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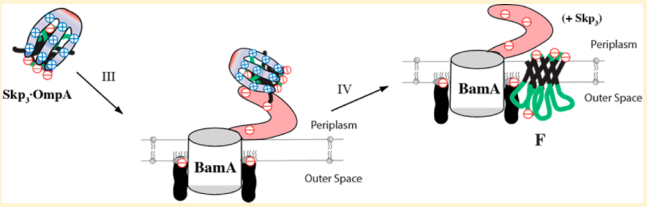
The Lipid Bilayer-Inserted Membrane Protein BamA of *Escherichia coli* Facilitates Insertion and Folding of Outer Membrane Protein A from Its Complex with Skp

Geetika J. Patel[†] and Jörg H. Kleinschmidt*[,†,‡]

[†]Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany

[‡]Abteilung Biophysik, Institut für Biologie, FB 10, Universität Kassel, D-34132 Kassel, Germany

ABSTRACT: Folding of β-barrel membrane proteins, either from a urea-unfolded form or from chaperone-bound aqueous forms, has been characterized for pure lipid bilayers. The impact of preinserted integral proteins from biomembranes has not been examined in biophysical comparisons, but this knowledge is important for the characterization of protein assembly machinery in membranes to distinguish specific effects from unspecific effects. Here, folding was studied for a β-barrel membrane protein, outer membrane protein A (OmpA) from *Escherichia coli*, in the absence and presence of two other preinserted integral proteins, BamA of the β-barrel assembly machinery complex (BAM) from *E. coli* and FomA from *Fusobacterium nucleatum*. Three different preformed lipid membranes of phosphatidylcholine were prepared to compare the folding kinetics of OmpA, namely, proteoliposomes containing either BamA or FomA and pure liposomes. Urea-unfolded OmpA folded faster into phosphatidylcholine bilayers containing FomA than into pure lipid bilayers, but the kinetics of OmpA folding and insertion were fastest for bilayers containing BamA. Incorporation of BamA into lipid bilayers composed of phosphatidylcholine and phosphatidylethanolamine greatly weakened the inhibiting effect of phosphatidylethanolamine on the folding of OmpA. Folding of OmpA from its complex with the periplasmic chaperone Skp into bilayers composed of phosphatidylethanolamine and phosphatidylcholine was inhibited in the absence of BamA but facilitated when BamA was present, indicating an interaction of Skp−OmpA complexes with BamA.



The mechanisms of insertion and folding of integral membrane proteins are not well understood. Because their average hydrophobicity is relatively low, outer membrane proteins (OMPs) from bacteria, mitochondria, and chloroplasts can be unfolded and solubilized in an 8 M solution of the chaotropic denaturant urea. OMPs fold upon dilution of urea in the presence of detergent micelles, which has allowed determination of their high-resolution structures. OMPs are β-barrel transmembrane proteins (TMPs) and can fold and insert spontaneously from their urea-unfolded forms into preformed lipid membranes in the fluid phase.[1,2] This has facilitated biophysical investigations of the mechanisms of TMP folding and insertion.[1,3−10] The studies have mostly focused on the folding of urea-denatured OMPs into pure lipid bilayers or detergent micelles and more recently into amphipathic polymers.[11,12] Outer membrane protein A (OmpA) from *Escherichia coli* has been a model for such biophysical studies.[2,5,7,13−16] OmpA is comprised of an eight-stranded transmembrane β-barrel domain (171 residues)[17,18] and a periplasmic domain (154 residues).[19] Urea-unfolded OmpA folds and inserts into lipid bilayers of either small unilamellar vesicles that are prepared by sonication[1] or into large unilamellar vesicles of short-chain phospholipids.[2] The folding kinetics are faster with an increased curvature elasticity[20] and an increased lipid bilayer flexibility.[2,21]

Periplasmic chaperones for OMPs (see ref 22 for a review), like the 17 kDa protein (Skp),[23,24] were discovered and characterized by genetic and microbiological studies. Skp from *E. coli* was shown to facilitate folding of OmpA from *E. coli* into lipid bilayers in the presence of negatively charged lipids or lipopolysaccharide.[25,26] Genetic studies also showed that the assembly of OMPs of bacteria, mitochondria, and chloroplasts into outer membranes requires the barrel assembly machinery (BAM) complex.[27] In *E. coli*, the BAM complex is composed of one transmembrane protein, BamA (also called YaeT or Omp85),[28] and four peripherally anchored lipoproteins (for recent reviews, see, for example, refs 29−32). Although thermophilic bacteria like *Thermus thermophilus* or *Thermosynechococcus elongatus* contain variants of BamA, they do not contain homologues of the lipoproteins.[33,34] BamA would then be a major factor for assembly of OMP in the outer membrane (OM) of bacteria. For *E. coli*, it has been demonstrated that the lipoprotein BamD is required for the assembly of OMP in the outer membrane.[35] BamA from *E. coli* (88 kDa) is composed of a C-terminal transmembrane domain (TMD) that forms a β-barrel (~385 residues) and an approximately equally sized N-terminal periplasmic domain (PD). The PD consists of five

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polypeptide transport-associated (POTRA) subdomains[36−40] (residues 1−405). For BamA of *E. coli*, it has been shown that the two N-terminal POTRA domains can be deleted, although strains grow poorly.[38] The transmembrane β-barrel and POTRA domains 3−5 of the periplasmic domain are required for the assembly of OMP in the OM.[38] In *Neisseria meningitidis*, the four N-terminal POTRA domains can be deleted with only slight defects in BamA function.[41] A recent report[42] indicated that the function of the *E. coli* BAM complex is preserved after reconstitution into *E. coli* lipid extract by detergent dilution. BAM function also required the presence of the periplasmic chaperone SurA, whereas energy sources like ATP were not necessary.

In this work, the effect of preinserted membrane proteins on the kinetics of folding and insertion of OmpA into preformed lipid bilayers was investigated for BamA from *E. coli* and FomA from *Fusobacterium nucleatum*. As a component of the BAM complex, we selected BamA to examine its role in the assembly of OmpA. To date, there are no reports whether the presence of a preinserted integral membrane protein in a lipid bilayer may affect bilayer properties that are important for membrane protein folding and insertion. To distinguish specific and unspecific effects, it is therefore necessary to compare the impact of BamA with that of another integral protein. We chose FomA for this comparison because it is not implicated in outer membrane protein assembly but functions as the major outer membrane porin of *F. nucleatum* and in biofilm formation.[43] The refolding and reconstitution of FomA into lipid bilayers have been established.[4,5,44] Here, a folding protocol was first established to obtain BamA in preformed lipid bilayers with its periplasmic domain oriented toward the outside of the lipid vesicles. The folding kinetics of OmpA were then compared for three different preformed lipid membranes of dilauroylphosphatidylcholine (diC$_{12}$PC), namely, proteoliposomes containing BamA, proteoliposomes containing FomA, and pure liposomes. Because phosphatidylethanolamine inhibits folding of OmpA into phosphatidylcholine bilayers, the folding kinetics of OmpA were also compared for bilayers containing dilauroylphosphatidylethanolamine (diC$_{12}$PE). Finally, to determine whether BamA supports membrane insertion of OmpA from a complex with the molecular chaperone Skp,[25,26,45] complexes of unfolded OmpA with Skp were formed and folding of OmpA from these complexes into lipid bilayers was monitored with or without integrated BamA.

MATERIALS AND METHODS

Overexpression of wt-BamA. The *bamA* gene was cloned between the NcoI and BamHI restriction sites of pET15b (Novagen) to obtain plasmid pET15_EcOMP85. The TSS method[46] was used to transform pET15_EcOMP85 into *E. coli* strain BL21(DE3) omp8 fhuA [F$^-$, *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3) Δ*lamB ompF*:: Tn5 Δ*ompA* Δ*ompC* Δ*fhuA*].[47] BamA with an N-terminal His$_6$-tag was then overexpressed in form of cytoplasmic inclusion bodies.

Overexpression of the TMD-BamA. The nucleotide sequence encoding the TMD of BamA (residues 404−810) was amplified by polymerase chain reaction (PCR) using the primers 5′-ATA ACA TAT GCG TGT TCC GGG TAG CC-3′ and 5′-GAC GGG ATC CTT ACC AGG TTT TAC CGA TG-3′ with the *bamA* gene as a template. To obtain plasmid pET15_BamAtmd, the PCR product was cloned into the pET15b vector (Novagen) between the NdeI and BamHI restriction sites. pET15_BamAtmd was transformed into *E. coli* strain BL21(DE3) omp8 fhuA for overexpression of TMD-BamA in inclusion bodies.

Isolation of wt-BamA and TMD-BamA. Overnight cultures of *E. coli* BL21(DE3), expressing either wt-BamA or TMD-BamA, were used to inoculate Luria-Bertani (LB) medium (containing 0.1 g/L ampicillin) at a ratio of 1:100. Cells were grown at 37 °C to an A_{600} of ∼0.6−0.7. For BamA expression, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.2 mM. After 5 h, cells were harvested by centrifugation for 30 min at 5000 rpm and 4 °C. The cells were then resuspended in buffer A [10 mM Tris (pH 8)], and lysozyme was added to a final concentration of 50 μg/mL. The mixture was stirred for 30 min at room temperature and then subjected to sonification using the macrotip of a W-450D Branson ultrasonifier (20% power, 50% pulse cycle) for 30 min while being cooled with an ice/water bath. Cells were then centrifuged at 3000g for 30 min at 4 °C. The supernatant was removed, and the pellet was resuspended in buffer B [20 mM Tris and 8 M urea (pH 8)]. The suspension was then centrifuged at 5000g for 30 min at room temperature. The supernatant was loaded onto a Q-Sepharose FF column (Amersham), and BamA was then eluted by applying a NaCl gradient (0−100 mM). The fractions were pooled and concentrated, and the concentration of BamA was determined.[48] The yields of either wt-BamA or TMD-BamA were very similar (∼30 mg/L of cell culture).

Purification of OmpA, FomA, Skp, and R-LPS. OmpA and FomA were overexpressed and purified in denatured form in 8 or 10 M urea as reported previously.[1,4] Skp was isolated from *E. coli* and purified as described.[26] The concentrations of OmpA, FomA, and Skp were determined using the method of Lowry et al.[48]

E. coli LPS (R2 core type; M_r ∼ 3900 g/mol) was a kind gift from O. Holst (Research Center Borstel, Borstel, Germany) and isolated as described.[25,49,50]

Preparation of Lipid Bilayers. Phospholipids 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (diC$_{12}$PC) and 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (diC$_{12}$PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Lipids were dissolved in chloroform, mixed at the desired molar ratio, dried under a stream of nitrogen, and desiccated under high vacuum for 3−5 h to prepare thin lipid films. The lipid films were hydrated in borate buffer (10 mM, pH 10, with 2 mM EDTA) and dispersed with a vortexer. LUVs were prepared by seven cycles of freezing and thawing of the hydrated lipids in liquid nitrogen and in a water bath at 35 °C. Lipid dispersions were extruded 30 times through polycarbonate membranes with a 100 nm pore diameter (Nucleopore, Whatman, Clifton, NJ) using a mini-extruder (Avanti Polar Lipids). LUVs prepared by this method have a size distribution of 100 ± 30 nm and are stable for at least 1 day.[51,52] LUVs were used for folding experiments on the day of preparation.

Folding of wt-BamA, TMD-BamA, and FomA into Lipid Bilayers. Oriented insertion and folding of BamA were initiated by 21-fold dilution of a concentrated solution of unfolded BamA in 8 M urea. The dilution was performed with a dispersion of preformed lipid bilayers (LUVs) in borate buffer (10 mM, pH 10) containing 2 mM EDTA (without urea). The final concentrations in the folding reaction mixtures were 6.3 μM BamA and 6.3 mM lipid, i.e., the lipid:BamA molar ratio was 1000. All samples were incubated for 24 h at 40 °C under gentle shaking in a thermo mixer. To remove residual urea, samples were dialyzed against 1 L of 10 mM borate buffer (pH

10) and 2 mM EDTA in a cold room. The buffer was exchanged three times. Folding of TMD-BamA was performed like that of wt-BamA, but for ~48 h. The final concentrations in the folding reactions were 12.4 μM TMD-BamA and 12.4 mM lipid. The folded TMD-BamA samples were dialyzed against HEPES buffer (10 mM, pH 7, with 2 mM EDTA) to remove residual urea. Similarly, FomA membranes were prepared as described.[4] As shown in the Results, this procedure leads to oriented insertion of BamA and FomA.

Separation of Homogeneous BamA/Phospholipid Membranes by Sucrose Density Gradient Centrifugation. Membranes containing inserted and folded BamA were separated from unfolded BamA by sucrose density centrifugation. Linear gradients from 15% to 60% sucrose and from 8% to 40% sucrose were prepared for wt-BamA and TMD-BamA, respectively, in a 25 mL centrifuge tube using a gradient mixer (Universität Konstanz). 0.6 mg of folded BamA in proteoliposomes was layered on top of the gradient and centrifuged at 132000*g* and 8 °C for 4 h. Two layers of different density were obtained after centrifugation and collected separately with a Pasteur Pipette. The BamA and lipid contents of these layers were estimated using the protocols by Lowry et al.[48] and Rouser et al.,[53] respectively. The higher-density layer contained the membrane-inserted and folded BamA. This fraction was dialyzed against 10 mM HEPES buffer (pH 7) containing 2 mM EDTA and its final lipid/BamA ratio was determined.[48,53] Membranes containing folded FomA were separated from unfolded FomA using the same protocol.

Folding of OmpA. To examine the function of BamA or FomA to facilitate folding of OMPs into membranes, folding of OmpA into BamA- or FomA-containing lipid membranes was monitored as a function of time. OmpA was isolated in unfolded form in 8 M urea. BamA/lipid membranes (LUVs) containing either wt-BamA, TMD-BamA, or FomA were prepared as described above. For comparisons in parallel folding experiments, lipid membranes (LUVs) that did not contain protein were prepared as described above. Unless stated otherwise, these lipid bilayers were then also subjected to sucrose density gradient centrifugation followed by dialysis. Prior to OmpA folding experiments, all prepared liposomes and proteoliposomes were again extruded five times through polycarbonate membranes with a 100 nm pore diameter.

Membrane insertion and folding were initiated by rapidly mixing urea-unfolded OmpA with an excess volume of BamA/lipid membranes, or alternatively pure lipid bilayers, in urea free 10 mM HEPES buffer (pH 7) containing 2 mM EDTA. Folding of OmpA from its complex with the chaperone Skp was performed by first adding the unfolded OmpA to urea free buffer containing Skp. Preformed lipid bilayers (LUVs) were then added to initiate folding and insertion. Alternatively, OmpA·Skp complexes were reacted with a solution of LPS in the same buffer, followed by the immediate addition of LUVs. The final concentrations in the folding reaction mixtures were 7.1 μM OmpA and, where present, 28.4 μM Skp and 35.5 μM LPS, corresponding to an OmpA:Skp:LPS molar ratio of 1:4:5. In experiments with diC$_{12}$PC, the lipid:OmpA molar ratio was 300. In experiments with diC$_{12}$PC/diC$_{12}$PE (8:2) membranes, the lipid:OmpA molar ratio was 191. The final urea concentration was 0.66 M in all experiments. Folding kinetics of OmpA were monitored at 40 °C for 120 min after addition of the membranes. Samples were analyzed by SDS–PAGE,[54,55] but without heat denaturation of the samples as described.[26]

Analysis of the Folding Kinetics of OmpA. Experimental time courses were analyzed using the kinetic model described in ref 25. Briefly, based on the observation that unfolded OmpA titrates in an aqueous solution between at least two forms with different fluorescence spectra,[45] the model assumes that OmpA folds via parallel pathways, characterized by slow and fast rate constants. The time dependence of the mole fraction of folded OmpA is then given by

$$X_{FP}(t) = 1 - [A_f \exp\{-k_f t\} + (1 - A_f) \exp\{-k_s t\}] \quad (1)$$

where A_f is the contribution of the faster phase in the last folding stage to the overall folding yield of OmpA and k_s and k_f are the rate constants of the slower and faster folding phases, respectively. In SDS–PAGE, only the final folding step can be monitored, because folding intermediates typically are not stable against exposure to SDS and therefore are usually not observed on gels. The kinetic model for the last folding step also includes the possibility that not all OmpA folds. In this case, $k_s = 0$ and eq 1 can then be transformed to

$$X_{FP}(t) = A_f(1 - \exp\{-k_f t\}) \quad (2)$$

where A_f corresponds to the final folding yield of OmpA and folding kinetics follow a single-step folding phase. All curve fits of eqs 1 and 2 were performed using IGOR Pro version 6.2 (Wavemetrics, Lake Oswego, OR).

CD Spectroscopy. Far-UV CD spectra were recorded using a Jasco 715 CD spectrometer (Jasco, Gross-Ulmstadt, Germany) and a cuvette with a 0.5 mm path length. Six scans were accumulated from 190 to 250 nm with a response time of 8 s, a bandwidth of 1 nm, and a scan speed of 20 nm/min. Background spectra without the protein were subtracted. The concentrations of wt-BamA and TMD-BamA were determined for each sample.[48] The CD spectra were recorded at room temperature and normalized to obtain the mean residue molar ellipticity, $[\Theta](\lambda)$:

$$[\Theta](\lambda) = 100\,\Theta(\lambda)/(cnl) \quad (3)$$

where l is the path length of the cuvette in centimeters, $\Theta(\lambda)$ is the recorded ellipticity in degrees at wavelength λ, c is the concentration in moles per liter, and n is the number of amino acid residues of wt-BamA (790) or TMD-BamA (408). Spectra were analyzed using DICHROWEB (see ref 56 and references therein). For analysis, three deconvolution algorithms, CONTIN,[57] SELCON3,[58] and CDSSTR,[59] were used. For the recorded spectra, application of these three methods resulted in very similar compositions of the secondary structures of BamA and TMD-BamA.

Proteolysis. wt-BamA and TMD-BamA protein (180 μL, 0.26 mg/mL) were incubated with 2.34 μL of trypsin (Fluka) (2 mg/mL) at 37 °C while being gently shaken. Proteolysis was stopped by adding 1 μL of trypsin inhibitor (0.5 mg/mL) at various time points up to 8 h. Samples were analyzed by SDS–PAGE.

RESULTS

BamA and the Separately Isolated TMD of BamA Fold Orientedly into Dilauroylphosphatidylcholine Bilayers. To examine the role of the transmembrane protein BamA in insertion and folding of an unfolded OMP like OmpA, BamA (791 residues, ~88.4 kDa) and TMD-BamA (408 residues, from position 383 to 790; M_r = 45.6 kDa) were first isolated in their unfolded forms in 8 M urea. Conventional reconstitution

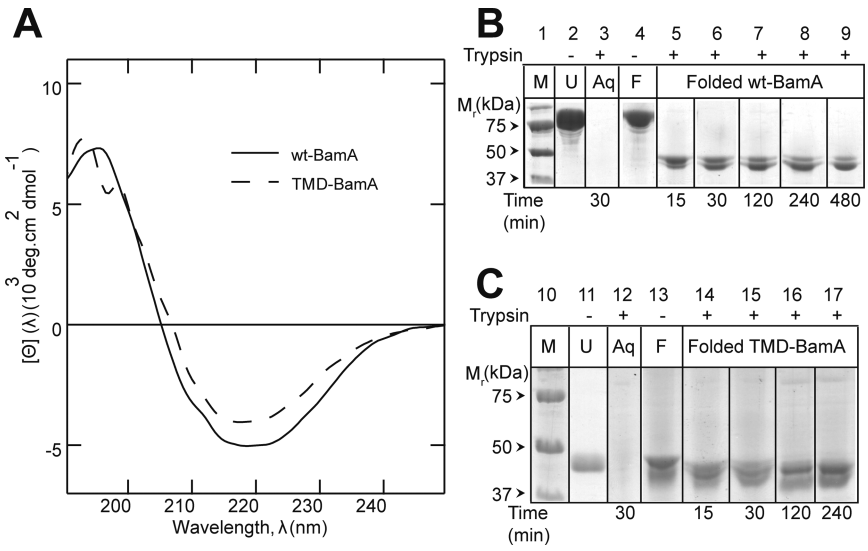

Figure 1. BamA inserts and folds into lipid membranes. (A) Far-UV CD spectra of wt-BamA (—) and TMD-BamA (– – –) obtained after folding into *di*C_{12}PC bilayers indicate predominantly β-sheet secondary structure. (B and C) SDS–PAGE analyses of folding of wt-BamA and TMD-BamA, respectively. Protein standards are shown in lanes 1 and 10. (B) Unfolded wt-BamA migrated at ~88 kDa (lane 2), and aqueous wt-BamA was completely cleaved by trypsin (indicated by a plus) within 30 min (lane 3). Folded wt-BamA (lane 4) migrated like unfolded BamA (lane 2) at ~88 kDa and was cleaved by trypsin into two major fractions, migrating at ~45.5 and ~43.5 kDa. The 45.5 kDa fragment was further cleaved to the 43.5 kDa fragment (lanes 5–9). This fragment of folded wt-BamA remained intact for at least 8 h and corresponded in size to the β-barrel domain, which is protected against proteolysis through its integration into the lipid membrane. (C) Unfolded TMD-BamA migrated at ~46.5 kDa (lane 11), and aqueous TMD-BamA was completely cleaved by trypsin within 30 min (lane 12). Folded TMD-BamA (lane 13) migrated like its unfolded form. Similar to wt-BamA, folded TMD-BamA was cleaved by trypsin into two major fractions, migrating again at ~45.5 and ~43.5 kDa (lanes 14–17). Folded TMD-BamA was protected for at least 5 h.

methods with detergents often result in proteoliposomes, in which the protein is present in two orientations, with the PD inside or outside the liposome.[1] Direct folding of OMPs previously led to the correct orientation with the periplasmic domain outside the vesicles, e.g., as shown by protease digestion for OmpA[1] and hVDAC1.[6] Therefore, BamA was first folded into a 1000-fold molar excess of large unilamellar vesicles (LUVs) of phospholipids by urea dilution. Lipids with different chain lengths were used for bilayer preparations, and the best folding yields of BamA were obtained with *di*C_{12}PC bilayers.

To analyze the homogeneity and yields of wt-BamA and TMD-BamA after folding, sucrose density gradient centrifugation (SGC) was performed. Two layers containing BamA were obtained (not shown). The lower-density layer contained misfolded forms, while the higher-density (HD) layer contained the folded form of either wt-BamA or TMD-BamA, as indicated by circular dichroism (CD) spectroscopy and protease digestion (Figure 1). The CD spectra of the HD layer (Figure 1A), recorded after removal of the sucrose by dialysis, indicated folding of BamA into a β-barrel. The analyses of the spectra[56−59] resulted in ~59% β-sheet and ~10.6% α-helix for folded wt-BamA and ~62% β-sheet and ~7.6% α-helix for folded TMD-BamA. The β-sheet content is slightly larger than that reported for BamA (Omp85$_{Tt}$) from *Thermus thermophilus* isolated in native form. Omp85$_{Tt}$ contained 55% β-sheet and 16% α-helix as determined by CD spectroscopy.[33] The transmembrane β-barrel domain (residues 209–554) of FhaC from *Bordetalla pertussis* is composed of ~66% β-sheet and ~34% random coil, as calculated from the X-ray crystal structure.[60] Interestingly, the analysis of the secondary structure of FhaC by CD spectroscopy[61] reported a content of ~59% β-sheet and 9% α-helix, which matches well with our CD data for wt-BamA.

The BamA β-barrel is far less stable than OmpA, and a shift in its electrophoretic mobility from ~88 kDa to ~55 kDa (folded) was reported only when electrophoresis was performed in a cold room without SDS in the stacking and running gels.[62] Since all SDS–PAGE experiments were performed with standard protocols at room temperature, folding and membrane insertion of BamA were confirmed by proteolysis. The HD-layer obtained after SGC was dialyzed to remove sucrose, treated with trypsin, and then analyzed by SDS–PAGE (Figure 1B). Aqueous wt-BamA (lane 3) was completely cleaved within 30 min, indicating that the PD, which folds in aqueous solution, is cleaved by trypsin. wt-BamA in *di*C_{12}PC membranes (lane 4) was first cleaved into two major fragments, one of ~45.5 kDa and another of ~43.5 kDa (lane 5). The 45.5 kDa fragment was gradually cleaved further to the 43.5 kDa fragment (Figure 1B, lanes 5–9). The size of the cleavage product corresponded to ~49% of the size of the entire wt-BamA, which is consistent with the size of the TMD. This fragment was protected against further trypsinolysis for at least 8 h. A similar SDS–PAGE analysis of the proteolysis of TMD-BamA is shown in Figure 1C. Unfolded TMD-BamA migrated at ~46.5 kDa (lane 11), and aqueous TMD-BamA was completely cleaved by trypsin within 30 min (lane 12). A change in the mobility of TMD-BamA after folding (lane 13) was not observed, but refolded TMD-BamA was cleaved by trypsin into two major fractions. The larger one, migrating at ~45.5 kDa, was gradually cleaved to the smaller ~43.5 kDa fragment (lanes 14–17). This fragment was protected from further proteolysis for at least 5 h. The proteolytic fragments suggested that the TMD-BamA expressed for this study contained the entire membrane-inserted transmembrane

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domain and in addition ~25−30 C-terminal residues of the slightly larger N-terminal PD. Cleavage of either wt-BamA or TMD-BamA resulted in the same two fragments migrating at 43.5 kDa and 45.5 kDa, suggesting that the PD is always completely cleaved, indicating that it is not protease-protected by the lipid bilayer or by the interior of the lipid vesicle. The trypsin digestion experiments shown in panels B and C of Figure 1 therefore indicate an oriented insertion as observed previously for OmpA in similar trypsin digestion experiments.[1] In these membranes, the lipid:protein ratios were ~600 for wt-BamA and ~360 for TMD-BamA, as determined by phosphate[53] and protein assays.[48] The yields of membrane-inserted folded wt-BamA and TMD-BamA were ≥90% as determined by protein estimation.[48]

Bilayer-Inserted BamA Facilitates Insertion and Folding of OmpA. To investigate the impact of BamA on the folding and bilayer insertion of urea-unfolded OmpA, folding experiments with three preparations of lipid membranes were performed in parallel. These were lipid bilayers containing either wt-BamA or TMD-BamA, both prepared as described above, and pure diC$_{12}$PC bilayers (LUVs, 100 nm). The latter were subjected to the same SGC and dialysis steps as the membrane preparations containing BamA. Insertion and folding of OmpA were monitored at 40 °C over 120 min and then analyzed by SDS−PAGE without the samples being boiled prior to electrophoresis, as described in previous studies (reviewed in ref 5). Unfolded OmpA migrates at 35 kDa, while the 30 kDa form strictly corresponds to folded and membrane-inserted OmpA.[5] When BamA was absent, approximately half of the OmpA folded after 30 min (Figure 2A, gel *a*). Up to 20% of the folded and unfolded forms of OmpA migrated as dimers as estimated by densitometry. The fractions of folded OmpA were very similar in the monomeric and dimeric forms. The formation of OmpA dimers at low lipid:OmpA ratios has been reported recently.[63] The SDS−polyacrylamide gels (Figure 2A) and their densitometric analyses (Figure 2B) demonstrated that OmpA folded faster when BamA was present. At a diC$_{12}$PC:OmpA molar ratio of 300 and an OmpA:BamA molar ratio of 2 and within 8 min, ~50% of OmpA was folded (Figure 2A, gel *c*), while only ~15% of OmpA was folded in the absence of BamA at the same diC$_{12}$PC:OmpA ratio (Figure 2A, gel *a*). This indicated a 3−4-fold greater folding rate over this time period in the presence of wt-BamA. At a reduced BamA content, that is at a ratio of OmpA:BamA of 6 (Figure 2A, gel *b*), half of OmpA had folded within 16 min, i.e., in approximately half the time compared to the time required for OmpA folding in the absence of BamA (Figure 2A, gel *a*). The effect of bilayer-inserted BamA on the folding of OmpA therefore depended on the concentration of BamA in the proteoliposomes (see also Table 1A for a kinetic analysis of these and additional experiments). Interestingly, at an OmpA:TMD-BamA ratio of 2 (Figure 2A, gel *d*), TMD-BamA increased the rate of folding of OmpA ~2-fold. This increase in the folding rate was smaller than that observed for full-length BamA at the same OmpA:BamA ratio (Figure 2A, gel *c*).

Membrane-Integrated FomA Affects Folding and Membrane Insertion of OmpA. Integration of a protein like BamA may affect the biophysical properties of the lipid bilayer that are relevant for folding and insertion of TMPs like OmpA. To the best of our knowledge, there have been no previous studies of how the kinetics of folding and insertion of membrane proteins into lipid membranes may be affected by

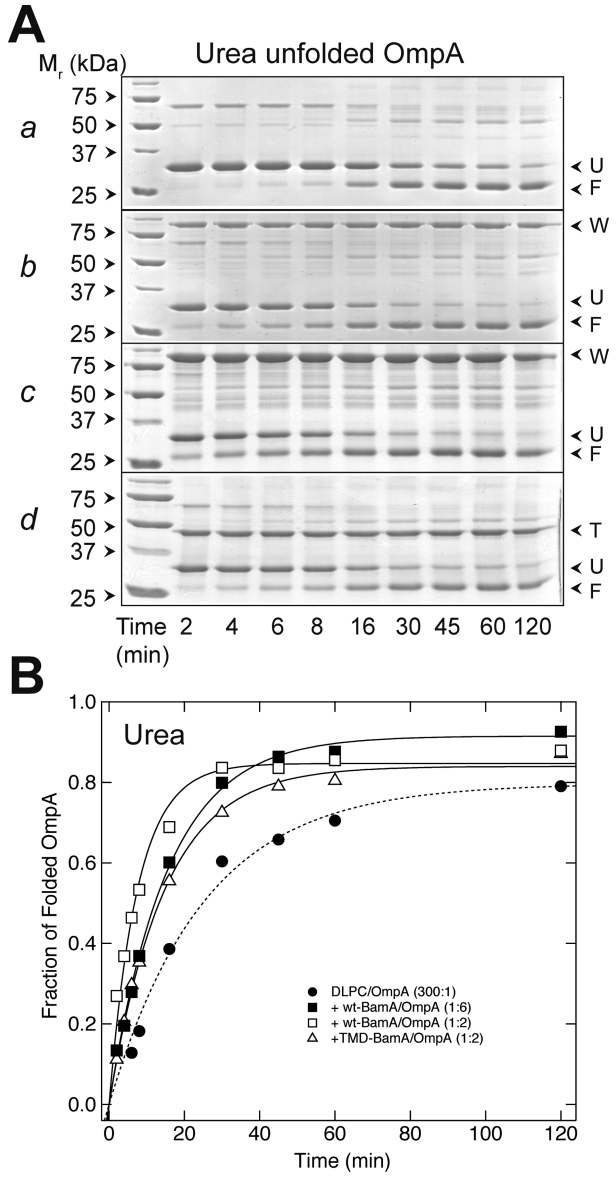

Figure 2. BamA, inserted and folded into diC$_{12}$PC lipid bilayers (Figure 1), facilitates folding and insertion of OmpA. In each experiment, unfolded OmpA in 8 M urea was reacted with a preparation of diC$_{12}$PC membranes under concurrent strong dilution of urea to a final OmpA concentration of 7.1 μM. The diC$_{12}$PC:OmpA ratio was 300:1 at a diC$_{12}$PC concentration of 2.14 mM in all experiments. Four folding experiments were performed in parallel and analyzed by SDS−PAGE and densitometry of the gels. (A) OmpA was folded into pure diC$_{12}$PC bilayers (a), into diC$_{12}$PC bilayers containing wt-BamA at diC$_{12}$PC:wt-BamA ratios of 1800 (b) and 600 (c), and into diC$_{12}$PC bilayers containing TMD-BamA at a diC$_{12}$PC:TMD-BamA ratio of 600 (d). The representative gels show unfolded OmpA (U), folded OmpA (F), wt-BamA (W), and TMD-BamA (T) migrating at ~35, ~30, ~88, and ~45 kDa, respectively. OmpA folding was monitored over 120 min at pH 7 and 40 °C. (B) Analysis of the gels by densitometry. The fraction of the folded monomeric OmpA to the total monomeric OmpA was plotted vs incubation time. Time courses derived from the gels depicted in panel A are shown for folding of OmpA into diC$_{12}$PC bilayers (●), into wt-BamA/diC$_{12}$PC bilayers at diC$_{12}$PC:wt-BamA ratios of 1800 (■) and 600 (□), and into TMD-BamA/diC$_{12}$PC bilayers at a diC$_{12}$PC:TMD-BamA ratio of 600 (△). The solid lines represent single-exponential fits (eq 2) to the experimental data.

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Table 1. Rate Constants of the Kinetics of Folding of OmpA into $diC_{12}PC$ Lipid Bilayers in the Absence and Presence of Bilayer-Inserted BamA[a]

(A) Folding of Urea-Unfolded OmpA into $diC_{12}PC$ Bilayers with or without Inserted BamA

membrane	c_{OmpA} (μM)	c_{lipid} (mM)	c_{BamA} (μM)	OmpA:BamA	k_f[b] (min^{-1})	A_f[c]
lipid only	7.1	2.1	0	–	0.040 ± 0.004	0.79 ± 0.03
lipid with wt-BamA	7.1	2.1	0.4	18	0.047 ± 0.005	0.89 ± 0.03
lipid with wt-BamA	7.1	2.1	1.2	6	0.065 ± 0.002	0.92 ± 0.01
lipid with wt-BamA	7.1	2.1	1.8	4	0.078 ± 0.003	0.91 ± 0.01
lipid with wt-BamA	7.1	2.1	3.6	2	0.132 ± 0.010	0.85 ± 0.02
lipid with TMD-BamA	7.1	2.1	3.6	2	0.068 ± 0.003	0.84 ± 0.01

(B) Folding of Urea-Unfolded OmpA into $diC_{12}PC/diC_{12}PE$ (4:1) Bilayers Containing BamA

membrane	c_{OmpA} (μM)	c_{lipid} (mM)	c_{BamA} (μM)	OmpA:BamA	k_f[b] (min^{-1})	A_f[c]
lipid with wt-BamA	7.1	1.36	1.2	6	0.017 ± 0.005	0.74 ± 0.01

(C) Folding of Skp-Bound OmpA into $diC_{12}PC/diC_{12}PE$ (4:1) Bilayers with or without Inserted BamA

membrane	c_{OmpA} (μM)	c_{lipid} (mM)	c_{BamA} (μM)	OmpA:BamA	k_f[b] (min^{-1})	A_f[c]
lipid only	7.1	1.36	0	–	0.0028 ± 12	0.54 ± 0.03
lipid with wt-BamA	7.1	1.36	7.1	1	0.025 ± 0.001	0.68 ± 0.02

(D) Folding of Skp-Bound OmpA into $diC_{12}PC/diC_{12}PE$ (4:1) Bilayers with or without Inserted BamA in the Presence of LPS

membrane	c_{OmpA} (μM)	c_{lipid} (mM)	c_{BamA} (μM)	OmpA:BamA	k_f[d] (min^{-1})	k_s[e] (min^{-1})	A_f[f]
lipid with LPS	7.1	1.36	0	–	0.15 ± 0.01	0.005 ± 0.001	0.49
lipid with wt-BamA and LPS	7.1	1.36	7.1	1	0.21 ± 0.01	0.010 ± 0.002	0.68

[a]The time courses of folding of OmpA into membranes with different compositions, shown in Figures 2–5, were fit to two kinetic models. In the absence of LPS (parts A–C), a single folding step of first order was used, described by a single-exponential function (eq 2). In the presence of LPS (part D), a kinetic model of two parallel first-order processes was used, described by eq 1. [b]Rate constant of OmpA folding obtained from fits to eq 2. [c]Yield of folded OmpA obtained from fits to eq 2. [d]Rate constant of the faster folding process of OmpA obtained from fits to eq 1. [e]Rate constant of the slower folding process of OmpA obtained from fits to eq 1. [f]Contribution of the faster folding process, obtained from fits to eq 1.

prior incorporation of membrane proteins, which do not have a specific function as a translocon or "insertase". To determine whether the increased folding rates observed in the presence of BamA are a specific effect or a consequence of altered bilayer properties caused by the incorporated protein, the major outer membrane protein of *F. nucleatum*, FomA, served for comparison. FomA, which functions as a porin and has not been implicated in facilitating folding of TMPs, was folded into $diC_{12}PC$ vesicles as described previously.[4] Proteoliposomes were again subjected to SGC and dialysis as described above for BamA. OmpA was then folded either into lipid bilayers containing FomA or into lipid bilayers containing BamA in parallel experiments. The FomA:lipid and BamA:lipid ratios were 900 in these membranes and determined by protein[48] and phosphate[53] assays. Gels *b* and *c* of Figure 3A show folding of OmpA into these membrane preparations, again at lipid:OmpA ratios of 300. The gels indicated that OmpA folds faster into lipid bilayers containing BamA than into lipid bilayers containing FomA (Figure 3A, gels *b* and *c*). Two, four, and six minutes after the initiation of OmpA folding, this effect was largest. Within the first 4 min, folding was more than twice as fast in the presence of BamA than in the presence of FomA. The relative intensities of the bands of the folded form of OmpA relative to the bands of its unfolded form were higher for folding experiments with membranes containing BamA (Figure 3A, gel *c*) than for those with membranes containing FomA (Figure 3A, gel *b*). After 10–15 min, more than half of the OmpA was folded and the differences in the fractions of folded OmpA between the two folding reactions were smaller. In comparison, formation of the folded form of OmpA in pure lipid bilayers (Figure 3A, gel *a*) displayed the slowest time course. In this experiment, the lipid bilayers were not subjected to SGC and dialysis (Figure 3A, gel *a*). As the comparison indicates, SGC followed by dialysis of the pure lipid vesicles led to slightly slower folding kinetics of OmpA (Figure 2A, gel *a*) compared to those of vesicles that were not subjected to these treatments (Figure 3A, gel *a*). This was observed despite the fact that the centrifuged and dialyzed vesicles were again extruded through membranes with a 100 nm pore size after the dialysis. Figure 3B shows the analysis of the time courses by densitometry. This figure also shows the time course of another control experiment of folding of OmpA, performed with a second preparation of pure lipid bilayers, without the SGC and dialysis steps. The major result of this experiment is that preinserted TMPs like FomA, which are not implicated in membrane protein folding, may lead to faster kinetics of TMP insertion and folding in comparison to those of pure lipid bilayers (Figure 3). While the effect of BamA was greater than the effect of FomA (the TMDs are of similar size), it remained unclear whether this difference is caused by specific interactions of OmpA with BamA or simply by differences in the biophysical properties of the two membrane preparations. A different experimental approach will be necessary to address this question in a future study. However, a recent NMR study indicated that peptides of another OMP, PhoE, bind to residues in the PD.[39]

BamA Drastically Reduces the Phosphatidylethanolamine-Induced Inhibition of OmpA Folding. The OM of *E. coli* contains ~80% phosphatidylethanolamine (PE) and ~20% phosphatidylglycerol (PG) and cardiolipin (CL) in its inner leaflet. To examine whether BamA supports membrane insertion of OmpA into lipid membranes containing PE, bilayers composed of $diC_{12}PC$ and $diC_{12}PE$ (4:1) were prepared and BamA was folded into these bilayers at a lipid:BamA ratio of 1000. After SGC, again two layers were obtained (not shown). Circular dichroism spectra and biochemical estimations of the lipid and BamA content[48,53] indicated that ~50% of BamA was present in the folded and bilayer-inserted form in the HD-layer. This was much less than the 92% folded BamA observed after folding of BamA into pure $diC_{12}PC$ bilayers, but consistent with previous observations that PE retards the folding kinetics of OMPs like OmpA.[25] The

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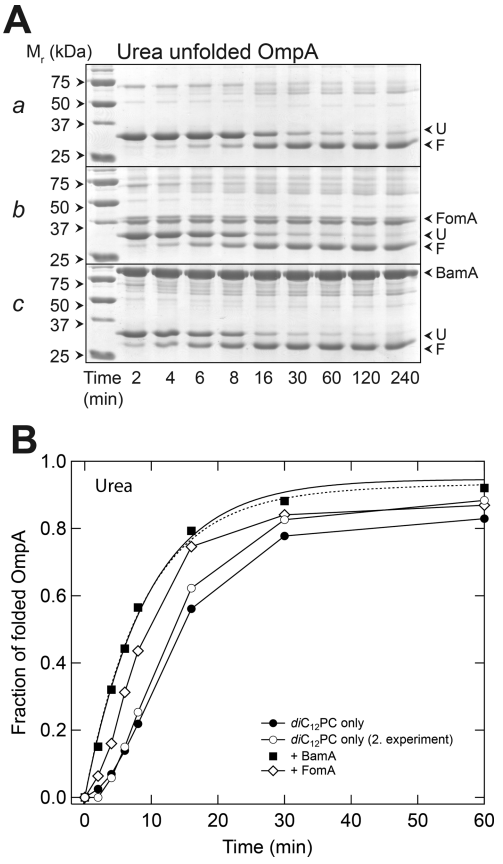

Figure 3. (A) Inserted FomA (gel *b*) and BamA (gel *c*) in *di*C_{12}PC bilayers facilitate folding and insertion of OmpA in comparison to those in pure *di*C_{12}PC bilayers (gel *a*). Urea-unfolded OmpA (U) was rapidly diluted into the preformed membrane preparations to initiate folding. The formation of folded OmpA (F) was monitored over 240 min at pH 7 and 40 °C. The *di*C_{12}PC:OmpA ratio was 300:1 at 2.14 mM *di*C_{12}PC in all experiments, which were performed in parallel. The three folding experiments were analyzed by SDS−PAGE and densitometry of the gels (B). The fraction of the folded monomeric OmpA to total monomeric OmpA was plotted vs incubation time. Time courses derived from the gels depicted in panel A are shown for folding of OmpA into *di*C_{12}PC bilayers (○ and ●), into FomA/*di*C_{12}PC bilayers (◇), and into wt-BamA/*di*C_{12}PC bilayers (■) at an OmpA:wt-BamA molar ratio of ∼3 and an OmpA:FomA molar ratio of ∼3. Equation 2 (—) and equation 1 (···) were only fit to the folding kinetics of OmpA obtained in the presence of BamA (■), as this was the only time course that did not exhibit a visible lag phase.

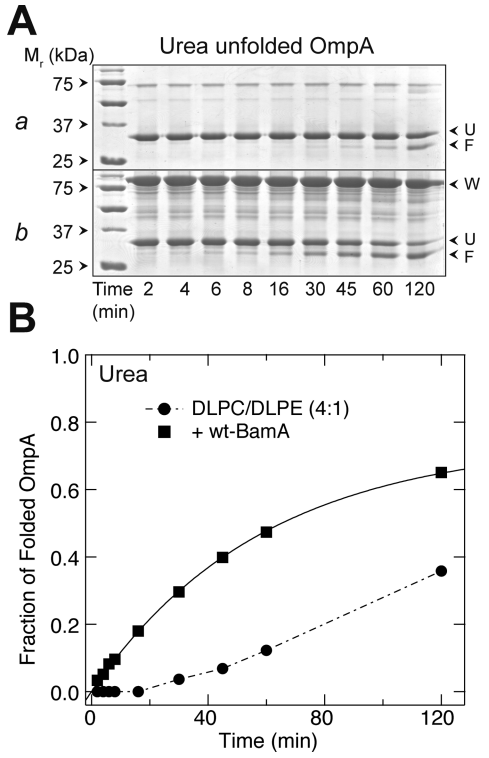

Figure 4. BamA facilitates folding of OmpA into lipid bilayers containing phosphatidylethanolamine, *di*C_{12}PE. Folding of OmpA was initiated by mixing OmpA with bilayers (LUVs) composed of *di*C_{12}PE and *di*C_{12}PC at a molar ratio of 1:4 either without or with bilayer-inserted wt-BamA at a lipid:BamA ratio of ∼191. A molar OmpA:BamA ratio of 1 was selected. The final OmpA concentration was 7.1 μM. Folding of OmpA was monitored over 120 min at pH 7 and 40 °C. (A) SDS−PAGE analyses of the folding of OmpA in the absence (gel *a*) and presence of bilayer-inserted wt-BamA at a BamA:OmpA ratio of 1 (gel *b*). The gels show unfolded OmpA (U), folded OmpA (F), and wt-BamA migrating at ∼35, ∼30, and ∼88 kDa, respectively. (B) Densitometric analyses of the gels shown in panel A were used to plot the time courses of the fraction of folded OmpA in the absence (●) and presence of wt-BamA (■). A single-exponential fit (eq 2) to the time course of OmpA folding into bilayers containing wt-BamA is shown (—). In the absence of wt-BamA, a pronounced lag phase is observed.

lipid:BamA molar ratio of the BamA proteoliposomes was 191, which was lower than the lipid:BamA ratio obtained with pure *di*C_{12}PC bilayers. For comparison, BamA-free bilayers composed of *di*C_{12}PC and *di*C_{12}PE (4:1) were prepared, including the SGC and dialysis steps. The pure lipid vesicles were of low density and floated on top of the sucrose gradient.

To examine the effect of BamA on the insertion and folding of OmpA into BamA-liposomes containing PE, urea-denatured OmpA was folded into *di*C_{12}PC/*di*C_{12}PE (4:1) membranes with and without integrated BamA. Both experiments were performed in parallel at a lipid:OmpA ratio of 191. This ratio was chosen to examine the effect of BamA at an OmpA:BamA ratio of 1. Folding was again monitored by SDS−PAGE (Figure 4A), and the gels were analyzed by densitometry (Figure 4B). After 2 h, ∼36% of OmpA was folded when BamA was absent. This was much less than the 80% of folded OmpA observed in the absence of *di*C_{12}PE (Figure 2A, gel *a*) but larger than the ∼10% of folded OmpA previously observed for *di*$C_{18:1}$PC/*di*$C_{18:1}$PE (7:3) bilayers after incubation for 4 h.[25] Although the lipid:OmpA ratio (191) was also lower than that for pure *di*C_{12}PC (300), the strongly retarded folding kinetics of OmpA confirmed that the PE headgroup inhibits the folding and insertion of OMPs like OmpA and BamA.

The incorporation of BamA into *di*C_{12}PC/*di*C_{12}PE (4:1) membranes dramatically increased both, folding rates and yields of OmpA. A pronounced ∼16 min lag-phase of OmpA folding in the absence of BamA was not observed in the presence of BamA. In the absence of BamA, folding yields of OmpA did not exceed 10% within the first 40 min. In contrast, at least 4 times as much OmpA was folded in the presence of BamA after 40 min (Figure 4A), and ∼65% of the OmpA was folded after 2 h, corresponding to a 1.8-fold increase compared to pure *di*C_{12}PC/*di*C_{12}PE (4:1) membranes (Figure 4). In conclusion, the incorporation of BamA at a lipid:BamA ratio of ∼191 led to faster kinetics of folding of OmpA into *di*C_{12}PC/*di*C_{12}PE (4:1) bilayers at the same lipid:OmpA ratio.

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BamA Facilitates Insertion and Folding of Skp-Bound OmpA. In Gram-negative bacteria, molecular chaperones facilitate the transport of outer membrane proteins across the periplasm (for reviews, see, for example, refs 22 and 64). The Skp trimer forms stable 1:1 complexes with several unfolded OMPs with nanomolar affinity.[45,65] Skp facilitates folding of OmpA into lipid bilayers containing negatively charged phosphatidylglycerol (PG) but inhibits folding into lipid bilayers composed of zwitterionic PC or PE.[25] The addition of small stoichiometric amounts of negatively charged lipopolysaccharide (LPS) also supported folding of OmpA into lipid bilayers from a complex with Skp at LPS:Skp ratios between 1 and 3.[25,26]

The N-terminal periplasmic domain of BamA carries a negative net charge, and the periplasmic part of the entire BAM complex is negatively charged. Since in the absence of negatively charged lipids, Skp inhibited folding of OmpA, it was interesting to examine whether BamA supports folding of OmpA from a complex with Skp into the neutral *di*C_{12}PC/*di*C_{12}PE (4:1) bilayers. Aqueous Skp·OmpA complexes were formed by reacting unfolded OmpA with a 4-fold molar excess of Skp under concurrent strong urea dilution as described previously.[25,26,45] Folding of OmpA was then initiated by mixing these complexes with *di*C_{12}PC/*di*C_{12}PE (4:1) bilayers either with or without bilayer-inserted BamA (Figure 5A, gels *a* and *b*). These experiments were performed in parallel with the folding reactions of urea-denatured OmpA shown in Figure 4. At the same time, fifth and sixth experiments were performed with Skp-bound OmpA in the presence of LPS, either in the absence or in the presence of bilayer-inserted BamA (Figure 5A, gels *c* and *d*). The latter two experiments were included because LPS previously affected kinetics of OMP folding in lipid bilayers[25,26] and in *E. coli*.[66]

Folding of Skp-bound OmpA into *di*C_{12}PC/*di*C_{12}PE lipid bilayers was very slow when BamA and LPS were both absent (Figure 5A, gel *a*), and very little OmpA folded within 120 min. This is consistent with earlier results that showed that Skp inhibits folding of OmpA into *di*$C_{18:1}$PC or *di*$C_{18:1}$PC/*di*$C_{18:1}$PE (7:3) bilayers.[25] In contrast, when Skp-bound OmpA was incubated with BamA/*di*C_{12}PC/*di*C_{12}PE bilayers, more than half of the OmpA folded within 60 min (gel *b*), at a lipid:BamA ratio of 191 and a BamA:OmpA ratio of 1. This demonstrated that BamA interacts with Skp−OmpA complexes at least transiently and facilitates the subsequent insertion and folding of OmpA into the lipid bilayer. In the presence of LPS at a low LPS:OmpA ratio of 5, OmpA folded even faster into lipid bilayers with incorporated BamA (gel *d*).

The gels shown in Figure 5A were analyzed by densitometry (Figure 5B). The kinetics of OmpA folding from a complex with Skp into *di*C_{12}PC/*di*C_{12}PE bilayers in the absence of BamA (●) led to only ~15% of folded OmpA after 120 min. This was less than half the yield obtained for urea-unfolded OmpA in the absence of Skp under otherwise identical conditions (Figure 4B). When BamA was present in the membrane, the folding yields of Skp-bound OmpA were greatly improved to ~66% (■). When Skp-bound OmpA was first mixed with a 5-fold molar excess of LPS and then incubated with *di*C_{12}PC/*di*C_{12}PE (4:1) bilayers in the absence of BamA, similar folding yields were obtained, but with faster kinetics (◆). Folding of OmpA from a complex with Skp was fastest for *di*C_{12}PC/*di*C_{12}PE (4:1) bilayers containing BamA in the presence of LPS (▲), leading to more than 90% folded OmpA. In this case, the half-time of OmpA folding was only ~5 min (Figure 5B). Interestingly, the effects of BamA and LPS were additive.

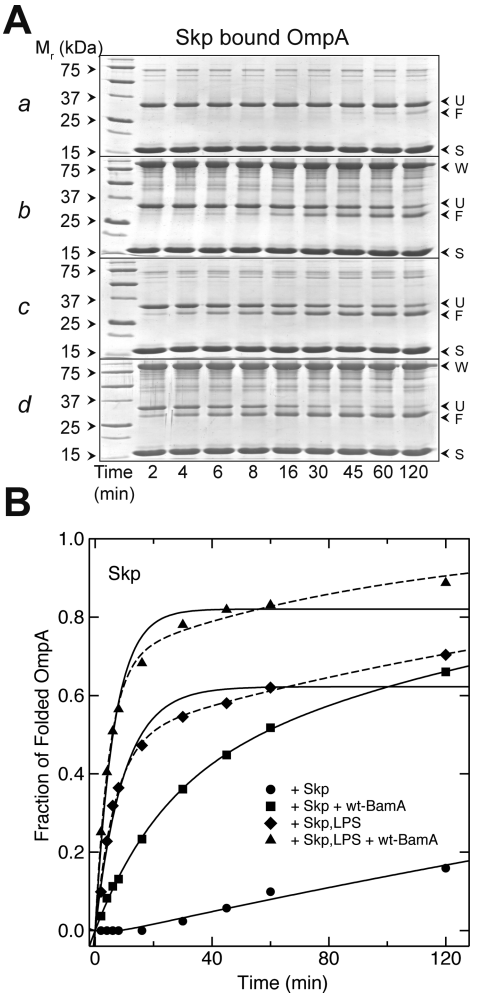

Figure 5. Bilayer-inserted BamA facilitates folding of OmpA from complexes with the periplasmic chaperone Skp. (A) OmpA was first reacted with a 4-fold molar excess of Skp under a strong dilution of the urea and then folded from its complex with Skp into *di*C_{12}PC/*di*C_{12}PE bilayers (4:1) either in the absence (gel *a*) or in the presence (gel *b*) of membrane-inserted BamA. In two additional experiments, a 5-fold molar excess of LPS was added to Skp·OmpA complexes, and samples were then incubated with *di*C_{12}PC/*di*C_{12}PE bilayers (4:1) in the absence (gel *c*) and presence of BamA (gel *d*). The gels show Skp (S) at ~17 kDa, unfolded OmpA (U) at ~35 kDa, folded OmpA (F) at ~30 kDa, and wt-BamA (W) at ~88 kDa. The final concentrations in the reaction mixtures were 7.1 μM OmpA, 1.36 mM lipid (191 lipids/OmpA), 28.4 μM Skp, and, where present, 7.1 μM BamA and 35.5 μM LPS. (B) Analysis of the gels by densitometry. The time courses are shown for folding of Skp-bound OmpA into pure *di*C_{12}PC/*di*C_{12}PE bilayers (4:1) (●) or *di*C_{12}PC/*di*C_{12}PE bilayers (4:1) with inserted BamA (■) and for Skp-bound OmpA that was first incubated with LPS and then folded into either pure lipid bilayers (◆) or bilayers containing BamA (▲). Single-exponential (—) or double-exponential (---) functions of the form of eq 2 or 1, respectively, were fit to the experimental data.

■ DISCUSSION

To investigate the impacts of TMPs on the folding and insertion of β-barrel membrane proteins, we established a model system. This system was composed of a lipid bilayer with a defined composition and an orientedly inserted TMP, like

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BamA or FomA. While FomA functions as a porin, BamA is known as the central component of the BAM complex. Several new observations were made. (1) Both wt-BamA and TMD-BamA folded and inserted with a defined orientation into lipid membranes, indicating that the oriented insertion is part of the folding of the β-barrel into the lipid bilayer. (2) When lipid bilayers contained preinserted β-barrel membrane proteins like wt-BamA, TMD-BamA, and FomA, the folding and insertion of OmpA were faster in comparison to those of pure lipid membranes. (3) wt-BamA, which contains five polypeptide transport-associated (POTRA) subdomains in its PD, showed the strongest acceleration of folding and insertion of OmpA, supporting a catalytic role for the PD. (4) BamA facilitated folding of Skp-bound OmpA into BamA/*di*C_{12}PC/*di*C_{12}PE membranes, indicating that wt-BamA interacts with Skp–OmpA complexes and mediates OmpA folding and insertion, which in the absence of BamA is strongly retarded by *di*C_{12}PE (this study) and *di*$C_{18:1}$PE.[25] Furthermore, the established model membranes containing BamA may also provide a basis for examining the formation of the BAM complex at the membrane–water interface by sequential addition of the participating lipoproteins, BamB, BamC, BamD, and BamE, in their post-translationally modified forms.

Integrated TMPs Accelerate the Kinetics of Insertion and Folding of OmpA into Lipid Bilayers. The incorporation of either TMD-BamA or FomA into lipid bilayers leads to faster insertion and folding of OmpA. Because FomA does not share sequence homology with BamA and has never been implicated to have a role in facilitating insertion and folding of β-barrel membrane proteins, nonspecific effects of preinserted proteins must be considered in studies of the function of protein assembly machineries. Inserted proteins perturb the structure of the lipid bilayer. Therefore, comparisons with pure lipid bilayers to demonstrate the function of the proteins of the BAM complex must be made with caution. Our data show only small differences between TMD-BamA and FomA regarding their effects on the insertion and folding of OmpA. A requirement of the TMD for the folding of OMPs cannot be completely ruled out as the stronger effect seen for wt-BamA compared to that for TMD-BamA may still depend on the connection of the PD of BamA with its TMD. In previous work, the β-barrel of BamA could not simply be replaced by the β-barrel of OmPlA.[41] However, OmPlA might not have been the best replacement for the TMD of BamA as it forms dimers while most current observations suggest that BamA is a monomer. Dimer formation of an OmPlA/PD–BamA fusion construct could have interfered with the formation of the BAM complex as the differences between the TMDs of BamA and OmPlA may also have an impact on the binding of the lipoproteins in the complex.

The PD of BamA Contributes to the Kinetics of Folding of OmpA into Membranes. The data shown in Figure 2 indicate that the PD facilitates folding of OMPs, consistent with a recent report that BamA directly binds OMPs via their C-terminal signature sequence[62] and another study showing that peptides derived from the OMP PhoE of *E. coli* bind to POTRA 1 and 2 of BamA.[39] In cells, this effect of the PD may be stronger as previous work suggested the BAM complex is stabilized by its lipoproteins.[35,67] The loss of BamB, BamC, or BamE also led to a decrease in the concentrations of OMPs like LamB and OmpA in the OM.[27,68,69] It appears to be possible that each protein of the BAM complex incrementally contributes to the faster kinetics of folding of OMPs into the OM, although only BamA and BamD are essential in *E. coli*.

wt-BamA Reduces the Effect of PE as an Inhibitor for the Folding and Insertion of β-Barrel TMPs. BamA partially canceled the inhibition of OmpA folding by *di*C_{12}PE and eliminated the lag phase (Figures 2 and 4). This alone may not be sufficient for OMP folding in cells, as the OM contains up to 80% phosphatidylethanolamine (PE). Interestingly, the lipoprotein BamE (SmpA) was recently shown to bind to phosphatidylglycerol (PG),[70] a phospholipid of the inner leaflet of the outer membrane that supports folding of OmpA.[25] Another study indicated that PE and PG are segregated into different domains in bacterial membranes.[71] It is possible that the BAM complex is part of a membrane domain rich in PG. Both integral[44,72] and peripheral membrane proteins[73] display lipid selectivity, which could explain the formation of these domains. Folding of OMPs into a PG-enriched domain would likely be much less retarded than folding into homogeneous PE/PG lipid bilayers.

The Kinetics of Folding of OmpA into Membranes Containing BamA Determined by Electrophoresis Are First-Order Kinetics. A kinetic model[25] was fit to the time courses obtained by densitometry (Figures 2–5). In most cases, the kinetics followed a single-exponential time course with two free fit parameters (eq 2), the rate constant and the folding yield (Table 1). This model is consistent with previous results for folding of OmpA into *di*$C_{18:1}$PC bilayers at neutral or basic pH[25] and with the pseudo-first-order kinetics observed for a range of short-chain phosphatidylcholine lipids.[2] For folding of urea-denatured OmpA into *di*C_{12}PC bilayers, wt-BamA increased the rate constant >3-fold, from 0.04 to 0.132 min^{-1} at a *di*C_{12}PC/wt-BamA ratio of 600 (Table 1), which is consistent with the half-times of folding observed on the gels (Figure 2A, gels *a* and *c*). At the same lipid and OmpA concentrations, the rate constant of OmpA folding increased with BamA content from 0.047 min^{-1} at a *di*C_{12}PC:wt-BamA ratio of 5400 to 0.132 min^{-1} at a *di*C_{12}PC:wt-BamA ratio of 600. In comparison, TMD-BamA also facilitated folding of urea-unfolded OmpA, but only with a rate constant of 0.068 min^{-1} at a *di*C_{12}PC:TMD-BamA ratio of 600, which is still ~70% faster than folding of OmpA into *di*C_{12}PC membranes in the absence of BamA. Folding intermediates of β-barrel membrane proteins are not as stable as the fully folded β-barrels and thus cannot be distinguished from unfolded forms by cold SDS–PAGE. Similarly, less stable but natively folded β-barrel membrane proteins like VDAC cannot be distinguished from their unfolded forms by cold SDS–PAGE.[6] Therefore, our experiments indicated only the last folding step, leading to a stable β-barrel. Double-exponential fit functions with three free fit parameters (eq 1) were obtained only in the presence of LPS (Figure 5 and Table 1D) and indicated two parallel last folding processes as reported for FomA[4] and for OmpA in either negatively charged bilayers[25] or the negatively charged amphipol A8–35.[11] These could be originating from coexisting aqueous conformations of OmpA observed by fluorescence in the titration regions of the basic side chains of OmpA[45] as double-exponential kinetics were also observed in the absence of BamA [Figure 5B (◆)]. The rate constants obtained from the fits (Table 1D) suggest that both the slower phase and the faster phase are accelerated by the presence of BamA, which also increases the contribution of the faster phase, A_f.

BamA-Mediated Folding of OmpA from Its Complex with the Periplasmic Chaperone Skp. Skp forms stable

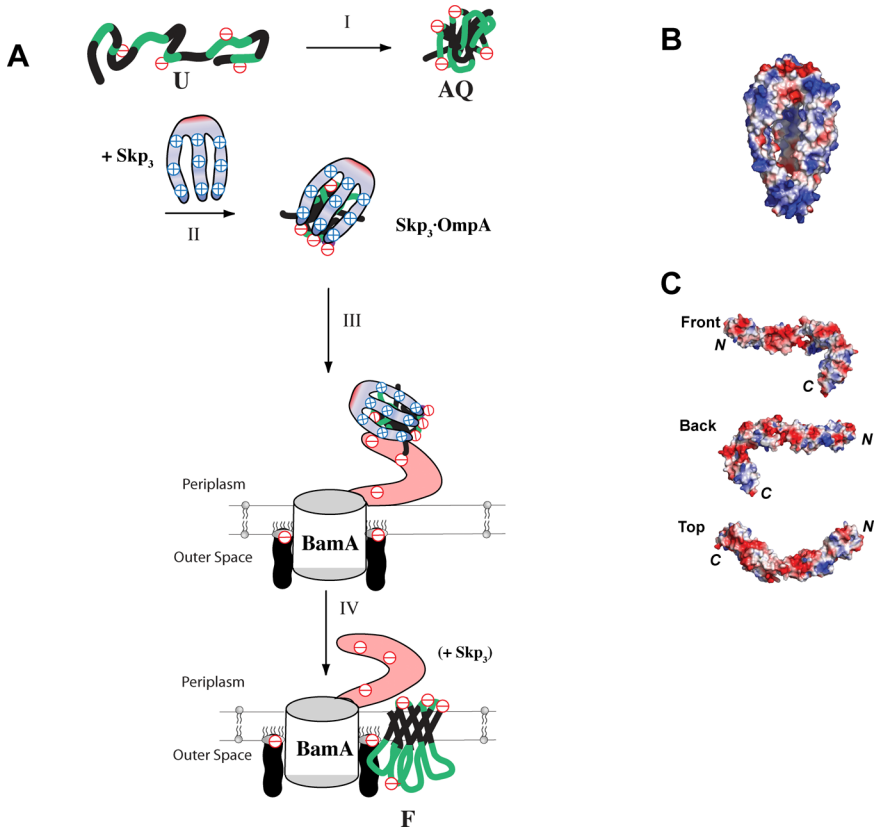

Figure 6. (A) Tentative scheme for the interactions of Skp_3·OmpA complexes with BamA. (I) Unfolded OmpA in 8 M urea (U) is a random coil and negatively charged above its pI of ~5.5. Polar segments are colored green and transmembrane segments black. Upon removal of urea, OmpA collapses into a compact aqueous form (AQ) with charged or polar residues on the surface and hydrophobic residues oriented to the inside.[65] (II) Positively charged Skp_3 and aqueous negatively charged OmpA form stable complexes (Skp_3·OmpA).[26,45] Skp_3 preserves OmpA in a folding competent form. (III) Skp_3·OmpA complexes may interact with BamA, releasing OmpA from its complex with Skp and facilitating its folding into the membrane (IV). (B) Crystal structure of the Skp trimer (PDB entries 1sg2 and 1u2m).[75,76] The trimer forms a large dipole, and the positive residues cover the surface of the Skp tentacles down to their tips. (C) Crystal structure of the periplasmic N-terminal domain (residues 23−347) of BamA (PDB entry 3efc) of *E. coli*. The surface of this domain contains several patches of two or more acidic residues. The domain extends from the membrane surface into the periplasm and may bind Skp_3·OMP complexes transiently through electrostatic interactions.

complexes with aqueous forms of unfolded OMPs at nanomolar affinity[45] (Figure 6, steps I and II). Folding of OmpA from these complexes into bilayers of zwitterionic lipids like PC and PE is inhibited,[25] while negatively charged PG or LPS[25,26] facilitate folding and insertion. The study presented here indicates that BamA supports OmpA folding from its complex with Skp into membranes of PE and PC, i.e., in the absence of negatively charged lipids (Figure 5). Inspection of the structure of the PD of BamA [Protein Data Bank (PDB) entry 3efc[37,38]] indicated that this domain contains several patches of two or more negatively charged residues and is overall negatively charged (Figure 6C). These patches may serve as interaction sites for positively charged residues of the Skp trimer, which is highly basic (Figure 6, step III). The tip region of the Skp (Figure 6B) tentacle domain alone has a calculated net positive charge of +15. Electrostatic interactions between the PD of BamA and Skp may facilitate the release of the negatively charged OmpA from its complex with Skp into the membrane (Figure 6, step IV).

Interestingly, it has been suggested that the highly negatively charged BamB may bind to positive residues in POTRA 3 of BamA through electrostatic interactions,[67] thereby increasing the negative net charge of the BAM complex. Here, addition of negatively charged LPS further increased the folding rates of OmpA [Figure 5 (▲)]. The negative charges of the PD of BamA alone may be only one factor in maximizing the rates of dissociation of OmpA from its complex with Skp in the periplasm. The periplasmic part of the entire BAM complex has a negative surface potential, and above pH 5.5, lipoproteins BamB, BamC, and BamD of *E. coli* are all negatively charged while BamE has a pI of ~7. Binding of OMPs like OmpA to Skp and the release of OMPs from Skp into lipid membranes involve electrostatic interactions.[25,45] In the absence of other negatively charged membrane components, like PG or the BAM lipoproteins, BamA therefore mediates folding and insertion into lipid membranes of PC and PE (Figure 6, step IV). The possibility that BamA is not the primary interaction partner for Skp cannot be excluded, because small stoichiometric amounts of LPS show a much stronger effect on the folding kinetics of OmpA than on those of BamA (Figure 5). BamA and BamD may both be required for a more efficient release of OmpA from its complex with Skp as the other lipoproteins are not essential in *E. coli*. Because Skp is also interacting with OMPs near the cytoplasmic membrane,[74] it may directly transport OMPs from the inner membrane to the BAM complex.

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AUTHOR INFORMATION

Corresponding Author

*Universität Kassel, Abt. Biophysik, FB 10 Heinrich-Plett-Str. 40, D-34132 Kassel, Germany. Telephone: +49-561-804-4041. Fax: +49-561-804-934041. E-mail: jhk@uni-kassel.de.

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ABBREVIATIONS

BAM, barrel assembly machinery; BamA, barrel assembly machinery protein A; CD, circular dichroism; *di*C_{12}PC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; *di*C_{12}PE, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine; FomA, major outer membrane protein from *F.nucleatum*; HD, high-density; IM, inner membrane; IPTG, isopropyl 1-thio-β-D-galactopyranoside; LB, Luria-Bertani; LD, low-density; LPS, lipopolysaccharide; LUVs, large unilamellar vesicles; OM, outer membrane; OMP, outer membrane protein; OmpA, outer membrane protein A of *E. coli*; PD, periplasmic domain; PG, diacyl-*sn*-glycero-3-phosphoglycerol; POTRA, polypeptide transport-associated; SGC, sucrose density gradient centrifugation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Skp, 17 kDa protein; SurA, survival factor A; TMP, transmembrane protein; wt-BamA, wild-type barrel assembly machinery protein A; TMD, transmembrane domain; TMD-BamA, transmembrane domain of BamA.

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