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From Chaperones to the Membrane with a BAM!

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Abstract

Outer membrane proteins (OMPs) play a central role in the integrity of the outer membrane of gram-negative bacteria. Unfolded OMP (uOMP) transit across the periplasm and subsequent folding and assembly are key for cellular biogenesis. Chaperones and the essential BAM complex facilitate these processes. *In vitro* studies suggest that some chaperones sequester uOMPs in internal cavities during their periplasmic transit to prevent deleterious aggregation. Upon reaching the outer membrane, the BAM complex acts catalytically to accelerate uOMP folding. Complementary *in vivo* experiments have shown the localization and activity of the BAM complex in living cells. Completing an understanding of OMP biogenesis will require a holistic view of the interplay amongst the individual components discussed here.

Keywords

Outer Membrane Proteins; Chaperones; BAM complex

Outer membrane protein assembly is a prerequisite for membrane integrity

The outermost membrane of gram-negative bacteria is the ultimate protective barrier of the cell, serving as the first line of defense that guards against extracellular threats. Composed of both lipids and thousands of **outer membrane proteins (OMPs)** (See Glossary), biogenesis of **outer membrane (OM)** components and consequent OM integrity is essential for cell viability [1]. Targeting these processes is a promising route for directed drug design against bacterial pathogens. Understanding of the OMP assembly machinery in *Escherichia coli* has grown immensely due to recent discoveries using several orthogonal techniques that include the publications of crystal structures of key proteins in the past 12 months. In this Review, we highlight recent discoveries involving the roles of chaperones and OM-localized folding factors in OMP assembly, as understanding the underlying mechanisms for these processes will facilitate their efficient therapeutic targeting.

β -barrel OMPs take a long and winding road to their native environment

Typical OMPs share a β -barrel folded topology composed of a closed cylinder of antiparallel β -strands. The hydrophilic interiors of OMP β -barrels are often water-accessible, while the exteriors are hydrophobic and lipid-exposed. Adjacent β -strands interact through hydrogen

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bonding and are covalently connected by short periplasmic turns and longer extracellular loops. Accurate OMP biogenesis is a prerequisite to the formation of this native, functionally active, conformation.

The biological assembly pathway of OMPs is particularly challenging because these polypeptides must cross multiple compartments in an unfolded form to reach the OM (Figure 1, Key Figure). OMPs are synthesized by ribosomes in the cytoplasm of gram-negative bacteria. The open reading frames of OMP sequences contain an N-terminal signal sequence that targets these newly translated chains to the **SecYEG** (Teal in Figure 1) translocon via interactions with **SecB** [2]. Subsequently, **unfolded OMP chains (uOMPs)**, Red in Figure 1) are secreted across the bacterial inner membrane through SecYEG using the energy of the **SecA** motor [3]. After entry into the periplasm, uOMPs remain unfolded until they reach the asymmetric outer membrane into which they fold (Figures 1 and 2). It is critical to note that uOMPs require a lipid bilayer to fold into their native conformation, which means that they must traverse the 165 Å aqueous periplasm in an unfolded, yet folding-competent state [4]. Herein lies a dilemma: uOMPs are prone to aggregate in aqueous environments [5,6] and the thermodynamically favorable, yet kinetically slow process of uOMP aggregation directly competes with the productive folding pathway of uOMPs. This potential dead end fate for an unfolded OMP is avoided *in vivo* by the presence of periplasmic chaperones. This issue of uOMP aggregation is confounded by the fact that the periplasm lacks ATP [7]. Therefore, periplasmic chaperones must prevent uOMP aggregation and its associated cell stress in the absence of an external energy source. To accomplish this important cellular feat, the thermodynamics and kinetics of chaperone-uOMP interactions must be fine-tuned to maintain uOMP proteostasis in the absence of ATP [8–11].

How do periplasmic chaperones safeguard uOMPs without an external energy source?

One mechanism periplasmic chaperones employ to prevent aggregation is to protect uOMPs by sequestering them within a defined internal uOMP- cavity. **Seventeen-kilodalton protein (Skp)**, light green in Figure 1) and the serine endoprotease **DegP** (cyan in Figure 1) are both oligomeric chaperones with defined internal binding cages that accommodate uOMPs (Figure 1) [12–14]. Most structural studies have focused on uOMP-Skp interactions [8,9]. In the apo (i.e. uOMP free) state, three α -helical Skp polypeptide chains associate to form a trimeric moiety with an internal cavity [15,16]. It was shown via fluorescence-based approaches that the uOMP client initially contacts the Skp trimer via the positively charged monomer tips [17]. **Nuclear Magnetic Resonance (NMR)** analyses suggest that the uOMP has high mobility and makes many weak local interactions within the internal cavity of Skp [18]. These numerous contacts sum to yield nanomolar dissociation constants for binding between Skp and uOMP clients [8].

Small-angle Neutron Scattering (SANS), See Glossary) experiments have demonstrated that the α -helical arms of Skp are flexible to accommodate the unstructured uOMP [19]. Structural modeling of uOMP bound to Skp revealed that the uOMP is not entirely

encapsulated but protrudes from the Skp cavity (Figure 3). In addition, modeling of Skp bound to a uOMP with a folded soluble domain suggests that uOMPs bind to Skp as an ensemble with the soluble OMP domain in a variety of orientations outside of the Skp trimer. Overall, it seems that both the bound uOMP and Skp exist in multiple conformations in the complex, all of which shield the uOMP from the aqueous periplasm and prevent deleterious uOMP aggregation. This type of uOMP capturing mechanism has also been observed for the periplasmic chaperone/protease DegP, which binds uOMPs in an internal cavity as evidenced by cryo-electron microscopy [14]. Whereas Skp is a functional trimer, DegP can form a holo-complex composed of up to 24 monomers. Interestingly, this oligomeric state is likely large enough to span the entire periplasm (Figure 1).

The second mechanism for safeguarding uOMPs from aggregation involves chaperones that are not known to form defined cages. The periplasmic prolyl-isomerases **survival protein A (SurA, magenta in Figure 1)** and **FkpB binding protein A (FkpA, yellow in Figure 1)** both lack internal cavities but are still known to bind uOMPs (Figure 1) [20–23]. It is currently unclear how these chaperones bind to uOMP clients to prevent their aggregation, although the stoichiometry of binding of SurA to one uOMP client (i.e. FhuA) was determined to be 2 SurA:1 uOMP [24]. Recent studies have suggested that both SurA and FkpA may exist in a variety of conformations that play a role in uOMP binding. Crosslinking and genetic experiments suggest that the two parvulin domains of SurA explore several conformations and these structural changes mediate SurA activity by modulating populations of “open” and “closed” SurA states [25]. Flexibility in one of the N-terminal helices of FkpA may allow this chaperone to exist in many uOMP-binding conformations [26]. Future work will be required to gain more detailed structural insight into how SurA and FkpA bind to uOMPs in a manner that prevents aggregation without having defined internal cavities and in the absence of ATP.

A view of the periplasmic OMP biogenesis pathway to scale (Figure 1) makes it tempting to suggest an additional rationale for the lack of a requirement of external energy for uOMP transport across the periplasm. As shown in Figure 1, the width of the periplasm is on the same length scale as the size of several relevant chaperone proteins. With thermal energy driven diffusion, chaperone-uOMP complexes may easily make the short journey from the IM to the OM.

In addition to binding uOMPs and consequently preventing off-folding-pathway aggregation, the periplasmic chaperones Skp and SurA have also been implicated in functioning in the folding pathway of uOMPs [27–30]. Studies have suggested that SurA binds to the majority of uOMP flux through the periplasm, with known clients including OmpA, LptD, and FhuA via recognition of aromatic motifs [31–34]. Proteomics studies have identified Skp clients as OmpA, LamB, and BtuB, among other periplasmic proteins [35]. The recent application of single molecule atomic force microscopy (AFM-SMFS) has parsed out the effects of both chaperones on the folding pathways of uOMPs. It was observed that Skp prevents the misfolding of a representative uOMP, while SurA promotes reinsertion of native β -hairpins into the bilayer. The unique roles of these chaperones in the folding pathways of uOMPs may stem from differences in the structural manner in which they bind uOMPs, as Skp has an internal cavity and SurA does not. Interestingly, the

reported fast kinetics of chaperone:uOMP binding and unbinding suggest that these complexes are short-lived and dynamic in nature [9]. The mechanistic relationship between chaperone-uOMP structure, binding kinetics, and chaperone roles in uOMP folding pathways is currently an active area of research.

The BAM complex: an enzyme that catalyzes uOMP folding

Once uOMPs complete the journey across the periplasm, they must fold into the OM. The OM-localized β -barrel BamA protein is an evolutionarily conserved protein from bacteria to eukaryotes that plays an essential role in uOMP assembly [36]. In *E. coli*, BamA associates with four lipoproteins: BamBCDE to form the β -Barrel Assembly Machinery complex (**BAM**, See Figure 1 and Glossary) [37–40]. While each of these accessory lipoproteins has been implicated in maintaining OM integrity, only BamA and BamD are required for cell viability [39,41]. The essentiality of the BAM complex likely stems from its role in the assembly of uOMPs into the bacterial OM.

The composition of the OM of *E. coli* plays a crucial role in BAM-mediated uOMP folding. *In vivo* uOMP folding occurs into the inner leaflet of the outer membrane, which contains lipids with both **phosphoethanolamine (PE**, See Glossary) and **phosphoglycerol (PG**, See Glossary) head groups [42,43]. This is consequently also similar to the composition of the bacterial IM. Paradoxically, these biological head groups retard uOMP folding *in vitro*, creating a pronounced kinetic barrier for unassisted uOMP folding [44]. This kinetic barrier may effectively prohibit uOMP folding into the IM because uOMP folding into the IM would certainly lead to dissipation of the proton gradient essential to sustain cell viability. Yet for uOMP folding into the OM, the BAM complex must overcome this same kinetic barrier - another remarkable feat that must be completed in the absence of an external energy source. Many recent studies have aimed to understand this process.

Because the biologically functional *E. coli* BAM complex contains five proteins, *in vitro* experiments have aimed to deconvolute the effects of these components by studying the individual BAM proteins in isolation. *In vitro* studies of BamA have revealed that this BAM component alone can accelerate uOMP folding, albeit slower than the entire BAM complex [44,45]. Because uOMPs spontaneously insert into synthetic lipid bilayers *in vitro* and BamA accelerates this process, BamA exhibits properties of an enzyme in that it reduces the activation barrier to uOMP folding into biological membranes. The recognition of uOMP substrates by BamA likely involves the conserved C-terminal aromatic signature of uOMP sequences because removal of this sequence inhibits BamA-assisted folding *in vitro* [44]. Still, the apparent K_m for this catalytic process was found to be high ($>20 \mu\text{M}$) suggesting the possibility that thermodynamically weak interactions occur between BamA and the uOMP client [11,44]. These results raise the question: how can BamA accelerate uOMP folding if it only weakly interacts with the uOMP? This conundrum can be addressed by realizing that these weak protein-protein interactions are only one facet of the role of BamA. BamA-accelerated uOMP folding is in fact a second order process, because BamA interacts with both the uOMP client and the surrounding lipid membrane. It is entirely plausible that BamA promotes the formation of a membrane defect. Because such membrane inhomogeneities are known to accelerate uOMP folding, this has been suggested as one

possible catalytic mechanism for uOMP-BAM mediated folding [46]. Such a membrane defect is poorly defined structurally but may be represented by a hydrophobic mismatch between the surrounding lipid bilayer and the BamA β -barrel (Figure 4) [11,47]. Together, both biochemical and structural findings suggest that BamA and the surrounding lipid bilayer work together to facilitate uOMP folding.

Comparisons between the activity of BamA alone and the BAM complex have yielded similarities in the catalytic activity: both BamA and the entire BAM complex have been shown to facilitate multiple rounds of catalysis with turnover numbers in both cases equal to approximately 1.5 [48,49]. These findings suggest that BamA itself undergoes a cyclic catalytic mechanism that is accelerated *in vivo* by the additional lipoproteins BamBCDE. Current studies are focusing on understanding how the different components of the BAM complex collaborate with the membrane to facilitate uOMP folding *in vitro* and biogenesis *in vivo*. The most recent progress towards these goals has been driven by the publication of several crystal structures of BamA alone and the BAM complex.

Caught in the act? BamA β -barrel has a unique seam

In 2013, the first crystallographic structure of a full-length bacterial BamA was solved [47]. This structural model contains the two domains of BamA: one transmembrane 16-stranded β -barrel domain and a series of five soluble **P**OLYPEPTIDE-**T**RANSport-**A**SSOCIATED (**POTRA**) motifs (Figure 5). Each of these structural subunits has unique features that may contribute to the catalytic ability of BamA to accelerate uOMP folding. In particular, the β -barrel domain structure revealed a surprising result: instead of exhibiting a geometry that maximizes the number of hydrogen bonds between the N- and C-terminal β -strands, the crystal structure of the *Neisseria gonorrhoeae* BamA revealed that the N- and C-terminal β -strands of the β -barrel interact with only two hydrogen bonds to close the β -barrel, and the C-terminal β -strand is twisted and bends into the β -barrel. This “open” β -barrel conformation has since been observed in crystallographic studies of *E. coli* BamA [50].

The non-canonical β -barrel seam of the *N. gonorrhoeae* BamA prompted the investigation of the role of movement of the N- and C-terminal β -strands in the function of this BamA homologue. Indeed, **molecular dynamics (MD)** (See Glossary) simulations of the *N. gonorrhoeae* BamA in dimyristoyl-glycero-3-phosphatidylethanolamine gel-phase lipids suggest that β -strands 1 and 16 laterally open in the absence of uOMP client (Figure 4) [47]. Crosslinking experiments that covalently link β 1 to β 16 of *E. coli* BamA were found to be detrimental to cell viability, suggesting that lateral opening of these β -strands may play a pivotal role in the function of BamA [51].

Lateral opening: The new old functional mechanism for β -barrels

Lateral opening was first proposed as a functional mechanism for the OMP PagP over 10 years ago [52]. Since then, several OMPs have been suggested to laterally open (e.g. TamA, PagP, FadL, OmpW, LptD) [52–56]. These β -barrels range in size from 8 to 16 β -strands and have a variety of substrates, including phospholipids and **lipopolysaccharide (LPS)**. The unique crystallographic evidence for the open conformation of the BamA β -barrel differs

from the previous structural and biochemical data for other OMPs that have functional mechanisms involving lateral opening. Interestingly, the potential similarities for insertion of LPS and OMPs by lateral opening of LptD and BamA, respectively, suggests the two major components of the OM may be assembled by similar mechanisms. The observation that BamA laterally opens has prompted the suggestion that BamA provides a template to assist uOMPs in folding [53,57], however no experimental evidence has been published to date that supports this mechanistic interpretation. Additionally, BamA is unlikely to provide conformational instructions for uOMP folding, as uOMPs can attain their native folded states in the absence of BamA *in vitro*.

Because lipids in the OM surround OMPs, this lateral gating hypothesis for the functional mechanism of BamA seems contradictory to the basic principles of thermodynamics. The BamA β -barrel interior is large enough to accommodate hundreds of water molecules, which would be exposed to hydrophobic lipids as the BamA β -barrel laterally opens. This process should result in a large energetic penalty that must be overcome by another compensating source of energy. Reconciling these thermodynamic considerations with the available structural information must be accomplished to elucidate the catalytic mechanism of BamA.

How do the soluble POTRA motifs contribute to BamA function?

The POTRA motifs of BamA have a conserved $\beta 1-\alpha 1-\alpha 2-\beta 2-\beta 3$ architecture and have been suggested to interact with client uOMPs through exposed β -strands [58]. Genetic experiments have suggested that the three POTRA motifs closest to the β -barrel (POTRA 3–5) are required for BAM function *in vivo* [59]. Recent studies have indicated that the presence of all five BamA POTRA motifs accelerates uOMP folding *in vitro* more than the presence of only one POTRA motif [44]. Together, these findings suggest that at least some of the POTRA motifs play a role in facilitating uOMP folding or these motifs are important for folding of BamA itself. Crystallographic, NMR, and **Small-angle X-ray Scattering (SAXS)** analyses of truncations of the POTRA motifs (e.g. POTRA 1–2) in the absence of the BamA β -barrel suggest that the five POTRA motifs may be divided into two rigid bodies (POTRA 1–2 and 3–5) connected by a flexible linker [60,61]. Binding of the BamA POTRA domain to the lipid membrane may also play a role in selecting accessible conformations [62]. Rotational and lateral flexibility of these POTRA motifs modulated by both the remaining BAM subunits and the membrane surface is likely involved in the catalytic cycle of BAM-mediated uOMP folding.

The POTRA motifs of BamA also interact with the BamBCDE lipoproteins (Figure 5). Crystal structures of individual POTRA motifs fused to BAM lipoproteins revealed that BamB and BamD interact with POTRA motifs 3 and 5, respectively [63,64]. The recently solved crystal structures of BamACDE and the entire BAM complex suggest that the most extensive contacts between BamA and the BAM lipoproteins occurs between BamA POTRA 5 and BamD [50,65,66]. This interaction between BamA and BamD may be functionally important, because outcompeting this interaction with a BamD-derived peptide inhibits BAM complex formation [67].

One possible conformation of the BamA POTRA domain was elucidated in recent crystallographic studies of BamACDE and the BAM complex where it was observed that the BamA POTRA motifs encircle the periplasmic face of the BamA β -barrel and were suggested to create a funnel to direct the uOMP client to the β -barrel of BamA (Figure 5) [50,65,66]. However, the observed conformation of POTRA 5 is inconsistent with this hypothesis. POTRA 5 occludes the β -barrel of BamA from the periplasm, and the ~ 15 Å opening at the periplasmic face of the BamA β -barrel is too small to accommodate a uOMP client [65]. Additionally, it is difficult to exclude the idea that the compactness of the POTRA domain in the crystal structure may result from crystal contacts between neighboring BAM proteins. Indeed, MD of BamABCDE suggests the BamA POTRA motifs sample a variety of less compact states [50]. Although the reported conformation of the POTRA domain may have implications for the function of the BAM complex, structural studies that are non-crystallographic in nature will be required to further understand the role of the POTRA domain and BAM lipoproteins in determining the conformations accessible to BamA.

Overall, crystal structures of the BAM complex and its individual components provide valuable snapshots of the catalytic cycle of BAM-mediated uOMP folding. The crystallographic conformations observed likely represent stable reaction intermediates; if so, these crystallographic data inherently only provide insight into local energetic minima along the functional pathway of BAM. Piecing these glimpses together to envision the entire mechanistic picture of this process will require orthogonal experimental techniques, both *in vitro* and *in vivo*.

Zoom out: OMP biogenesis in living *E. coli*

While the molecular details of BAM-mediated uOMP assembly are being elucidated by atomistic-level structural and biochemical analyses, the macroscopic whole-cell localization and diffusion properties of the BAM complex in cells have been investigated. Complementary studies have suggested that two OMPs, LamB and BtuB, are inserted in discrete punctate spots along the mid-cell of *E. coli* [68,69]. Because these OMPs are assembled by BAM, these results raised an intriguing question: is the BAM complex localized to certain regions of the *E. coli* OM? Tagging BamA and BamC with fluorescently labeled antibodies revealed that both BAM proteins were colocalized to OMP “islands” [69]. New BamC-containing “islands” originate at non-polar regions. As BamBCDE facilitate BamA folding, one may speculate that BamA is likely also inserted at mid-cell [70,71]. However, OMP “islands” are not strictly localized to mid-cellular regions, therefore neither is the BAM complex [69]. The existence of the BAM complex throughout the OM of *E. coli* seems at odds with the spatial restriction of OMP insertion to the mid-cell regions. To explain this apparent contradiction, it has been suggested that the activity of the BAM complex is non-uniform throughout the cell and that active BAM complexes are limited to the *E. coli* mid-cell [69]. Although it is tempting to speculate that heterogeneous cellular lipid distributions may play a role in spatial localization of BAM activity, no conclusive link between these two factors has been shown. Further studies are required to determine any factors that may modulate and spatially constrict BAM activity.

In addition to localized insertion of OMPs, time-resolved tracking of OMP populations revealed that OMPs display limited diffusion and long-range immobility in living bacteria [69,70]. In particular, OMPs were confined to corrals within which their diffusion coefficients were consistent with those in phospholipid bilayers *in vitro*. The limited, long-range diffusion of OMPs must therefore be due to higher order protein-protein interactions or protein-lipid interactions. This macroscopic finding agrees well with the observed limited mobility of OMPs in a biological asymmetric bilayer in MD simulations (Figure 2) [72]. In sum, the spatial organization of the OM of *E. coli* is determined by inherent properties of both OMPs and lipids and may be modulated by the location of active BAM complexes. An intriguing consequence of these findings is that this lateral membrane organization appears to be heritable and may play a role in epigenetic inheritance in bacterial populations.

Concluding Remarks

Several questions involving the OMP biogenesis pathway have been addressed in recent years. We now have a basic understanding of how uOMPs are bound to chaperones with internal cavities as well as a structural model for the BAM complex. However, the mechanistic details of OMP biogenesis mediated by both chaperones and the BAM complex are still lacking. Future work must include experimental tools that integrate information from individual components together to create a more holistic understanding of OMP biogenesis. The multi-scale nature of current studies that combine many orthogonal experimental techniques will reveal the secrets of OMP biogenesis in ways that structural biology or biochemistry alone cannot. Particularly exciting will be the discovery of the catalytic mechanism for BAM-mediated OMP folding that explains how uOMPs move from chaperones to the membrane through BAM (See Outstanding Questions).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Glossary

Atomic Force Microscopy-based Single Molecule Force Spectroscopy (AFM-SMFS)

a branch of scanning probe microscopy in which the tip of a cantilever mediates mechanical unfolding of a single folded protein molecule

β -Barrel Assembly Machinery (BAM)

E. coli OM-localized multi-protein complex composed of the OMP BamA (formally YaeT, Omp85) and lipoproteins BamBCDE (formally YfgL, NlpB, YfiO, and SmpA, respectively)

DegP

E. coli serine endoprotease, a periplasm-localized oligomeric protein that is known to bind to uOMPs and degrade misfolded OMPs

FkpB binding protein A (FkpA)

E. coli periplasm-localized dimeric *cis/trans* prolyl isomerase that is known to bind to uOMPs

Lipopolysaccharide (LPS)

a polymer of sugars that is covalently linked to the outer leaflet of the outer membrane of Gram-negative bacteria

Molecular Dynamics (MD)

these simulations are computationally derived trajectories that apply Newton's laws of motion to calculate movements of systems of molecules

Nuclear Magnetic Resonance (NMR)

a solution-based spectroscopic technique in which electromagnetic radiation is used to probe an atomic chemical environment and discern information about biomolecular structure

Outer Membrane (OM)

region of Gram-negative bacteria; this membrane is asymmetric in that the inner leaflet is composed of phospholipids and the outer leaflet contains Lipid A/ lipopolysaccharide

Outer Membrane Proteins (OMPs)

β -barrel proteins that reside in the outer membrane of Gram-negative bacteria

Phosphoethanolamine (PE)

a zwitterionic lipid head group that contains a primary amine and phosphate linked by two carbons

Phosphoglycerol (PG)

a negatively charged lipid head group that contains a glycerol moiety linked to phosphate

Polypeptide-Transport Associated (POTRA)

periplasmic-localized ~70 residue soluble motifs with conserved architecture in *E. coli* BamA protein

Small-angle Neutron Scattering (SANS)

a solution-based technique in which neutron scattering of biomolecules yields structural information

Small-angle X-ray Scattering (SAXS)

a solution-based technique in which x-ray scattering of biomolecules yields structural information

SecA

A peripheral inner membrane protein-export protein. SecA acts as a receptor for SecB and utilizes the energy of ATP hydrolysis to help translocate uOMPs through the SecYEG translocase

SecB

A cytoplasmic protein-export protein. SecB recognizes the periplasmic export signal sequence of OMPs and binds to OMPs post-translationally, maintaining them in a translocation-competent conformation

SecYEG

An inner membrane localized protein translocase complex. The α -helical transmembrane protein SecY works with SecA to mediate passage of OMPs across the inner membrane of Gram-negative bacteria

Seventeen Kilodalton Protein (Skp)

An *E. coli* periplasm-localized trimer that is known to bind to uOMPs

Survival factor protein A (SurA)

An *E. coli* periplasm-localized *cis/trans* prolyl isomerase that is known to bind to uOMPs

Unfolded Outer Membrane Proteins (uOMPs)

OMPs in a precursor conformation to folded state that lacks structure

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Box 1**Misfolding or Refolding: The effect of periplasmic chaperones**

Advances in single molecule AFM have allowed for the tracking of force-mediated unfolding of individual OMPs [73,74]. These experiments provide novel insight into biophysically interesting questions regarding the unfolding mechanism of OMPs, as it was discovered that OMPs unfold under force via a stepwise mechanism of β -hairpin removal from the membrane. More relevant for the biological context, Thoma *et al.* find the chaperones SurA and Skp have differential effects on the refolding trajectories of the OMP FhuA: SurA promotes the reinsertion of single native β -hairpins into the lipid membrane, while Skp substantially decreased the population of misfolded polypeptide conformations [24,75]. These data suggest that Skp acts primarily in binding uOMPs to prevent aggregation and SurA functions in both binding uOMPs and somehow affects the uOMP folding pathway. Future work should aim to deconvolute the effect of BAM on this proposed folding pathway at a single-molecule level.

Figure 1, Key Figure. Depiction of Outer Membrane Protein biogenesis components in *E. coli* shown to scale

Unfolded OMPs enter the periplasm via the SecYEG translocase (teal, PDB: 2CFQ), which is located in the bacterial inner membrane. Once in the periplasm, unfolded OMPs interact with several chaperones, including: DegP (cyan, PDB: 3CSO), FkpA (yellow, PDB: 1Q6U), SurA (magenta, PDB: 1M5Y), and Skp (pale green, PDB: 1U2M). The outer-membrane localized BAM complex (BamABCDE shown in blue/green/magenta/pink/yellow respectively, PDB: 5D0O) facilitates uOMP folding into the OM (e.g. OmpLA; red, PDB: 1QD5). The multi-protein Acr complex (tan, PDBs: 1EK9, 2F1M, 2DHH) was used to position the inner and outer membranes. The structures of these membranes were derived from MD simulations of smaller patches that were concatenated to make this image [63]; phosphate atoms of lipid head groups are shown in orange spheres. Arrows indicate known interactions, although the exact sequence and mechanisms of these interactions are unknown. Note the peptidoglycan is excluded from this figure. This image was created in Pymol [76].

Figure 2. OmpLA forms extensive interactions with the outer membrane of *E. coli* [63]

This membrane is asymmetric, as the inner and outer leaflets (e.g. blue and purple sphere lipids, respectively) are composed of phospholipids and LPS, respectively. Phosphate atoms of lipid head groups are shown in orange spheres. All-atom MD simulations of an OMP embedded in an *E. coli* outer membrane have revealed that divalent ion-mediated interactions create electrostatic networks that impart rigidity and limited mobility in the LPS leaflet of the bilayer. This image was prepared in VMD [77].

Figure 3. Structural model showing uOMP encapsulated by Skp

The uOMP protrudes from the internal binding cavity of Skp. This model was constructed from SANS experiments of Skp bound to unfolded OmpW [18]. Skp is depicted as green cartoon, while uOMP is shown as grey surface. This figure was created in VMD [77].

Figure 4. Molecular Dynamics Simulation Snapshot of *N. gonorrhoea* BamA barrel in membranes (A) BamA β -barrel (blue) in dimyristoyl-glycero-3-phosphatidylethanolamine gel-phase lipids reveals that the β -barrel seam (strands 1 and 16, shown in green) of BamA is distorted [40]. The phosphorus atoms of the phospholipids are shown as orange spheres. (B) β -strands 1 and 16 form a non-canonical barrel seam, as these strands only form two hydrogen bonds in this particular barrel conformation. (C) The hydrophobic thickness on the two sides of the BamA barrel, indicated by two arrows, are different in this conformation. This image was created in VMD [77]. Trajectory file kindly provided by Prof. JC Gumbart.

Figure 5. A crystallographic structure of the BAM complex

(A) The structure of the BAM complex (PDB: 5D0O) suggests how the BAM lipoproteins bind to the BamA POTRA motifs [43]. BamD forms extensive contacts with BamA. BamABCDE are shown in blue, green, magenta, pink, and yellow respectively. (B) The BamA POTRA motifs encircle periplasmic face of the β -barrel; the BAM lipoproteins are excluded from this view. POTRA motifs are colored red, orange, tan, green, and light blue (P1–5). This image was created in VMD [77].