impairing its assembly and function in outer membranes [24]. Dornmair et al. [25] showed that OmpA could be extracted from the outer membrane and denatured and solubilized in 6–8 M urea and that the protein spontaneously refolded into detergent micelles by rapid dilution of the denaturant. They subsequently showed that urea-unfolded OmpA can also be quantitatively refolded into preformed lipid bilayers by rapid dilution of urea [26]. OmpA and other Omps can also be expressed in inclusion bodies and subsequently be refolded in various detergents [27], which has become an important tool in Omp structural biology.

We recently achieved the complete and reversible refolding of OmpA in lipid bilayers [28]. In this work, we showed that OmpA can be reversibly extracted from lipid bilayers by the addition of urea and then refolded into lipid bilayers by removal of the denaturant. The unfolded state of OmpA is completely dissociated from the membrane and its circular dichroism (CD) spectrum indicates that little or no residual secondary structure is left in this state (Fig. 2B). The refolded form reproduces the CD spectrum of the native state, which indicates its predominant β-sheet secondary structure. The formation of tertiary structure of OmpA (and many other β-barrel membrane proteins) is conveniently monitored by a shift of the apparent molecular mass when the proteins are run on sodium dodecyl sulfate (SDS) polyacrylamide gels without boiling the samples prior to loading on the gels. Unboiled samples of OmpA run on SDS-gels at an apparent molecular mass of 30 kDa if the protein was completely folded, but at 35 kDa if it was unfolded or incompletely folded. This shift on SDS gels has proven to be a very useful assay for tertiary structure formation of OmpA and other outer membrane proteins [29]. Complete refolding as measured by the SDS-polyacrylamide gel electrophoresis (PAGE) shift correlates with the re-acquisition of the ion channel activity of OmpA [20]. For OmpA reconstituted into small unilamellar vesicles composed of 92.5 mol% 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (POPC) and 7.5 mol% 1-palmitoyl-2-oleoyl-*sn*-phosphatidylglycerol (POPG), we observe a gradual shift from the folded to the unfolded state that is centered at approximately 3 M urea (Fig. 2A). The transition from the folded to the unfolded state could also be monitored by CD spectroscopy and Trp fluorescence spectroscopy and occurred at the same urea concentration independent of the method. This and other lines of evidence prove that the urea-induced unfolding of OmpA in these lipid bilayers is a coupled two-state membrane partition-folding reaction. Therefore, the plots of the fraction unfolded f_u vs. the

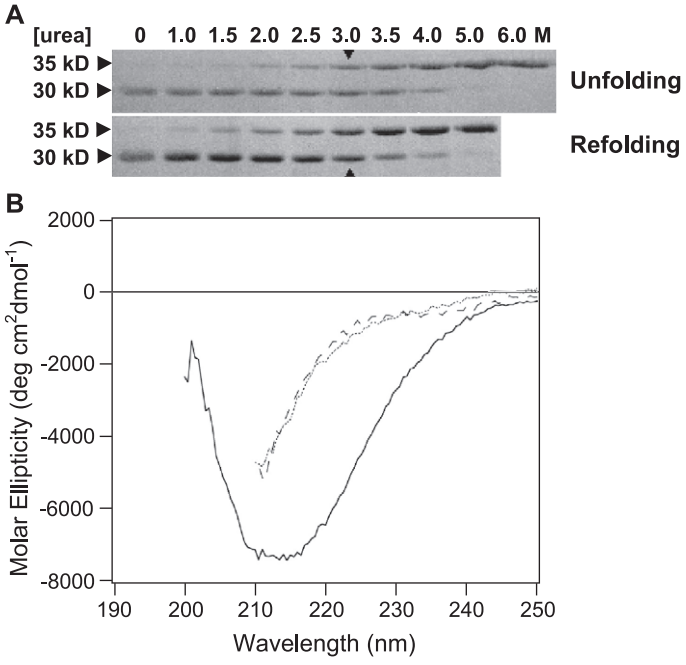

Fig. 2. Reversible folding of OmpA into bilayers composed of 92.5% POPC and 7.5% POPG. (A) SDS-PAGE of unboiled samples as a function of urea concentration in unfolding and refolding reactions. The 30 kDa form represents the native state, and the 35 kDa form represents unfolded states. The approximate transition midpoints are indicated with arrows. (B) CD spectra of native membrane-inserted OmpA (solid line), denatured OmpA obtained by treating membrane-inserted OmpA with 6 M urea (dashed line), and denatured OmpA in the absence of lipids (dotted line; from Ref. [28]).

concentration of urea can be evaluated with standard procedures to extract the free energy of unfolding, $\Delta G^0_{u,H_2O}$ and the m-value, which is a measure of the cooperativity of unfolding.

$$f_u = \frac{\exp\left(\left\{m[\text{urea}] - \Delta G^0_{H_2O}\right\}/RT\right)}{1 + \exp\left(\left\{m[\text{urea}] - \Delta G^0_{H_2O}\right\}/RT\right)} \quad (1)$$

These equilibrium folding experiments on OmpA in lipid bilayers represent the first example of an integral membrane protein, for which a quantitative measurement of the thermodynamic stability could be obtained. An example of equilibrium folding curves measured by Trp fluorescence and analyzed by Eq. (1) is shown in Fig. 3. These data show that $\Delta G^0_{u,H_2O}$ and the m-value are 4.5 kcal/mol and 0.9 at pH 7. $\Delta G^0_{u,H_2O}$ decreases as a function of increasing pH and reaches 3.0 kcal/mol at pH 10. The m-value is constant or increases very slightly in this pH range. The rather small $\Delta G^0_{u,H_2O}$ of OmpA, which is of the same order of magnitude as that of water-soluble proteins, is perhaps surprising in view of the quite extreme heat resistance of this and other β-barrel membrane proteins. However, if one simply calculates the free energy of transfer of all residues that are transferred into the lipid bilayer with the augmented Wimley and White hydrophobicity scale [30], one finds that the net $\Delta G^0_{u,H_2O}$ amounts to only about 1 kcal/mol. This value may be further increased by adding a few kcal/mol for unfolding, which would bring the prediction close to the experimentally determined value. The take-home message from these measurements and theoretical considerations is that the overall stability of membrane proteins is not as large as one might have anticipated, but rather similar in magnitude to that of soluble proteins of similar size. As is true for soluble proteins, the thermodynamic stability of this and perhaps most membrane proteins is determined by the sum of many relatively large thermodynamic contributions that ultimately cancel to yield a relatively small net free energy of folding.

The TonB-dependent transporters are monomeric 22-stranded β-barrels, whose lumina are filled with N-terminal globular 'plug' domains consisting of α-helical and β-sheet secondary structure. These plug domains are tightly inserted into the barrels, where they make extensive salt bridge and hydrogen bond contacts with the inner barrel wall. The mechanism of substrate transport through these transporters is presently not well understood. The thermal stability of FhuA in micelles was studied by DSC [31]. These studies demonstrated that the plug domain unfolds independently from the surrounding β-barrel. In the absence of the bound substrate ferrichrome, a reversible transition centered at 65 °C was well separated from an irreversible transition centered at 74 °C. The binding of the substrate increased the lower transition temperature to 71 °C, while the higher transition temperature was not changed. Since the higher

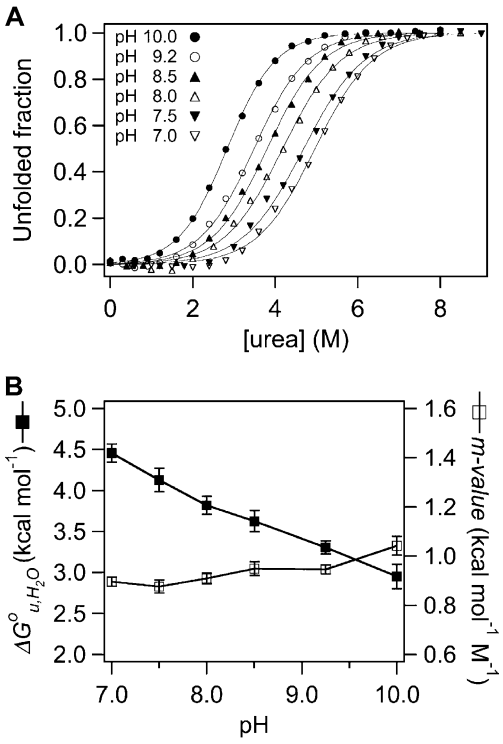

Fig. 3. Thermodynamic stability of OmpA in POPC/POPG (92.5/7.5) bilayers as a function of pH. (A) Unfolding curves measured by Trp fluorescence. The data were fit to the two-state equilibrium folding model (Eq. (1)). (B) Dependence of $\Delta G^0_{u,H_2O}$ and m-value of OmpA in POPC/POPG bilayers on the pH.

temperature transition was accompanied with a significant change of the CD signal at 198 and 215 nm, this transition has been assigned to the denaturation of the β-barrel. The lower temperature transition has been assigned to the denaturation of the plug domain and neighboring loops emerging from the β-barrel because antibodies that recognize unfolded outer loops bound to FhuA only above the lower transition temperature of 65 °C. A deletion mutant (Δ21–128) that lacked the plug domain underwent a thermal denaturation at a lower temperature (T_m=62 °C) than the wild-type protein implying that the presence of the plug stabilizes the barrel structure.

The stability of FepA in Triton X-100 micelles was probed by solvent-induced denaturation and EPR spectroscopy [32,33]. In these studies, FepA was functionally refolded from the denatured state by dialyzing the denaturant in the presence of TX-100 micelles. When guanidine hydrochloride (GdnHCl)- and urea-induced denaturation was monitored by site-directed spin-label EPR spectroscopy at an extracellular loop site, unfolding occurred in a sharp transition at 2.0 M GdnHCl or 5.5 M urea, respectively. The free energies of unfolding were approximately 6 kcal/mol with both denaturants. The rate of unfolding of the substrate-bound protein was significantly smaller than that of the free protein. This confirms that the substrate has a stabilizing effect on FepA as has also been observed by DSC with FhuA. When residues

pointing towards the center of the barrel were spin-labeled, their EPR spectra indicated completely mobile residues in 4 M GdnHCl. However, residues facing the detergent were still quite immobilized at this GdnHCl concentration. The authors argued that denatured FepA retained substantial residual hydrophobic interactions with the detergent micelle. This observation is reminiscent of residual hydrophobic interactions that have been observed in soluble proteins at high denaturant concentrations [34]. However, in these studies with FepA, the degree of denaturation was unfortunately not recorded by a global method such as CD spectroscopy and therefore, it is not clear whether the transition is two-state and whether fully denaturing conditions have been reached in this work.

As discussed above, the long loop L3 of the porins folds back into the lumen of the pores where they engage in a hydrophobic contact with a few residues inside the barrel. However, the shorter loop L2 forms a 'latch' that reaches over from one subunit to a neighboring subunit and thereby stabilizes interactions between the subunits in these trimeric proteins. Porins are extremely stable towards heat denaturation, protease digestion, and chemical denaturation with urea and GdnHCl [4]. For example, extraction of OmpF from the *E. coli* outer membrane requires the heating of outer membranes in mixtures of isopropanol and 6 M GdnHCl at 75 °C for more than 30 min [35]. The trimer structure itself is maintained up to 70 °C in 1% SDS [36]. A mutagenesis study combined with DSC and SDS-PAGE shift assays demonstrates that the inter-subunit salt bridge and hydrogen bonding interactions involving loop L2 contribute significantly to the trimer stability. For example, mutations breaking the salt bridge between Glu 71 and Arg 100 decrease the trimer–monomer transition temperature from 72 to 47–60 °C and ΔH_{cal} from 430 to 200–350 kcal/mol. A similar behavior was observed when residues 69–77 of L2 were deleted [36].

4. Mechanisms of β-barrel membrane protein folding

A quite detailed picture of the mechanism of folding and membrane insertion of OmpA has emerged from a variety of kinetic studies. In an early study, the kinetics of folding of OmpA into lipid bilayers composed of dimyristoyl-*sn*-phosphatidylcholine (DMPC) in the fluid phase were found to be rather slow, i.e. on the order of many minutes at 30 °C [37]. The kinetics of folding of OmpA into lipid bilayers composed of dioleoyl-*sn*-phosphatidylcholine (DOPC) were later studied over a wide temperature range from 2 to 40 °C [38]. This work revealed that membrane insertion and folding occurred in three distinct kinetic phases. The fastest phase detected by Trp fluorescence changes and attributed to the initial binding of the unfolded protein to the bilayer surface had a time constant of 6 min and was rather independent of temperature. A second phase was strongly temperature-dependent and had time constants in the 15 min to 3 h range. The activation energy determined from an Arrhenius plot was 11 kcal/mol. This phase has been assigned to a deeper insertion, but not yet complete translocation of the β-strands in the lipid bilayer. The slowest phase was observed by the SDS gel-shift assay, which reports on the completion of the β-barrel. Complete folding in DOPC bilayers was only observed at temperatures greater than 30 °C, had a time constant of about 2 h, and took about 6 h to go to completion at 37 °C.

This three-step mechanism of OmpA insertion into bilayers was subsequently further refined by time-resolved Trp fluorescence quenching (TDFQ) [39]. This technique permits a direct observation of the translocation of Trps of the protein across the lipid bilayer. Briefly, quenchers of Trp fluorescence are placed at different depths in the membrane and the time course of passage of Trps past these zones of quenchers is followed. Phospholipids that are selectively labeled with bromines or nitroxide spin labels at defined acyl chain positions are conveniently used as depth-specific fluorescence quenchers. The technique is most useful and the results are best interpreted if used with proteins that have only single Trp residues [40]. Examples of some TDFQ time courses of single Trp mutants of OmpA are shown in Fig. 4. Trp 7, which is located on the periplasmic side of the β-barrel, enters the *cis* membrane interface, but does not translocate. In contrast, Trp 143, which is located on the extracellular side of the β-barrel, enters the membrane (with unresolved kinetics at 30 °C) and then translocates to the *trans*-side of the membrane with the kinetics shown in Fig. 4. In order to separately measure the translocation rates of the individual β-hairpins, single Trp mutants of OmpA were made with the Trps individually placed at the beginnings of the outer loops of each of the four hairpins. When the membrane translocation rates were measured, it was found that each of four β-hairpins of OmpA crossed the membrane with the same time course [40]. Therefore, OmpA inserts and folds into lipid bilayers by a mechanism, in which the individual TM β-hairpins are translocated in a concerted fashion. In fact, the kinetics of the final portion of secondary structure formation and closure of the β-barrel are synchronized in various lipid backgrounds [41]. This result is in accordance with the notion that inter-strand hydrogen bonds and the barrel itself have to form while the protein translocates across the membrane. This mechanism of β-barrel insertion and folding also contrasts with the two-stage mechanism that has been proposed for α-helical membrane proteins [42]. In the two-stage model, individual TM α-helices are thought to insert independently of each other in stage I and then form lateral associations only in stage II.

Since the positions of individual Trps could be located by TDFQ in the membrane at different time points of folding and membrane insertion, it was also possible to characterize crude ensemble structures of the folding intermediates by this technique. The following intermediates were characterized in the in vitro folding pathway (Fig. 5): the unfolded state U hydrophobically collapses into intermediate I_W in

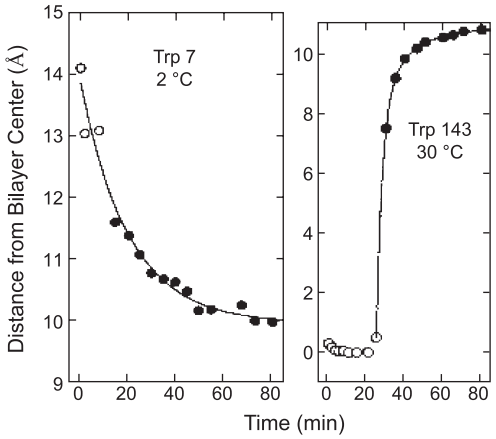

Fig. 4. Measurement of the translocation rate of single tryptophans of OmpA into and across DOPC bilayers by TDFQ. (A) Time course of the movement of Trp 7 of OmpA into lipid bilayer at 2 °C. This Trp stays on the *cis* side and does not cross the bilayer. (B) Time course of the movement of Trp 143 across the lipid bilayer at 30 °C. This Trp translocates across the bilayer with a time constant of 3.3 min at 30 °C. The solid lines are fits of the data to exponential functions (adapted from Ref. [40]).

water. This intermediate binds to the membrane surface, where it forms intermediate I_{M1} with disordered Trps. I_{M1} proceeds to intermediate I_{M2} with all Trps located at ~10 Å from the bilayer center. Some fraction of β-structure is developed at this stage. Since correct tertiary contacts are not yet formed and since the secondary structure elements lie in the interface, this state has been termed a "molten disk" state. Next, the four Trps on the four β-hairpin loops translocate to about the center of the lipid bilayer. This intermediate state, I_{M3}, is more globular, but has still not achieved the correct tertiary fold and has therefore been termed the "molten globule" analog of membrane proteins. I_{M3} then proceeds to the native state N, in which all Trps are located at ~10 Å from the bilayer center, which is in good agreement with the crystal structure of OmpA. These experiments showed very clearly that folding and membrane insertion are two coupled processes, quite similar to the partition-folding coupling that has been discussed for some helical peptides (see, e.g. Ref. [43]). It is also clear that the membrane interface is intimately involved in the folding process, as is also commonly assumed to be the case for the spontaneous insertion of helical toxins into lipid bilayers (see, e.g. Ref. [44]). The finding that certain elements of the phosphoporin PhoE fold while still exposed to the periplasm is consistent with the mechanism proposed in Fig. 5. The results of Eppens et al. [45] can be explained if the engineered disulfide bonds are formed in intermediates I_{M2} or I_{M3} of PhoE. The requirement that the barrel needs to be completely folded in order to translocate across the membrane probably also explains why the time constants of this process are so slow and the activation energies are so high. Folding and translocation of the barrel into lipid model membranes requires the creation of a large defect in order to

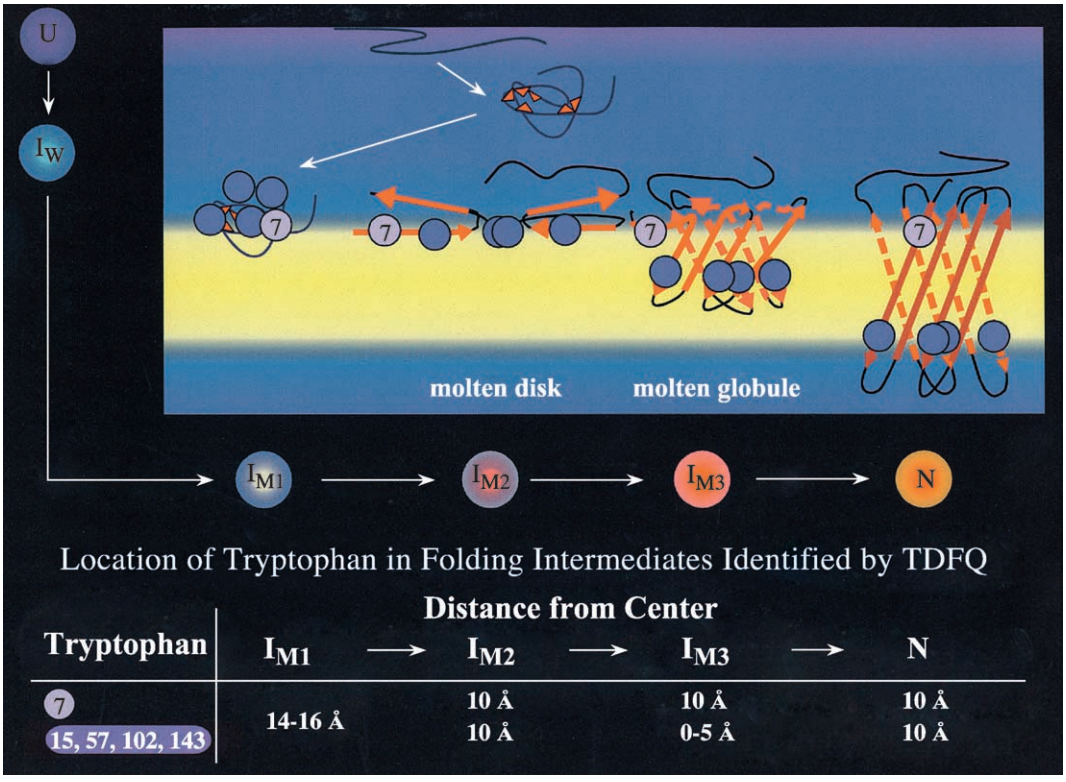

Fig. 5. Model of folding and membrane insertion of OmpA. The unfolded, hydrophobically collapsed water-soluble intermediate, three membrane-bound intermediate, and the native states are shown. See text for a more detailed description of the folding intermediates. The distances of the Trps from the bilayer center as measured by TDFQ are shown in the lower part of the figure.

insert a barrel of the size of OmpA. Moreover, the membrane provides a 100–1000-fold more viscous environment than water for folding and inserting membrane proteins. Chaperones—lipids and proteins—may accelerate this process in vivo as will be discussed in the following sections.

5. Assisted folding of β-barrel membrane proteins

Folding of outer membrane proteins in vivo clearly occurs on a much faster time scale than described in the preceding section. For example, pulse-chase experiments show that about half of the pool of newly synthesized PhoE assembles in the outer membrane within about 30 s and the other half in about 10 min [46]. Two periplasmic proteins have been proposed to act as folding catalysts for outer membrane protein assembly. Skp is a 17 kDa basic protein that binds several outer membrane proteins including OmpA, OmpC, OmpF, PhoE, and LamB [47]. Although Skp is water-soluble, it also binds peripherally to phospholipid bilayers [48]. Therefore, it is likely that soluble and membrane-bound pools also exist in the periplasm and that Skp could shuttle between the inner and outer membrane. Skp binds to newly synthesized and unfolded Omps immediately after they are translocated across the inner membrane as demonstrated for OmpA and PhoE [49,50]. Three to four molecules of Skp bind to each molecule of unfolded OmpA [51]. Therefore, Skp appears to fulfill all requirements of a passive chaperone that keeps Omps in an unfolded form and prevents them from aggregation in the periplasm. However, it does not appear to accelerate the folding and insertion into the outer membrane. Rather to the contrary, the efficiency and rate of membrane insertion are inhibited in the presence of Skp unless LPS is also present [51]. Therefore, Skp does not appear to be an active folding catalyst. Possible effects of LPS on the folding and insertion of Omps will be further discussed in the next section.

SurA is a periplasmic peptidyl-prolyl isomerase that has been shown to assist the folding of several outer membrane proteins including OmpA, OmpF, and LamB [52]. The peptidyl-prolyl isomerase activity resides in one of two parvulin-like domains in the C-terminal half of the protein. The N-terminal domain of SurA has a different chaperone-like activity and is required for the selective recognition of Omps by SurA [53]. This chaperone function of the N-terminal domain may be even more important than the peptidyl-prolyl isomerase activity of the parvulin domains because deletion of the latter did not significantly affect the maturation of Omps. The crystal structure of SurA reveals a 50-Å-long cleft that can accommodate unfolded peptides [54]. Sequences containing aromatic–random–aromatic motifs appear to bind particularly well to SurA [55]. This motif is frequently found at the edges of outer membrane protein β-barrels where they form the well-known aromatic girdles that also interact with the interfaces of lipid bilayers.

Several other folding catalysts and chaperones have been identified in the periplasmic space, but they have either no role in the folding of Omps or their roles with respect to Omps have not yet been assessed. Disulfide bond reductases of the Dsb system are probably not important because most Omps lack cysteines. DsbA catalyzes the formation of a disulfide bond in OmpA, which however is located in the periplasmic globular domain of this protein [56]. Other periplasmic peptidyl-prolyl isomerases include RotA and FkpA and other periplasmic binding proteins include MglB, OppA, and PapD, which is specialized to interact with unfolded pili proteins.

A proteinaceous machinery for the insertion of Omps in the outer membrane is not known, but a potential candidate, Omp85, has been recently identified in *N. meningitidis* [57]. This essential outer membrane protein of 85 kDa has homologs in all Gram-negative bacteria and is predicted to form a 12-stranded β-barrel (residues 483–797) that is preceded by a very large periplasmic domain (residues 1–482). A large variety of different Omps including porins and Omp enzymes do not assemble properly in Omp85-depleted cells. Omp85 can be cross-linked with unfolded porins, but how it mediates the insertion of Omps into the outer membrane is unknown. Perhaps, the periplasmically exposed N-terminus acts as a chaperone that is required to strip Omps from chaperones like Skp that prevent aggregation, but inhibit membrane insertion and folding.

6. Lipid effects of β-barrel membrane protein folding

The kinetic and equilibrium in vitro folding studies described in the previous sections were carried out in lipid bilayers composed primarily of phosphatidylcholines (PC). Since bacterial membranes do not contain PC, but are mostly composed of PE and about 20% PG and CL, one might ask whether these lipids facilitate the insertion and folding of Omps. For comparison, the assembly of the helical membrane protein lactose permease in the inner *E. coli* membrane has been shown to be assisted by PE [58]. For Omps, we also need to consider LPS, which is present only in the outer leaflet of the outer membrane. Therefore, one might expect LPS to contribute to the insertion and folding of Omps only at a late stage, unless it is co-inserted with the protein into the outer membrane.

We examined the effect of the different lipid classes on the thermodynamic stability of OmpA in lipid model membranes. Fig. 6 shows the effect of including increasing amounts of 1-palmitoyl-2-oleoyl-*sn*-phosphatidylethanolamine (POPE) in a bilayer composed of POPC and 7.5% POPG on the urea-induced unfolding curves [28]. The thermodynamic stability and thus $\Delta G^0_{u,H_2O}$ of OmpA increased from 3.2 to 5.1 kcal/mol when POPE was included up to 40 mol%. Similarly, the *m*-value increased from 1.1 to 1.8 upon addition of 40% POPE. When the POPE content was further increased to the physiological

~80%, OmpA became so stable that the reversible folding experiment could no longer be carried out. Similarly, we were unsuccessful in measuring the refolding kinetics into lipid bilayers consisting mostly of PE (unpublished results). Including PG or CL up to 30 and 15 mol%, respectively, stabilized OmpA and increased the cooperativity of folding (unpublished results).

Inclusion of LPS in small unilamellar vesicles inhibited the insertion and folding of OmpA (unpublished results). However, this result is not conclusive because LPS is only present in the outer leaflet of outer membranes and would not be encountered in the inner leaflet by unfolded OmpA. Therefore, one would have to make vesicles with an asymmetric lipid distribution with LPS only present in the inner leaflet to reproduce the in vivo situation in these in vitro experiments. Unfortunately, such vesicles cannot be prepared for technical reasons. Nevertheless, there is some literature that assigns a role to LPS and its assistance in Omp assembly in the outer membrane. It has been reported that PhoE first folds as a monomer in LPS before the folded PhoE/LPS complex inserts as an entity into the outer membrane. We think that this conclusion is not warranted given the design of the experiment that lead to it. In these experiments, de Cock and Tommassen [59] refolded PhoE into Triton X-100 micelles with or without LPS before they inserted these complexes into outer membranes with a higher concentration of Triton X-100. Triton X-100 folds many membrane proteins, but is not present in the periplasm of Gram-negative bacteria. Moreover, it is unknown whether

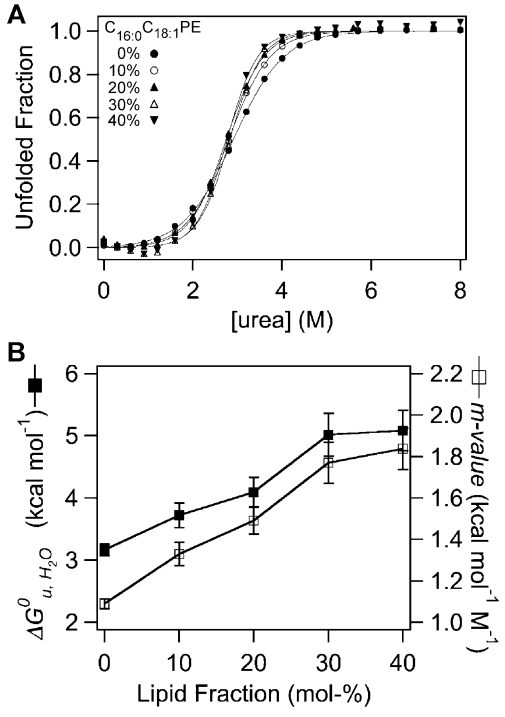

Fig. 6. Effect of increasing mol fraction of POPE in POPC/POPG (92.5/7.5) bilayers on the thermodynamic stability of OmpA. (A) Unfolding curves measured by Trp fluorescence. (B) Dependence of $\Delta G^0_{u,H_2O}$ and *m*-value of OmpA folding on the mol fraction of POPE (adapted from Ref. [28]).

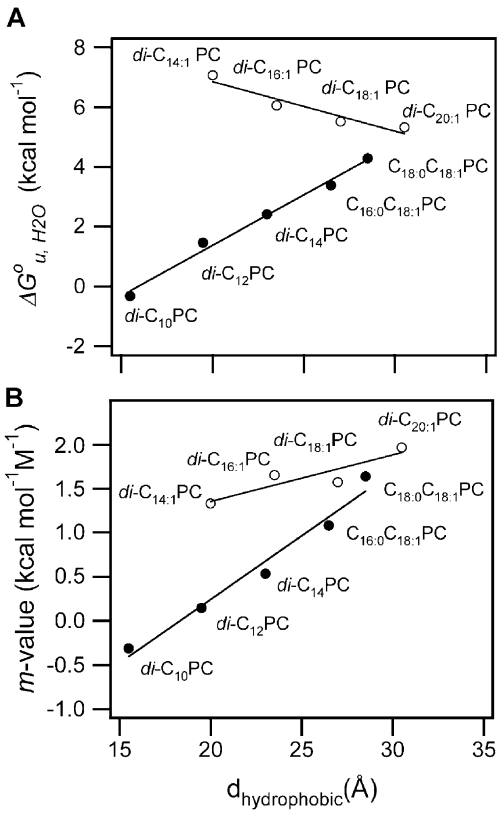

Fig. 7. Dependence of (A) the free energy of unfolding $\Delta G^0_{u,H_2O}$ and (B) the *m*-value on the hydrophobic thickness of phosphatidylcholine bilayers. Filled circles, saturated acyl chain series with no lateral bilayer pressure. Open circles, double *cis*-unsaturated acyl chain series with increasing lateral bilayer pressure as the hydrophobic thickness decreases (from Ref. [28]).

newly synthesized LPS is present in the periplasm and how it is shuttled from the inner to the outer membrane in the biogenesis of outer membranes [60]. Another study has implicated a role for LPS synergizing with the chaperone Skp [51]. However, the stimulation of folding and insertion was only about 19% and membranes had to be added quickly after the addition of LPS. The role of LPS, which is negatively charged, may have simply been to displace the basic Skp from unfolded OmpA and thus make it competent for membrane insertion and folding in these experiments. In summary, whether or not LPS is really involved in the folding and assembly of Omps in outer membranes (other than binding at a very late stage from within the outer leaflet) is in our opinion still an open question.

The effect of varying the bilayer thickness was first studied by measuring the rates of folding and insertion of OmpA into PC bilayers with different acyl chain lengths [41]. As one might expect from the material properties of lipid bilayers, the folding and insertion rates increase significantly as the bilayer thickness decreases. When the bilayers are sufficiently thin (i.e. PCs with 12-carbon or shorter acyl chains), insertion and folding of OmpA into large unilamellar vesicles is observed, whereas in average or thick bilayers complete insertion and folding occurs

only in small unilamellar vesicles. Small vesicles are more strained and therefore exhibit more defects than large vesicles, which facilitates quantitative refolding of OmpA in small, but not in large vesicles if the hydrocarbon thickness of the bilayer is more than 20 Å, i.e. that of DLPC. This effect of bilayer thickness on the kinetics of OmpA insertion and folding may have some physiological consequences because the hydrophobic thickness of outer bacterial membranes is thought to be thinner than that of the inner membranes [60].

The effect of thickness of the lipid bilayer on the thermodynamic stability of OmpA was also examined [28]. Increasing the thickness of the lipid bilayer increases the stability, i.e. increases $\Delta G^0_{u,H_2O}$ of OmpA in a linear fashion (Fig. 7). The *m*-value and thus the cooperativity of folding also increases linearly with bilayer thickness. The increase in $\Delta G^0_{u,H_2O}$ is 0.34 kcal/mol per Å of increased bilayer thickness, which converts to 4 cal/mol per Å^2 of increased hydrophobic contact area. This is only about 20% of the standard value for the hydrophobic effect [61]. It is very likely that elastic forces that arise from lipid deformation due to hydrophobic mismatch of the protein and lipid bilayer counteract the energy gain from the hydrophobic effect. If hydrophobic mismatch is the dominating reason for the discrepancy between the expected and measured values, we conclude that each angstrom of hydrophobic mismatch subtracts 1.4 kcal/mol from the thermodynamic stability of OmpA. Since about 24 lipids are expected to be in direct contact with OmpA, we conclude that the elastic deformation energy is about 60 cal/Å/mol of boundary lipid. This estimate is an upper bound because it neglects possible elastic deformations beyond the first shell of boundary lipid.

The favorable effect of POPE on the thermodynamic stability of OmpA that was discussed above could be due to the fact that this lipid is cone-shaped and thereby induces curvature stress and an internal lateral pressure on embedded membrane proteins [62], rather than a result of the different chemical nature of the PE compared to the PC headgroup. To further test this hypothesis, we performed thermodynamic stability measurements in PC bilayers with *cis*-unsaturated acyl chains of different chain length in the *sn*-1 and *sn*-2 positions. Since the relative cross-sectional area of the hydrocarbon compared to the headgroup region becomes larger as the chain length is decreased in this lipid series, curvature stress and the internal lateral pressure will increase as the chain length and bilayer

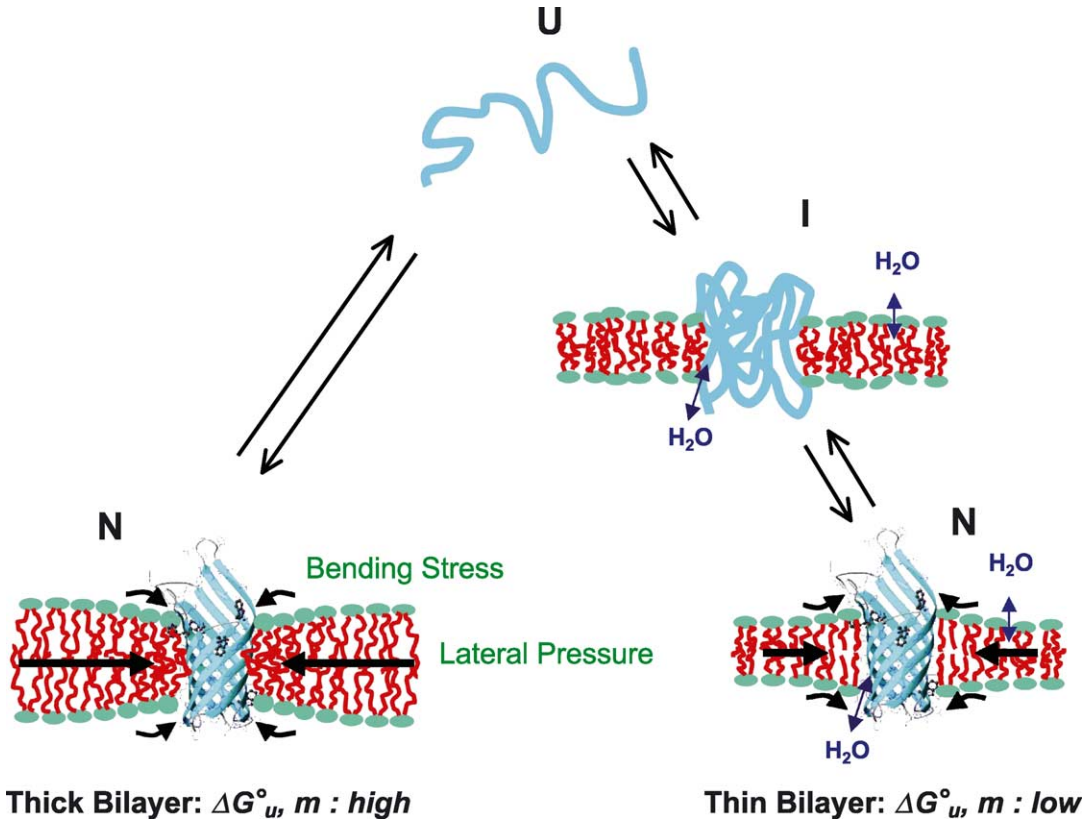

Fig. 8. Cartoon depicting the folding of OmpA into lipid bilayers. Left path, folding into most bilayers is a thermodynamic two-state process. Right path, folding into thin bilayers is multi-state, i.e. at least one equilibrium intermediate occurs. Bilayer forces acting on OmpA folding are indicated with arrows. The large black arrows indicate lateral bilayer pressure imparted on the lipid/protein interface in the hydrophobic core of the bilayer by intrinsic curvature strain. Increasing this pressure increases the thermodynamic stability of the protein. The small black arrows indicate lipid deformation forces caused by hydrophobic mismatch between the protein and unstressed bilayers. These forces decrease the thermodynamic stability of the protein. Water molecules penetrate more easily into the hydrophobic core of thin bilayers (blue arrows) and stabilize equilibrium intermediates until, in very thin bilayers, complete unfolding is no longer observed. The unfolded state in urea is dissociated from the membrane (from Ref. [28]).

thickness decrease. Consistent with the hypothesis that lateral pressure from intrinsic bilayer curvature stress can be a major stabilizing factor of membrane proteins, we found that the thermodynamic stability of OmpA increased with decreasing chain length in the double *cis*-unsaturated PC lipid series (Fig. 7). Contrary to the case of the saturated PC series where the *m*-value was correlated with the $\Delta G^0_{u,H_2O}$ increase, the *m*-value and cooperativity decreased when the bilayer pressure was increased in the double-unsaturated PC series. A summary of the various bilayer forces that act on OmpA and that modulate its thermodynamic stability in membranes is illustrated in Fig. 8. The lipid-contacting surface of OmpA is approximately hourglass-shaped. Therefore, cone-shaped lipids that increase the internal bilayer pressure stabilize OmpA. On the other hand, if the apolar portion of the bilayer is thicker or thinner than the ~26 Å hydrophobic thickness of the protein, lipid deformation occurs and bilayer bending stress opposes the stability of OmpA. If the bilayers are very thin, more water penetrates and folding intermediates are thermodynamically stabilized.

7. Concluding remarks

Beta-barrel membrane proteins serve many different functions including enzymatic, transport, and structural support in the outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts. Since the periplasm of Gram-negative bacteria lacks ATP as an energy source, outer membrane proteins have devised mechanisms to spontaneously insert into outer membranes after secretion into the periplasmic space. The β-barrel design is particularly well suited for spontaneous insertion because each TM segment is only moderately hydrophobic and because periplasmic chaperones keep these proteins soluble and prevent their aggregation before membrane insertion. The chaperones may be replaced with chemical denaturants to perform refolding studies of Omps in vitro. The TM domain of OmpA has served as an excellent model for such in vitro folding studies in the presence of a variety of different lipid model membranes. It has been possible to determine the thermodynamic stability of this protein in various lipid backgrounds and the contributions of many lipid properties including polar headgroup, acyl chains, and physical bilayer forces to the protein stability have been examined. Moreover, detailed kinetic studies have revealed a pathway for β-barrel membrane protein folding that is different from the two-stage model proposed for α-helical membrane proteins and that includes membrane-bound "molten-disk" and "molten-globule"-like folding intermediates. One major unresolved question is how the process of folding is accelerated in vivo compared to the rates that are currently achieved by reconstitution in vitro. An intriguing possibility is the involvement of the recently discovered folding catalyst Omp85 of *N. meningitidis*, which is an integral protein of the outer membranes of most if not all Gram-negative organisms [57].

Acknowledgments

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