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Function of the Omp85 Superfamily of Outer Membrane Protein Assembly Factors and Polypeptide Transporters

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Abstract

The Omp85 protein superfamily is found in the outer membrane (OM) of all gram-negative bacteria and eukaryotic organelles of bacterial origin. Members of the family catalyze both the membrane insertion of β-barrel proteins and the translocation of proteins across the OM. Although the mechanism(s) by which these proteins function is unclear, striking new insights have emerged from recent biochemical and structural studies. In this review we discuss the entire Omp85 superfamily but focus on the function of the best-studied member, BamA, which is an essential and highly conserved component of the bacterial barrel assembly machinery (BAM). Because BamA has multiple functions that overlap with those of other Omp85 proteins, it is likely the prototypical member of the Omp85 superfamily. Furthermore, BamA has become a protein of great interest because of the recent discovery of small-molecule inhibitors that potentially represent an important new class of antibiotics.

Contents

1. INTRODUCTION

The Omp85 superfamily is a group of proteins that are widely distributed in the outer membrane (OM) of gram-negative bacteria and organelles of bacterial origin, including mitochondria and chloroplasts. These exceptionally important proteins carry out various essential housekeeping functions. Like almost all bacterial outer membrane proteins (OMPs) and a subset of mitochondrial and chloroplast OMPs, Omp85 proteins contain an amphipathic β-sheet that is folded into a closed β-barrel structure that serves as a membrane-spanning domain (100). While β-barrels whose structure has been solved range in size from 8 to 36 β-strands (69, 87), all members of the Omp85 superfamily contain a 16-stranded β-barrel located at their C terminus (12, 17, 29, 33, 82). A comprehensive taxonomic analysis identified 10 distinct subfamilies within the Omp85 superfamily (39). Most subfamilies are defined by the presence of one to five soluble N-terminal polypeptide translocation associated (POTRA) domains (25, 96). Although the overall sequences of the β-barrels of the Omp85 paralogs vary considerably, they typically share characteristic sequence features such as a conserved motif (VRGF) found in an extended surface loop (loop 6) that is required for protein stability (25, 82). Some members of the superfamily are free-standing proteins, whereas others are associated with accessory proteins that contribute to function (39). As described in more detail below, the structures of several Omp85 proteins have been solved either in isolation or as complexes and differ significantly (**Figure 1**).

Although the function of many members of the Omp85 family is unknown, the Omp85 proteins that have been characterized to date catalyze the insertion of other β-barrel proteins into the OM, the translocation of proteins or protein domains across the OM, or both. In gram-negative bacteria, BamA is a universal and highly conserved Omp85 protein that is an essential component of the barrel assembly machinery (BAM), a heterooligomer that promotes the integration of β-barrel proteins into the OM (36, 119, 128). The bacterial OM is an asymmetric membrane that consists of phospholipids in the inner leaflet and a unique glycolipid called lipopolysaccharide (LPS) in the outer leaflet (47, 66). In addition to BamA, *Escherichia coli* BAM contains several lipoproteins (designated BamB–BamE) that are bound to the BamA POTRA domains on the periplasmic side of the OM [the space between the inner membrane (IM) and the OM] (30, 106, 128). In 2021, BamA was also shown to promote protein export across the OM

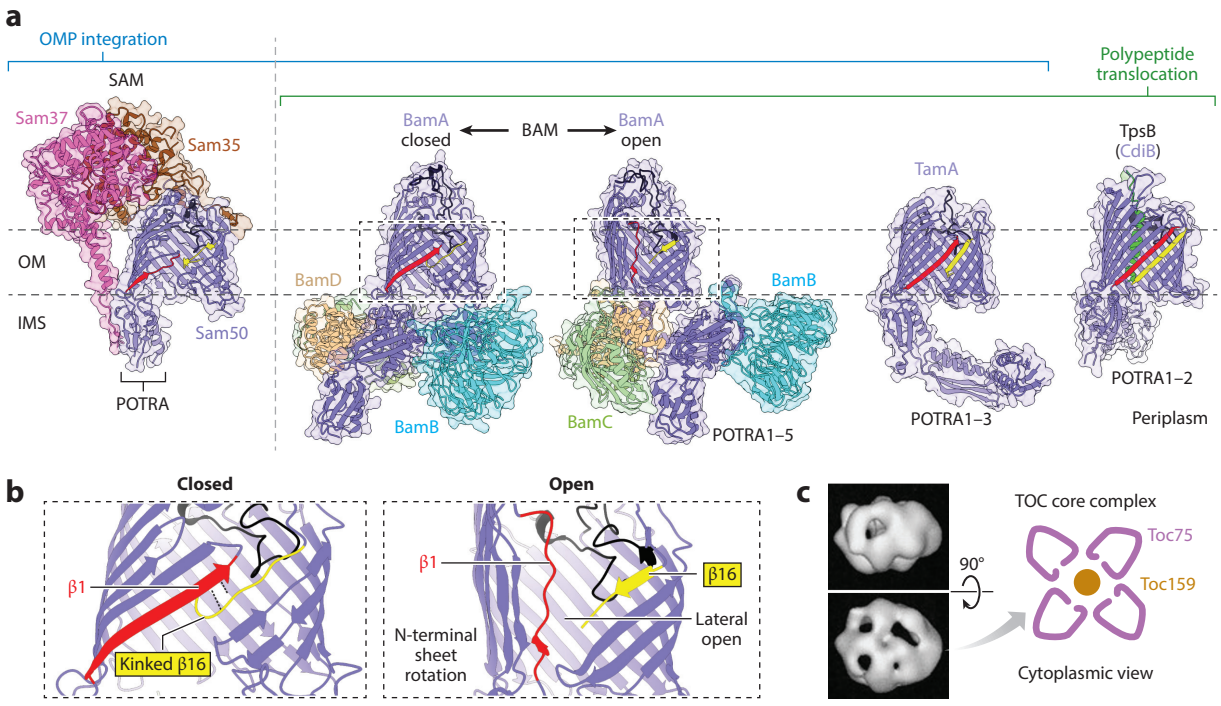

Figure 1

Structures of members of the Omp85 protein superfamily. (*a*) Representative high-resolution structures of Omp85 proteins (*purple*) and their accessory proteins are depicted. From left to right: the SAM complex from *Thermothelomyces thermophilus* consisting of Sam50 and cytoplasmic accessory proteins Sam35 and Sam37 (PDB ID: 6WUT) (17); the BAM complex from *Escherichia coli* consisting of BamA [shown in both lateral-closed (PDB ID: 5D0O) (30) and lateral-open (PDB ID: 5LJO) (49) conformations] and periplasmic accessory proteins BamB–BamE (BamE is not visible); TamA from *E. coli* (PDB ID: 4C00) (29) that forms the TAM with TamB; and the TpsB translocator-type Omp85 protein CdiB from *Acinetobacter baumannii* (PDB ID: 6WIL) (33). The POTRA domain(s) of each Omp85 protein and the luminal α-helix of CdiB (*green*) are indicated. The first and sixteenth β-strands (β1 and β16) that form the β-seam of each Omp85 protein are red and yellow, respectively, and loop 6 is black. BamA and TamA have been implicated in both OMP integration and polypeptide translocation reactions. (*b*) Magnified views of the β-barrel domain of *E. coli* BamA (*boxes*). The closed form has a weakly hydrogen-bonded β-seam (*dotted lines*) and a kinked β16. In the open form the N-terminal β-strands of BamA rotate outward to create a lateral opening. (*c*, *left*) A low-resolution (~32 Å) negative-stain electron microscopy reconstruction of the TOC core complex from *Pisum sativum* (pea) showing putative side view (*top*) and cytoplasmic view (*bottom*). (*Right*) The oligomeric model depicts four copies of the Omp85 family member Toc75 surrounding a single Toc159 accessory protein. Panel *c* adapted from Reference 99 with permission from the publisher. Abbreviations: β1, β-strand 1; BAM, barrel assembly machinery; IMS, intermembrane space; OM, outer membrane; OMP, outer membrane protein; POTRA, polypeptide translocation associated; SAM, sorting and assembly machinery; TAM, translocation assembly module; TOC, translocase in the outer chloroplast membrane.

(20). *Klebsiella* species contain a BamA-like protein designated BamK that rescues the function of a loss-of-function *bamA* mutant, and some *Pseudomonas* species contain a second copy of BamA (BamA2) (39, 118). Many *Proteobacteria* and *Bacteroidetes* also produce a nonessential Omp85 protein called TamA [translocation assembly module (TAM) subunit A; see **Figure 1**] and a similar uncharacterized protein called TamL (40). The exact function of TamA is unclear (1), but available evidence strongly suggests that it functions in association with TamB, a large periplasmic protein anchored in the IM, to promote the assembly of a subset of OMPs (41, 101–103, 113). Members of a wide variety of phyla (e.g., *Proteobacteria*, *Fusobacteria*, *Cyanobacteria*) produce a different type of Omp85 protein called TpsB that is a component of the so-called two-partner

secretion (TPS) system (39) (see **Figure 1**). Unlike the BamA and TamA families, TpsB proteins are devoted exclusively to the secretion of a specific exoprotein known as TpsA (32). TpsA proteins are typically large (100–500 kDa) virulence factors that fold into an extended β-helix and that are encoded in the same operon as their cognate TpsB transporter (58).

Members of several other Omp85 subfamilies are widespread but have remained largely uncharacterized (39). For instance, several bacterial lineages, including *Proteobacteria*, *Fusobacteria*, and *Bacteroidetes*, encode Omp85 proteins that contain an additional patatin-like domain attached to the N terminus of a single POTRA domain (15). A study of the *Pseudomonas aeruginosa* PlpD protein suggests that the patatin-like domain might be secreted and that this subfamily might therefore share properties with the TPS system (95). Given that other uncharacterized subfamilies contain N-terminal metalloprotease domains, BamB-like domains, or potentially N-terminal lipidated residues (39), functional domain variation at the N terminus appears to be a common theme among bacterial Omp85 proteins.

With respect to eukaryotic Omp85 proteins, mitochondria contain a conserved homolog of BamA called Sam50 (or Tob55) that is the essential subunit of the so-called sorting and assembly machinery (SAM) (16). Like BAM and TAM, SAM promotes the integration of β-barrel proteins into the OM (57, 85, 124). Sam50 differs from BamA, however, in that it contains only one POTRA domain (instead of five) and associates with two accessory proteins, Sam35 (Tob38) and Sam37 (Mas37), that are unrelated to the bacterial BAM lipoproteins and that are located on the cytosolic side of the OM (the equivalent of the extracellular space in bacteria; see **Figure 1**) (17, 116, 120). A study of SAM published in 2021 strongly suggests that at least in yeast it functions by a mechanism that is distinct from that of BAM (116) (see Section 2.2). Chloroplasts produce an essential Omp85 protein called OEP80 or Toc75-V (86) whose function has remained elusive, but in 2021 a study provided evidence that it plays an important role in facilitating β-barrel insertion (28). In addition to OEP80, chloroplasts produce a completely distinct Omp85 protein, Toc75, that has been well characterized. Toc75 forms a translocation channel that, in concert with several proteins exposed on the cytoplasmic side of the OM (Toc34, Toc64, and Toc159) that are likewise subunits of the translocase in the outer chloroplast membrane (TOC), provides the primary pathway for the import of nuclear encoded proteins into chloroplasts (23, 104).

Although the basic functions of members of the Omp85 superfamily have emerged during the past 20 years, the mechanisms by which they catalyze specific protein transport and insertion reactions are still not well understood. In this review we describe striking new insights into the function of these proteins that have recently emerged through a combination of structural and biochemical studies. We focus on BamA, which, perhaps because it plays a critical role in cell physiology and is essential for viability, is the best-studied member of the Omp85 superfamily. Furthermore, BamA has become a protein of great interest because it is the target of several recently described small molecules that have potent antibacterial activity against various pathogens (38, 53, 76, 79). We also speculate on the evolution of the superfamily based on phylogenetic analyses and recent functional studies of BamA and other Omp85 proteins.

2. MECHANISMS BY WHICH Omp85 PROTEINS CATALYZE THE ASSEMBLY OF OUTER MEMBRANE β-BARREL PROTEINS

Although recent studies have provided remarkable insights into the mechanisms by which BAM and SAM promote the assembly (folding and membrane insertion) of OMP transmembrane β-barrel domains into bacterial and mitochondrial OMs, a complete picture of their mode of action has yet to emerge. Nevertheless, the process can clearly be divided into three basic steps: (*a*) recognition, (*b*) folding and integration, and (*c*) release into the lipid bilayer. It is likely that all

three steps are facilitated by a unique property of the BamA and Sam50 β-barrel domains. Unlike most OMP β-barrels, which form exceptionally stable structures through hydrogen-bonding between their first and last β-strands (the β-seam; see CdiB in **Figure 1**) (100), the BamA and Sam50 β-barrels have an unstable β-seam between β-strand 1 (β1) and a kinked β-strand 16 (β16) that can open laterally (17, 75, 81, 82). For unknown reasons, TamA also exhibits this unusual β-seam structure (29). The dynamicity of the BamA β-seam is illustrated in several solved structures that show a lateral-closed conformation, in which the interior of the β-barrel domain is open to the periplasm, and a lateral-open form, in which the interior of the β-barrel domain is open to the external milieu (30, 49, 50). Based on genetic and cell physiology studies, the lipoprotein accessory subunits of BAM (especially BamD) likely play important supporting roles (8, 70, 78, 123), but their exact function is unclear. As noted in Section 2.2, Sam35 and Sam37 likewise appear to play important supporting roles by structurally stabilizing intermediate stages in the assembly of mitochondrial OMPs (116).

2.1. Recognition of β-Barrel Proteins

Bacterial OMPs and their mitochondrial and chloroplast counterparts reach their destination by distinct but conceptually related pathways. Bacterial OMPs are first translocated across the IM into the periplasm through the Sec machinery (66). Most mitochondrial and chloroplast OMPs (which are nuclear encoded proteins) are first translocated across the OM through the translocase in the mitochondrial outer membrane (TOM) or TOC complexes into the intermembrane space (IMS), which is the evolutionary derivative of the bacterial periplasm (17). Transport of OMPs into the periplasm/IMS in all of the systems is based on the recognition of an N-terminal signal peptide (or transit peptide in chloroplasts). Once in the periplasm, bacterial OMPs interact with molecular chaperones, including Skp (a jellyfish-like homotrimer), SurA (a parvulin-like peptidyl-prolyl isomerase), and DegP (a large cage-forming homooligomer), that presumably keep them in an insertion-competent conformation (66). One of these proteins (SurA) interacts with BamA (6, 107) and may play a role in targeting OMPs to BAM (see below). In contrast, available evidence suggests that in mitochondria β-barrel proteins are transferred from the TOM complex to SAM by the small translocase in the mitochondrial inner membrane (TIM) chaperones and by the formation of a supercomplex between the two complexes by Tom22 (46, 90, 125).

Despite the differences in targeting pathways and in the chaperones that interact with β-barrel proteins in bacteria and mitochondria, it has long been known that a C-terminal motif in β-barrels called the β-signal (G/ζ-Φ-ζ-Φ/Ω-ζ-Ω in bacteria, where ζ, Φ, and Ω represent hydrophilic, hydrophobic, and aromatic amino acids, respectively, and G-Φ-x-Φ/Ω-x-Φ/Ω in mitochondria) plays a critical role in assembly (52, 63, 68, 112). Recent evidence indicates that the β-signal targets OMPs to BAM in *E. coli* (121). Studies in which the interactions between arrested OMP assembly intermediates and BAM or SAM were precisely mapped by disulfide bond cross-linking showed that the β-signal binds tightly to BamA or Sam50 β1, whereas the N-terminal strand of the substrates binds weakly and dynamically to the C terminus of the Omp85 protein (19, 45). These studies strongly suggest that BamA and Sam50 recognize their client proteins by binding to the β-signal (**Figure 2**). Consistent with this notion, a cryo-electron microscopy (cryo-EM) study of the arrested assembly intermediate bound to BAM has clearly shown a direct interaction between the β-signal and β1 of a laterally open form of BamA (21) (see **Figure 3**). Furthermore, another structural study has shown that a naturally occurring antimicrobial peptide called darobactin that mimics a β-signal binds to the open BamA β-barrel at the same location and likely produces toxic effects by acting as a competitive inhibitor of the β-signal–β1 interaction (63).

The recognition of incoming OMPs likely involves more than the binding of the β-signal by Omp85 proteins. The findings that folded states affect the assembly of OMPs in vitro and that

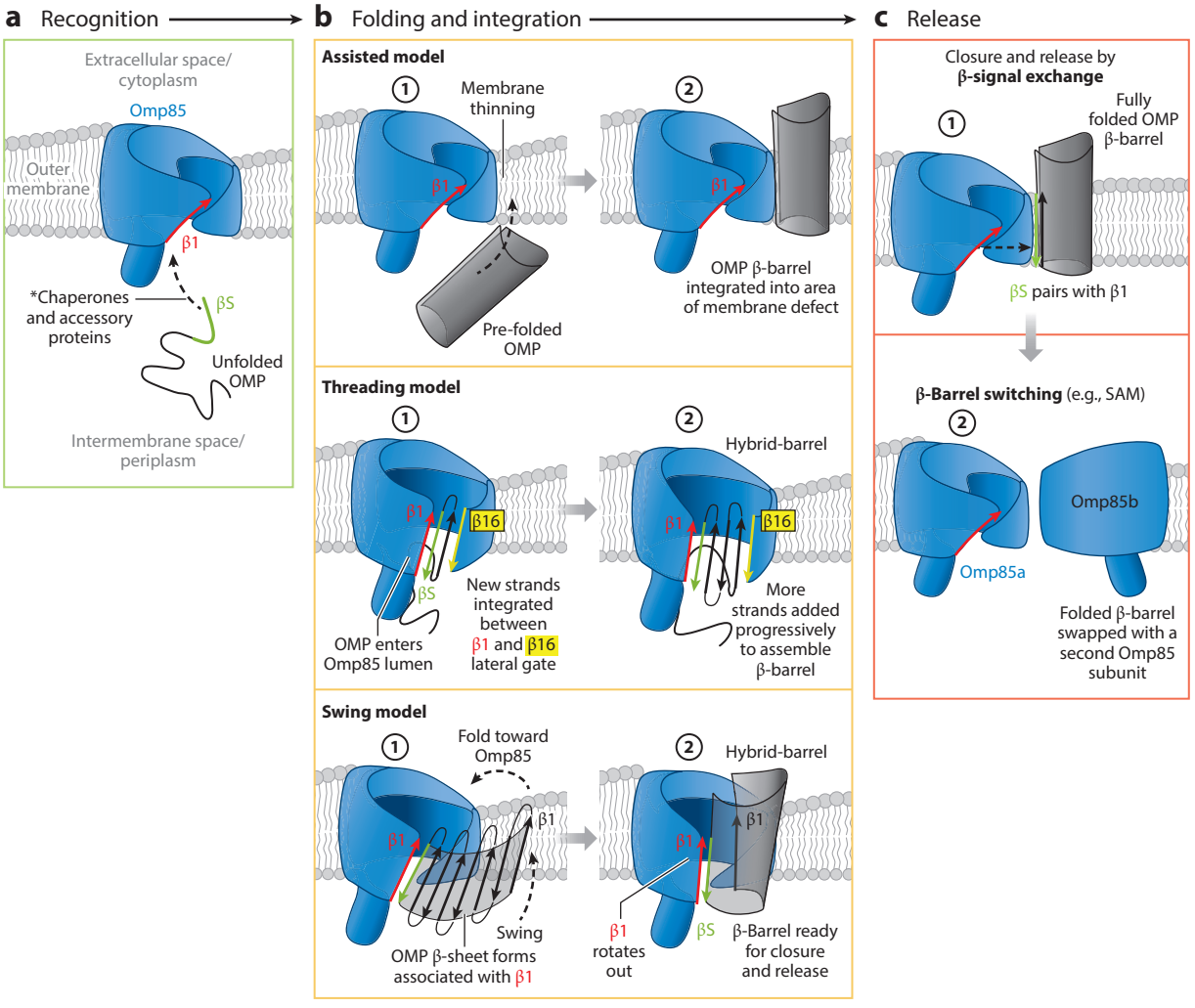

Figure 2

Stages of OMP β-barrel folding and membrane insertion by Omp85 integrases. (*a*) Recognition. Available evidence indicates that unfolded OMPs in the periplasm (bacteria) or IMS (mitochondria) are recognized by chaperones or accessory proteins and delivered to the Omp85 protein (e.g., BamA or Sam50) at the OM. The βS (*green*) at the C terminus of OMPs is bound by Omp85 β1 (*red*). (*b*) Folding and integration. Three models are depicted. In the assisted model (*top*), the OMP undergoes significant folding prior to membrane insertion (step 1). The Omp85 protein locally thins the OM near its C terminus to catalyze OMP integration (step 2). In the threading model (*middle*), unfolded OMPs enter the lumen of the Omp85 protein and a β-hairpin containing the βS is inserted into the OM between β1 and β16 of the lateral gate (step 1). OMP β-strands are threaded sequentially from C to N termini to form an integrated hybrid-barrel intermediate structure (step 2). In the swing model (*bottom*), the OMP forms a partially folded β-sheet that is bound only to β1 of the Omp85 protein via the βS (step 1). The rotation of the N-terminal β-strands of the Omp85 protein (into the lateral-open conformation) allows for a swinging action as the β-sheet is integrated. The β-sheet then folds toward the Omp85 protein to form an integrated hybrid-barrel intermediate structure (step 2). (*c*) Release. It has been proposed that hydrogen bonds formed between the OMP βS and the Omp85 β1 are exchanged with those formed within the OMP β-seam (βS-β1) to promote closure and release of the fully folded OMP (step 1). In the SAM system, it appears that the new β-barrel is displaced by a second copy of the Omp85 protein (Omp85b) (step 2). The BAM system does not require this second step. Abbreviations: β1, β-strand 1; βS, β-signal; BAM, barrel assembly machinery; IMS, intermembrane space; OM, outer membrane; OMP, outer membrane protein; SAM, sorting and assembly machinery.

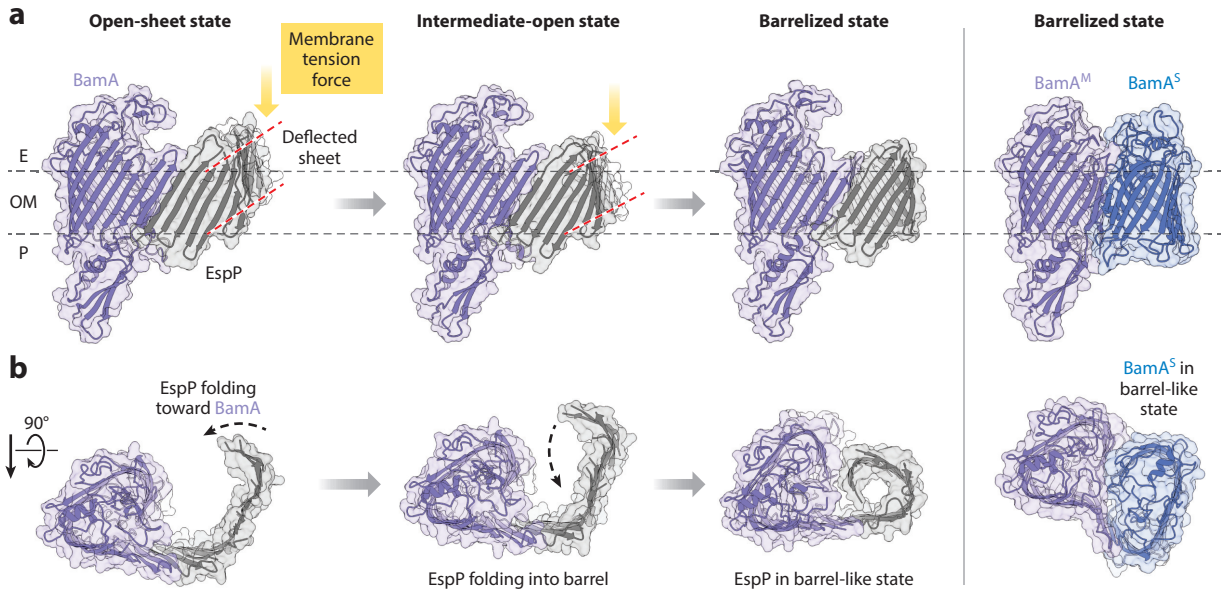

Figure 3

Model of BamA-catalyzed folding of bacterial β-barrels. Side views (*a*) and extracellular views (*b*) of BamA-OMP hybrid-barrel intermediate structures. Recent cryo-EM structures of BAM folding the β-barrel of the autotransporter EspP (open-sheet state, PDB ID: 7TT5; intermediate-open state, PDB ID: 7TT6; barrelized state, PDB ID: 7TT7) (21) suggest that EspP forms a β-sheet in the OM that would deflect the membrane (*far left* and *mid-left*). Current evidence suggests that the tension forces across the rigid OM would assist assembly by forcing β-sheet folding toward BamA and into a barrel-like structure (*mid-right*) that may precede the release stage of assembly. The recent cryo-EM structure of BamA ($BamA^M$) folding a new copy of BamA ($BamA^S$; PDB ID: 6V05) (*far right*) (117) may depict a stage of assembly that is similar to that of the BAM-EspP barrelized state. For clarity, accessory proteins of the BAM complex and BamA POTRA1–4 are removed from the displayed structures. Abbreviations: BAM, barrel assembly machinery; $BamA^M$, BamA subunit of the active BAM insertase; $BamA^S$, BamA substrate; cryo-EM, cryo-electron microscopy; E, extracellular space; OM, outer membrane; OMP, outer membrane protein; P, periplasm; POTRA, polypeptide translocation associated.

the presence of SurA can compensate for β-signal mutations in vivo (48, 121) suggest that the conformation of OMPs and their mere delivery to BAM by chaperones may significantly influence their recognition. There is also evidence that the BamAB and BamCDE subcomplexes participate in substrate recognition and that BamD recognizes the β-signal (36, 37, 92). Indeed a new study that investigated the assembly of the trimeric porin OmpC showed that the C-terminal region of BamD binds to the β-signal and that the N-terminal region binds to a newly identified conserved motif found in the fifth-to-last strand of OMPs (the −5 signal) (26). It is plausible that the −5 signal provides a means for the >20% of *E. coli* OMPs that lack a classical β-signal to bind to BAM. These proteins also contain other conserved C-terminal motifs (121) that in one case interact strongly with the N terminus of BamA (71, 117). In addition, the observation that the C terminus of chloroplast β-barrel proteins, which also lack a β-signal, contributes to membrane insertion (28) suggests that the final β-strand of OMPs has a potentially universal role in promoting recognition by Omp85 insertases.

2.2. Folding and Integration of OMP β-Barrels

Although the function of the BAM lipoproteins is largely unknown, several different models have been proposed to explain the mechanism by which BamA promotes the assembly of OMP

β-barrels. All of these models are based on the postulate that the opening of the unstable BamA β-seam plays an important role in the assembly process. Consistent with this notion, experiments have shown that locking the β-seam shut with disulfide bonds causes a lethal phenotype or inhibits OMP assembly in vitro (18, 49, 81). The original two models were based on different ideas about the significance of the lateral opening. The threading (or budding) model proposed that incoming β-barrel segments pass through the BamA pore in an unfolded conformation and then enter the OM through a lateral gate formed between the strands of the BamA β-seam (82). In this scenario, BamA β1 and β16 template the sequential formation of β-hairpins and lead to the assembly of an expanding hybrid-barrel composed of BamA and the substrate β-barrel (**Figure 2**). Ultimately, the new β-barrel buds off from BamA into the lipid bilayer. At the other extreme, the assisted model suggested that the unstable β-seam, as well as the uniquely wedge-shaped structure of BamA, facilitates the integration of partially or completely folded β-barrels by altering the thickness of the OM and thereby lowering the kinetic barrier imposed by lipid headgroups (27, 82, 98). Indeed, molecular dynamics simulations support the idea that the local membrane structure would be significantly perturbed by the opening of the BamA β-barrel (73, 82).

Recent experimental results have provided evidence that supports an alternative model for OMP assembly in *E. coli*. Of particular note, the study described above in which interactions between an arrested OMP assembly intermediate and BamA were mapped introduced the idea that incoming β-barrel proteins form an asymmetric hybrid-barrel with BamA during the insertion process (19) (see the swing model in **Figure 2**). This notion is strongly supported by the aforementioned cryo-EM analysis of a BAM-OMP assembly intermediate that reveals the dynamics of integration (21). The structural data strongly suggest that OMP assembly involves the membrane integration of a slightly curved β-sheet that is bound to BamA β1 via the β-signal but unbound at the N terminus (hence the asymmetry). The β-sheet then progressively folds toward BamA to form a nearly closed β-barrel-like structure (21) (see **Figure 3**). A remarkable degree of membrane remodeling and significant BamA conformational dynamics were observed for these intermediate states. Taken together with previous biochemical studies (19, 21), the results suggest that the lateral-open form of BamA binds to the β-signal and then promotes the integration of partially folded substrates into a locally perturbed membrane bilayer through a swing mechanism. The notion that OMP β-barrels pass through an asymmetric hybrid-barrel intermediate is also supported by another cryo-EM study that examined an atypical substrate captured at a late stage of assembly (117) (see **Figure 3**).

Despite these advances in elucidating the mechanism by which OMP β-barrels are inserted into the bacterial OM, the stage at which folding is initiated remains unclear. Experiments on a class of OMPs known as autotransporters that contain an extracellular (passenger) domain connected to the β-barrel by an embedded linker show that the linker becomes resistant to proteases and chemical modification prior to its insertion into the OM (51). In addition, an analysis of so-called trimeric autotransporters, a family of OMPs composed of three identical subunits that each contribute four β-strands to a single β-barrel and an extracellular segment connected to the β-barrel by an embedded linker, clearly demonstrated that the barrel begins to form in the periplasmic space (105). Perhaps analogously, a slow-folding version of LptD (an essential component of the LPS biogenesis pathway) incorporates an unlinked lipoprotein that acts as a plug in the fully folded protein into its β-barrel prior to membrane integration (71). In all of these cases, the linker or lipoprotein cofactor might nucleate a folding reaction, and it is unclear whether empty β-barrel proteins follow the same assembly pathway. Furthermore, it is not clear whether the initiation of folding occurs in the periplasm or through interactions with the periplasmic components of BAM. The recent observation that BamD can bind to specific β-strands within nascent OMPs supports the latter hypothesis (26). In principle, the POTRA domains of BamA might also promote folding by

providing a β-strand to template the formation of a β-sheet (otherwise known as β-augmentation; see 65). Alternatively, the elongation model proposes that folding and insertion are completed in a concerted reaction in which β-augmentation is initiated by the BamAβ1–β-signal interaction (98).

Several lines of evidence have suggested that there might be significant differences in the mechanisms by which SAM and BAM catalyze the assembly of OMPs despite an overall structural similarity. Besides the fact that the accessory proteins associated with BamA and Sam50 are entirely unrelated, mitochondrial OMP β-barrels typically contain 19 β-strands, whereas all known bacterial β-barrels contain an even number of β-strands. In addition, a disulfide cross-linking study that examined the interaction of mitochondrial β-barrel proteins containing N-terminal deletions with yeast Sam50 strongly suggested a threading mechanism of assembly (45). Remarkably, a structural study published in 2021 showed that in the resting state SAM consists mainly of two copies of Sam50 that are laterally open toward each other and a cytoplasmic cap consisting of a single copy of Sam35 and Sam37 (116). A fraction of SAM complexes, however, contain a completely closed β-barrel protein called Mdm10 in place of the second copy of Sam50. The structural data and evidence that incoming β-barrel substrates are bound to a complex that contains a single copy of Sam50, Sam35, and Sam37 led to a novel β-barrel switching model. In this model, substrate β-strands enter the pore of Sam50a in the Sam50 dimer and are threaded through the lateral gate in a stepwise fashion. During the process the substrate gradually displaces Sam50b. Following the release of some substrates the Sam50 dimer reforms; in other cases Mdm10 transiently replaces the substrate before it, in turn, is replaced by Sam50b. As predicted by the β-barrel switching model, a fully folded mitochondrial OMP (Tom40) that was fortuitously trapped on SAM in a heterologous expression system formed a structure that was stabilized by Sam37 (120). Although the biological advantage of the unique dimeric structure of SAM remains unknown, it is conceivable that the second Sam50 subunit (Sam50b) protects the mitochondrial membrane from deleterious effects of lateral opening.

2.3. Release of OMP β-Barrels from Omp85 Integrases

Regardless of the exact mechanism of integration, most of the available evidence now suggests that at some point β-barrels pass through an assembly intermediate in which at least the β-signal is tightly bound to the open form of the BamA or Sam50 β-barrel. The data therefore beg the question of how newly integrated β-barrel proteins are efficiently released into the lipid bilayer. A structural investigation of a special case in which the substrate is a new copy of the BamA β-barrel itself ($BamA^S$) revealed that the register of the hydrogen bonds between the C-terminal strand of $BamA^S$ and the N-terminal β-strand of the BamA subunit of the active BAM insertase ($BamA^M$) differs from those formed by the first and last β-strands of the fully folded BamA β-barrel (117). Moreover, the kinked structure of β16 (a unique feature of BamA and closely related Omp85 proteins) creates an overhang of the last few C-terminal residues of $BamA^S$ that results in an interaction with $BamA^M$ that is incompletely hybridized (75, 117). This and other observations led to the proposal that the release of new BamA β-barrels is triggered by the formation of hydrogen bonds between the overhang and the N-terminal β-strand of $BamA^S$, which causes the release of its C terminus from BAM (117). Although this strand-exchange model is thermodynamically favorable, typical OMPs that contain canonical structural features (e.g., a stable β-seam) cannot undergo an overhang-enhanced release mechanism. A simpler strand-exchange model stems from the observation that the β-seam of fully folded OMP β-barrels is stabilized by a larger number of hydrogen bonds than the BamAβ1–β-signal interaction is (21). In this model, both the asymmetric interaction between Omp85 proteins and their substrates and the greater number of hydrogen bonds in the β-seam of fully folded barrels create a stability gradient that promotes the

thermodynamically favorable closure of the new β-barrels by strand-exchange (19, 21). Regardless of the mechanism of release, it is clear that the timing and efficiency of release can be regulated by several factors. In this regard it is notable that an interaction between a surface-exposed lysine residue in an autotransporter β-barrel and LPS may contribute to its release from BAM (88).

2.4. Factors That Might Contribute to the OMP Assembly Function of BAM

Recent work has led to intriguing evidence that BAM is not uniformly distributed in the *E. coli* OM. Experiments in which the positions of newly synthesized BAM components and a model OMP (BtuB) were monitored by fluorescence-based methods revealed that both are located in ∼0.5-mm islands that initially form in nonpolar regions and then slowly diffuse toward the cell poles (91). The notion that OMPs are assembled primarily in nonpolar regions was supported by a microscopy study published in 2021 in which BAM and the Sec machinery colocalized with the divisome at the midcell site of septation (13). In light of recent evidence that the tension across the OM affects OMP folding and that the lipid environment itself plays a significant role in OMP assembly (see Section 5) (21, 47), it is conceivable that BAM needs to be localized to regions of the OM that have specific physical properties or lipid compositions to function optimally. Super-resolution imaging experiments indicate that BAM resides in ∼200-nm supercomplexes in situ that are held together by interactions between BamA and BamB and that expand during active protein synthesis (35). It is currently unclear, however, whether the formation of these BAM precincts significantly affects the efficiency of OMP assembly or whether they are required for the assembly of specific OMP families.

Although the function of TAM has not been established, several models have been proposed in which this complex works in conjunction with BAM to stimulate the assembly of at least a subset of OMPs (1). In one model, TAM and BAM act at distinct stages of assembly. In a second model, TAM is activated to assist BAM only under specific conditions. In a third model, TAM and BAM cooperate by transiently forming a larger complex. This model is supported by the observation that FimD, the OM channel component of the fimbrial usher secretion system, is assembled efficiently only when both BAM and TAM are present in the cell (113). The notion that Omp85 assembly machines associate and disassociate to synergistically enhance substrate folding is especially intriguing in light of the finding that the Sam50 β-barrel forms an open homodimer and a heterodimer with the Mdm10 β-barrel that appear to be important for OMP assembly in mitochondria (116). Indeed, it is conceivable that BamA and TamA, both of which have the potential to open laterally (29), form an analogous complex in bacteria. The curious observation that TamB interacts with BamA and promotes OMP assembly in *Borrelia burgdorferi*, an organism that lacks TamA, also suggests a possible functional connection between TAM and BAM (56). Nevertheless, the observation that a *tamA* knockout does not affect the assembly of abundant OMPs and affects growth only under specific conditions (62, 97, 109) strongly suggests that TAM has a highly specialized function in *E. coli*. As an aside, it is possible that TAM acts independently of BAM and that TamA also forms a hybrid-barrel with its substrate at an intermediate stage of assembly (29, 114).

3. MECHANISMS BY WHICH Omp85 PROTEINS CATALYZE PROTEIN TRANSLOCATION REACTIONS

Because the TpsB component of bacterial TPS systems transports polypeptides across rather than into the OM, it is not surprising that its structure differs in specific ways from those of BamA, TamA, and Sam50 (**Figure 1**). The β-barrels of the TpsB proteins differ from the insertases in that they contain a stable β-seam and an embedded α-helix (H1) that is connected to the N terminus

of the periplasmic POTRA domains (12, 33, 77, 82). TpsB proteins also contain long extracellular loops [in addition to loop 6 (L6), which is also found in BamA and other insertases] that may contribute to the diffusion of polypeptides across the OM.

Given that the TpsB β-barrel forms a constricted channel that is only ~3 Å in diameter in the resting state (12), it seems likely that exoprotein secretion would require large conformational changes. Consistent with this notion, electrophysiology and electron paramagnetic resonance spectroscopy experiments on the *Bordetella pertussis* TpsB protein FhaC indicated that the β-barrel cycles between a closed channel state and an open channel state, which is favored in the presence of its exoprotein substrate filamentous hemagglutinin (FHA), in which H1 moves from the lumen of the FhaC β-barrel to the periplasm (31). In a follow-up study, a similar approach demonstrated conformational changes in L6 that involve the disruption of interactions with the inner wall of the β-barrel to modulate channel opening (34). Conformational changes in the membrane-proximal POTRA domain were also affected by the presence of FHA, which had previously been shown to bind to the POTRA domains (44). Taken together, the results suggested a dynamic interaction between the substrate, the POTRA domains, and L6 that plays a key role in the transport reaction.

Significant insight into the translocation path of a TpsA exoprotein and a model for the mechanism of translocation emerged from a study in which the formation of disulfide cross-links between cysteine residues engineered into FHA fragments that were stalled at different stages of secretion and FhaC was monitored (4) (see **Figure 4**). As expected, the results indicated that the conserved N-terminal secretion domain (or TPS domain) of FHA interacted sequentially with the POTRA domains, the inner β-barrel surface, and the extracellular loops of FhaC. While the cross-linking data implied that the substrate does not enter the FhaC β-barrel by a single defined path, they showed that it interacts primarily with extracellular β-strands 5–8 (B5–B8) and loop 5 (L5) at the

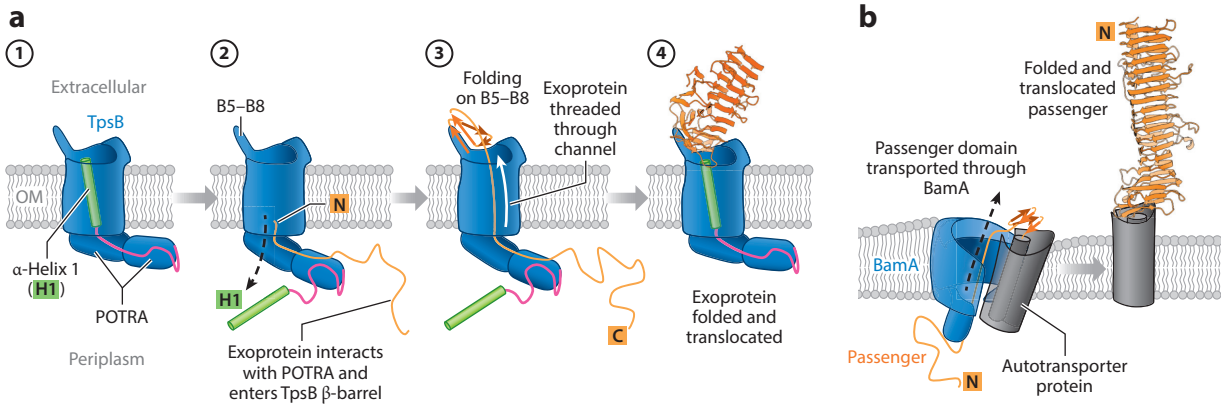

Figure 4

Models of polypeptide translocation by Omp85 proteins. (*a*) Export of exoproteins by the TpsB Omp85 subfamily (two-partner secretion). (Step 1) The TpsB β-barrel lumen is a channel that is plugged by an N-terminal α-helix (H1; *green*). H1 is linked to POTRA domain 1 (the linking region is shown in *pink*). (Step 2) H1 exits the channel and the N terminus (N) of the unfolded exoprotein (*orange*) interacts with the POTRA domains and the lumen of the TpsB β-barrel. (Step 3) The N terminus of the exoprotein begins to fold into a β-helix at the cell surface with the assistance of extracellular TpsB β-strands 5–8 (B5–B8). (Step 4) The folding of the β-helix energizes translocation through the channel and eventually leads to the complete export of the exoprotein. (*b*) Export of autotransporter passenger domains by BamA. During an intermediate stage of assembly in which BamA forms a hybrid-barrel with the autotransporter β-barrel, the unfolded passenger domain (*orange*) is translocated through the lumen of the BamA β-barrel in a C- to N-terminal direction. Similar to exoprotein transport, the folding of the passenger domain into a β-helical structure at the cell surface is believed to energize the export reaction. Abbreviations: OM, outer membrane; POTRA, polypeptide translocation associated.

exit site of the β-barrel. The observation that the shortening of B5–B8 impairs FHA secretion strongly suggests that the extracellular β-sheet plays an important role in the translocation reaction. Consistent with this interpretation of the data, surface-exposed β-strands present within the autotransporter β-barrel domain can also aid the folding of autotransporter passenger domains (129). Taken together with the studies mentioned above, the results led to a model in which the binding of the TPS domain of an exoprotein to the TpsB POTRA domains stabilizes an open conformation in which H1 moves from the lumen of the β-barrel to the periplasm (**Figure 4**). After the N terminus of the exoprotein is threaded through the pore, specific β-hairpins transiently bind to B5–B8, which facilitates folding possibly by β-augmentation. Formation of a β-helical structure prevents the backsliding of the exoprotein through the TpsB channel and promotes completion of the translocation reaction.

Recent work has demonstrated that BamA can also function as a translocase that facilitates the secretion of the large passenger domains of autotransporter proteins. An analysis of intermolecular disulfide bonding shows that the passenger domain traverses the BamA β-barrel lumen near the site of the β1–β-signal interaction in the hybrid-barrel assembly intermediate formed between BamA and the autotransporter (20) (see **Figure 4**). Because passenger domains are similar to TpsA exoproteins in that they almost always fold into β-helices, the stepwise folding of the polypeptide on the cell surface has been proposed to help drive translocation (60, 89). In principle, Omp85–substrate hybrid-barrel structures might be used more generally as channels to promote the surface localization of large loops found in other OMP families, but this possibility remains to be tested. A study published in 2021 has suggested that BamA segments located near the β-seam might also be involved in the import of specific toxins into *E. coli* (14).

The unique features of Toc75 strongly suggest that it promotes the transport of proteins from the cytoplasm into chloroplasts by a completely distinct mechanism. Unlike TpsB proteins, Toc75 translocates a wide variety of structurally unrelated proteins. In addition, Toc75 forms a stable TOC complex with two integral membrane proteins, Toc34 and Toc159, in a unique stoichiometric ratio of approximately 3:3:1–4:4:1 (9, 64, 99). Toc34 and Toc159 are GTPases that face the cytoplasm and that bear no relationship to proteins found in bacteria (24, 99). Available evidence indicates that Toc34 and Toc159 are the primary transit peptide receptors that regulate the early steps of import by using their GTPase activities to ensure the fidelity of targeting (7, 93, 126). Although it is clear that Toc75 plays an important role in translocation, its mechanism of action is poorly understood. Electrophysiology experiments in which Toc75 is reconstituted into planar lipid bilayers show that the protein exhibits cationic-selective channel activities that are altered by the presence of transit peptides that target proteins to the TOC (42, 43). The pore size was originally predicted to be ∼14–26 Å, which is sufficiently large to transport largely unfolded polypeptides, but the recent observation that folded polypeptides can be imported has suggested that an ∼30–35 Å pore may form through the transient joining of two adjacent Toc75 β-barrels into a structure that is analogous to the hybrid-barrels formed between Omp85 integrases and their substrates (23, 24). Elucidation of the function of Toc75 has been complicated by conflicting reports in which the POTRA domains have been localized to both the cytoplasm and the IMS. Nevertheless, the finding that the POTRA domains bind to a chloroplast preprotein suggests that they may play a role in promoting protein translocation that is similar to the role played by homologous domains in the TPS system (84).

4. EVOLUTION AND DIVERSITY OF THE Omp85 PROTEIN FAMILY

Two lines of evidence suggest that a BamA-like protein is the founding member of the Omp85 family. First, the protein plays an essential role in the physiology of gram-negative bacteria (from

which eukaryotic organelles are derived) and is universally conserved (39, 119, 128). Second, BamA is the only Omp85 protein that has been directly shown to promote both membrane protein integration and protein translocation reactions (20). Given that there is significant sequence divergence between the BamA and TpsB families (39), it seems unlikely that the nonessential TpsB family emerged first and that the BamA family acquired a translocation function later as a result of convergent evolution. Based on current evidence, the TpsB family and the TamA/TamL family probably branched off of a BamA-like ancestor that had both integrase and translocation functions to evolve more specialized secretion functions and to augment the function of BamA to compensate for deficiencies in BAM that arose in specific organisms or biological niches. Unlike TamA and TamL, TamB is widely distributed across gram-negative lineages and appears to have arisen early during bacterial evolution and later co-opted into TAM (40). The C terminus of TamB has a conserved β-taco structure that is potentially analogous to the β-sheet-based structures that are acted upon by BamA and TpsB proteins and that may play an important functional role (59).

Perhaps the most intriguing members of the Omp85 superfamily in bacteria are largely uncharacterized subtypes that contain identifiable Omp85 β-barrel domains (Pfam19143) but dramatically different N-terminal domains (39). The Omp85 lipoproteins (found in the phyla *Bacteroidetes* and *Chlorobi*) contain three POTRA domains but also sequence features that predict the presence of an N-terminal lipid anchor. A tether of this sort would likely constrain the movement of the POTRA domains that has been shown to be important for BamA function (122). The putative lipid anchor is especially curious in light of the observation that many lipoproteins in the *Bacteroides* are surface exposed (127). Another widespread class of Omp85 proteins contains one POTRA domain and a domain containing homology to patatin-like phospholipases (PLPs) (39). One study provided evidence that the PLP domain of PlpD is released from the cell surface, presumably after it is secreted through the covalently linked β-barrel domain (95). Omp85 families that contain no POTRA domains but instead possess N-terminal WD40-like or metalloprotease domains have also been identified. Although it seems unlikely that the β-barrel domains of these proteins simply serve as membrane anchors for periplasmic proteins that mediate basic physiological functions, the role of the β-barrels remains to be determined. Indeed, the existence of Omp85 proteins that lack an N-terminal domain provides strong evidence that the β-barrel itself can be functionally significant either as a free protein or perhaps as a component of a larger complex. It is certainly possible that β-barrel-only Omp85 proteins aid OMP assembly simply through lateral openings or by altering the structure of the OM.

It is likely that mitochondrial and chloroplast Omp85 proteins evolved from a BamA-like protein to adapt to the environment of eukaryotic cells and the nature of the substrates that they act on. It is noteworthy, however, that the POTRA domains of OEP80 may have changed orientation during the evolution of Toc75 (108). The TOC machinery is connected to a complex that transports proteins across the IM [the translocase in the inner chloroplast membrane (TIC)] by Tic236, an essential protein that is homologous to TamB (10). An evolutionary analysis indicates that Tic236 coevolved with Toc75 throughout the plant lineage and that core components of the chloroplast import machinery therefore likely evolved from a BamA/TamB machine present in ancient cyanobacteria. This observation is especially remarkable because BAM and TAM generally catalyze OMP membrane integration and polypeptide export reactions, whereas the TOC-TIC supercomplex mediates a protein import reaction that moves proteins in the opposite direction. The implication is not only that the Omp85 superfamily recruited new factors during the course of evolution but also that family members and associated proteins that arose early could also be repurposed.

5. THE ENERGETICS OF PROTEIN TRANSPORT BY THE Omp85 SUPERFAMILY

Because the bacterial periplasm lacks ATP and there is no membrane potential across the OM, the sources of energy used by BAM, TAM, and TpsB proteins to move client proteins into or across the membrane have remained a fascinating and enduring mystery. It has been known for many years that the bacterial OM differs from many other biological membranes in that it is extremely rigid (47, 115). Remarkably, a study published in 2018 showed that the OM (rather than the cell wall) is the predominant load-bearing element in gram-negative bacteria (94). Both the high protein concentration in the OM (OMPs have been estimated to fill at least half of the total volume of the OM) and LPS molecules, which are strongly held together by salt bridges with divalent cations, appear to contribute to membrane rigidity (5, 72, 80). Based on evidence that a mutation that truncates *E. coli* LPS and thereby increases membrane fluidity reduces BAM activity, the mechanical properties of the OM have been proposed to play a critical role in BAM function (110, 111). This idea was compellingly supported by a structural and biochemical study published in 2022 in which multiple stages of the folding of an OMP were observed (21). At an early stage, an open-outward conformer of BamA held the β-signal of an open OMP β-sheet at an angle that causes deflection of the membrane (**Figure 3**). At a later stage, the substrate formed a more closed hybrid-barrel with BamA but no membrane deflection was detected. The structures implied that BamA could harness the intrinsic tension in the OM as an energy source to force the β-sheets of incoming OMPs to close into β-barrels. Biochemical experiments showed that the rate of OMP assembly by BAM could be significantly slowed by indirectly relaxing OM tension and vice versa (21). The implication of this study is that the macrostructure of the bacterial OM can store energy that is useful at a molecular scale to power the functions of at least one Omp85 protein.

Two lines of evidence suggest that energy derived from the cytoplasm or the membrane potential across the IM might also play a role in the insertion of proteins into the OM. First, biochemical and cryo-EM studies indicate that the periplasmic domains of SecDF and YidC, two components of the Sec machinery in the IM, contact the periplasmic components of BAM and form an extended intermembrane complex (3). The data suggest that conformational changes in SecDF driven by the proton-motive force in the IM drive the passage of OMPs to BAM and possibly facilitate membrane insertion. Second, the observation that TamB is essential for TAM function and contains a conserved α-helix that anchors it in the IM (102) raises the possibility that the protein obtains energy from the proton-motive force that activates a lever arm in the TamA POTRA domains (or pushes against TamA to deform the membrane) to facilitate OMP insertion (101, 103).

In the absence of an obvious energy source, it has long been thought that translocation through TpsB transporters is driven by a vectorial folding process in which small unfolded segments of an exoprotein diffuse across the OM and then fold in a stepwise fashion into β-helical segments that cannot slide backward through the transport channel. Consistent with this notion, single-molecule atomic force microscopy experiments have shown that FHA unfolds in a stepwise, hierarchical process (2). With the use of this approach, a mechanically resistant, conserved N-terminal subdomain (which is the first segment of the protein that is secreted) was identified. Available evidence suggests that the passenger domains of autotransporters, which also fold into a β-helix, are secreted by a similar mechanism (60). Like the N terminus of TpsA proteins, the C terminus of autotransporter passenger domains (which likewise is the first segment of the polypeptide that traverses the OM) appears to function as an autochaperone that plays an especially important role in nucleating folding (83, 89). Curiously, mutations that impair the folding of the middle portion of a passenger domain do not affect secretion, and the surprising observation that an intrinsically disordered

polypeptide that replaces a native passenger domain is secreted efficiently indicates that sequential folding is not the only possible source of energy that drives secretion (61, 62). Other factors, such as charge interactions between secreted polypeptides and membrane lipids or the Donnan force across the OM, may also promote secretion. In any case, the remarkable similarity between TpsA proteins and autotransporter passenger domains implies that β-helical structures have a selective advantage for export reactions that are mediated by Omp85 proteins.

The energetics of protein transport through Toc75—in contrast to the transport of proteins through Omp85 proteins in bacteria—is well understood. Two IM proteins, Tic40 and Tic110, form a scaffold for the assembly of an ATP-dependent motor that drives the import of proteins into the stroma (11, 54). The motor itself contains members of the Hsp70 (cHsp70), Hsp90 (Hsp90C), and Hsp100 (ClpC/Hsp93) families of chaperones (22). Although the exact function of each protein is not yet clear, each protein appears to play a key role in the import process (55, 67, 74). The energetics of insertion of proteins into the OM of chloroplasts and mitochondria has not been investigated but may involve conformational changes in the factors that link Omp85 proteins to the IM.

6. FUTURE OUTLOOK

Although the Omp85 superfamily was discovered many years ago, research on these proteins has intensified recently. The renewed interest in these proteins can be attributed both to technological advances (e.g., in cryo-EM) that have significantly increased the feasibility of studying membrane proteins and to an increased recognition of the fundamental importance of Omp85 proteins in essential biological processes and in human health and disease. Although remarkable insights into the function of the Omp85 superfamily have emerged over the past few years, a multitude of questions remain. The role of accessory proteins in modulating the function of Omp85 proteins, especially factors such as the essential BamD lipoprotein and the envelope-spanning TamB protein, is an enigma that should be the focus of future experiments. The degree to which BamA (and perhaps TamA) is a multifunctional protein should also be further investigated by examining the assembly of various OMPs in diverse bacterial species. To fully elucidate the mechanism by which TpsB proteins, Toc75, and other Omp85 translocases promote protein export or import, it would be useful to trap secretion/import intermediates that are analogous to the OMP assembly intermediates that have proven to be of great value in clarifying the function of Omp85 insertases. Because the low-resolution structures of the large TOC complexes were generated nearly 20 years ago, it would also be exciting to reexamine these complexes using state-of-the-art cryo-EM methods to identify interactions between Toc75 molecules and accessory components. The energetics of protein transport into and across the bacterial OM remains poorly understood, but evidence published in 2022 that the membrane itself can, at least in part, power OMP folding should be replicated by examining various OMPs in multiple organisms that inhabit different environments that might affect the properties and/or composition of the OM. Furthermore, trans-envelope Sec-BAM, TAM, and TIC-TOC supercomplexes have been only recently discovered and their biological relevance should be further investigated with a particular focus on the possible role of conformational changes in the IM components in providing energy across the periplasm (or the IMS). Finally, there is a need to better understand the structural and functional differences between bacterial Omp85 proteins (especially BamA) and human mitochondrial Sam50 to promote the rational design of highly specific antimicrobial therapeutics that do not interfere with mitochondrial function.

It should of course also be of great interest to explore the functions of the families of Omp85 proteins that have not yet been characterized. At the moment, it is completely unclear why Omp85

proteins that contain putative enzymatic domains, N-terminal lipid anchors, or nothing more than a β-barrel have evolved. Resolving these mysteries will not only help define the functional range of Omp85 proteins but also provide fascinating new insights into the properties of the Omp85 superfamily as a whole.

DISCLOSURE STATEMENT

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