β-Barrel Membrane Protein Assembly by the Bam Complex

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Abstract

β-barrel membrane proteins perform important functions in the outer membranes (OMs) of Gram-negative bacteria and of the mitochondria and chloroplasts of eukaryotes. The protein complexes that assemble these proteins in their respective membranes have been identified and shown to contain a component that has been conserved from bacteria to humans. β-barrel proteins are handled differently from α-helical membrane proteins in the cell in order to efficiently transport them to their final locations in unfolded but folding-competent states. The mechanism by which the assembly complex then binds, folds, and inserts β-barrels into the membrane is not well understood, but recent structural, biochemical, and genetic studies have begun to elucidate elements of how the complex provides a facilitated pathway for β-barrel assembly. Ultimately, studies of the mechanism of β-barrel assembly and comparison to the better-understood process of α-helical membrane protein assembly will reveal whether there are general principles that guide the folding and insertion of all membrane proteins.

Contents

OM: outer membrane

IM: inner membrane

LPS: lipopolysaccharide

Sec: the inner membrane protein complex that inserts integral α-helical membrane proteins and translocates unfolded periplasmic, outer membrane, and extracellular proteins

OMP: outer membrane protein

Lol proteins: the proteins that extract OM lipoproteins from the IM, transport them across the periplasm, and insert them into the OM

Bam: the outer membrane protein complex that receives unfolded OMPs in the periplasm, folds them into β-barrels, and inserts them into the membrane

INTRODUCTION

There are only two known classes of integral membrane proteins in all prokaryotes and eukaryotes: α-helical proteins and β-barrel proteins. Because polypeptides consist of amide bonds, which are polar, all membrane proteins must internally satisfy the hydrogen bonds of the peptide backbone. Helical and β-barrel membrane proteins satisfy this requirement in different ways: α-helices make hydrogen bonds between proximal residues, whereas distal residues must form hydrogen bonds to close a β-sheet into a cylindrical barrel. Consequently, these proteins must be assembled in different ways. Hydrogen bonds can form sequentially as a polypeptide folds into a helical structure, and provided that the side chains of the amino acids are hydrophobic, the folded helix can be inserted into the membrane. A helical bundle can then form by association of the individually inserted α-helices. In contrast, β-barrels do not have a fully hydrophobic exterior until their tertiary structure is complete. The folding and membrane insertion of β-barrel proteins are therefore likely coupled, but the mechanism remains unknown.

The cellular machines that assemble α-helical and β-barrel proteins contain components that have been conserved from bacteria to humans. The majority of membrane proteins are α-helical, and their assembly has been the subject of much study (1, 2). β-barrel proteins are found in the outer membranes (OMs) of Gram-negative bacteria as well as in the mitochondria and chloroplasts of eukaryotes. In this review, we describe the components of the β-barrel assembly pathway and the current understanding of how they function together to facilitate the folding and insertion of these proteins. We focus on the pathway in *Escherichia coli* and highlight the features of this pathway that have been conserved across prokaryotes and in higher eukaryotic systems.

Gram-negative bacteria, including *E. coli*, contain both types of integral membrane proteins, segregated between two membranes. These bacteria contain a double membrane cell envelope (3). The cytoplasmic inner membrane (IM) is a phospholipid bilayer containing integral α-helical proteins and peripheral lipoproteins, which carry out energy-driven transport processes. The OM is an asymmetric bilayer composed of phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet; it is not energized but also contains lipoproteins and integral β-barrel proteins, which create pores in the membrane to allow nutrients and solutes into the cell and waste products out of the cell. The aqueous compartment between these membranes is named the periplasm, and it contains the peptidoglycan cell wall and soluble proteins. Since the 1960s when this cellular architecture was first revealed by electron microscopy (4), the biogenesis of the two different membranes has been of interest, and the machines that assemble proteins in these membranes have been identified. The Sec (secretion) machinery assembles α-helical proteins in the IM and is responsible for translocating periplasmic and outer membrane proteins (OMPs) from the cytoplasm (1, 2, 5, 6). The Lol (localization of lipoprotein) machinery transports lipoproteins from the IM to the OM (7), and the Bam (β-barrel assembly machine)

complex folds and inserts β-barrels in the OM (8–10). *E. coli* therefore serve as a simple model system in which the assembly of all the known classes of membrane proteins can be studied.

CYTOPLASMIC SYNTHESIS, TARGETING, AND SECRETION

All integral membrane proteins are synthesized in the cytoplasm with N-terminal signal sequences that direct them to the secretion machinery in the IM (11). However, following their synthesis, α-helical and β-barrel proteins are delivered to the Sec machine by distinct pathways (**Figure 1**). IM proteins are cotranslationally targeted to the Sec machinery. As these proteins are synthesized on the ribosome, a protein-nucleic acid complex, the signal recognition particle, binds to their N-terminal signal sequences and transports them to the Sec machine. OMPs, by contrast, are posttranslationally directed to the Sec machine (1, 12). To prevent cotranslational secretion of these proteins, an additional protein called trigger factor (TF) competes with the signal recognition particle for binding to their signal sequences as they emerge from the ribosome (13–18). The chaperone protein, SecB, then binds to OMPs as they elongate and subsequently directs them to the Sec machine (19, 20). Therefore, although both α-helical and β-barrel membrane proteins are directed to Sec, they are handled differently in the cytoplasm to ensure their proper targeting and assembly.

Although membrane proteins are more stable in the hydrophobic membrane environment, in all cells, protein complexes facilitate their insertion into membranes. The Sec machine inserts IM proteins using the energy provided by their synthesis on the ribosome. The mechanism by which these proteins are inserted into the membrane has received considerable attention (1, 21). Many of the details still require clarification, but the general model suggests that α-helical segments fold and are individually released laterally into the membrane through a gate in the channel (22). IM proteins therefore are never exposed to an aqueous environment during their biogenesis. OMPs are translocated through the Sec pore using the energy provided by an ATPase, SecA (23). The posttranslational targeting of nascent β-barrel proteins and the lower hydrophobicity of their sequences allow them to be translocated more rapidly, but this requires that they be kept in a soluble folding-competent state (24). These proteins are translocated in an unfolded state and then interact with chaperones in the periplasm to prevent them from aggregating or misfolding in this second aqueous compartment. There is no ATP outside the inner membrane; therefore, the proteins responsible for maintaining OMPs in folding-competent states in the periplasm and assembling them in the OM likely do so without using energy.

TF: trigger factor

Chaperone: a soluble protein that prevents unfolded proteins from misfolding or aggregating before they adopt their native state

PERIPLASMIC TRANSPORT

Proteins with a propensity to form β-sheet structures are also prone to aggregation given the stability of amyloid-like, multimeric structures. To transit the periplasmic compartment in an unfolded state, chaperones are required to bind OMPs after they transit the Sec channel and have their signal sequences cleaved by the signal peptidase (25). The periplasmic chaperone, SurA, has been shown to transport the bulk mass of OMPs to the OM. A parallel pathway that relies on two other periplasmic proteins, Skp and DegP, has been shown to compensate for the absence of SurA and may be more important for handling proteins that have fallen off the efficient assembly pathway (26).

SurA consists of an N-terminal domain, two peptidyl-prolyl domains (P1 and P2), and a C-terminal domain; crystal structures reveal that the N- and C-terminal domains and the P1 domain form a globular core with a crevice that can accommodate extended peptides. The P2 domain is connected to this core by 30-Å flexible linkers and demonstrates PPIase activity in vitro (27–29a). The core domain contains the chaperone activity of the protein and is structurally similar to the C-terminal domain of TF (14, 28). This striking structural similarity may reflect the fact that SurA and TF have both

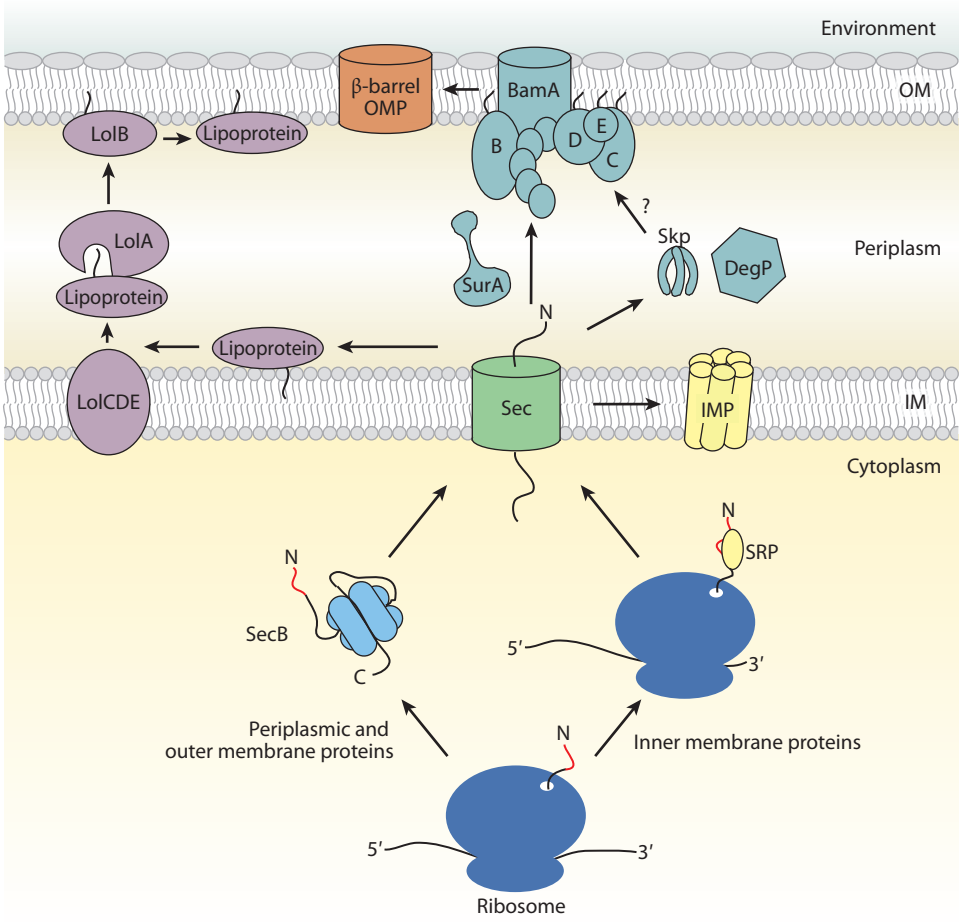

Figure 1

Cell envelope protein biogenesis. All proteins destined for the periplasm and two membranes are ribosomally synthesized in the cytoplasm. Inner membrane (IM) proteins (IMPs) are cotranslationally directed to Sec and inserted into the membrane, whereas periplasmic and outer membrane proteins (OMPs) are posttranslationally translocated. Soluble periplasmic proteins then fold in this second aqueous compartment. Outer membrane (OM) lipoproteins are lipidated at the outer leaflet of the IM and then transported to the OM by the Lol pathway. β-barrel OMPs transit the periplasm in unfolded states with the help of chaperones (primarily SurA) and are then folded and inserted into the OM by the five-protein Bam complex. BamA, -B, -C, -D, -E, β-barrel assembly machine components; DegP, periplasmic protease and putative chaperone; LolA, -B, -CDE, localization of lipoprotein machinery components; Sec, secretion machinery; SecB, cytoplasmic chaperone; Skp, periplasmic chaperone; SRP, signal recognition particle; SurA, periplasmic chaperone.

evolved to recognize features of unfolded proteins and prevent them from misfolding in their respective compartments.

SurA has been shown to bind to model peptides and unfolded OMPs with low micromolar affinity and to prefer substrates rich in aromatic residues arranged in alternating sequences (Ar-X-Ar) (30–32). These sequences are much more commonly found in OMPs than in soluble or IM proteins (30, 32). Strains lacking SurA exhibit defective OM phenotypes and have decreased levels of the major OMPs,

including OmpA, LamB, OmpC, and OmpF (26, 29, 33, 34). Kinetic analysis of the assembly of LamB indicated that SurA is important in the transformation of unfolded LamB monomers to folded monomers and affects the rate of signal sequence cleavage, suggesting that SurA may interact with unfolded OMPs while they are translocating across the IM (35). Although the bulk mass of OMPs are transported by SurA, it is not an essential protein, and most OMPs can utilize other chaperones (34).

The minor periplasmic chaperone, FkpA, also possesses separable PPIase and chaperone activities, but unlike SurA, genetic inactivation of this protein does not produce observable defects (36–39). However, simultaneous deletions of FkpA and members of the Bam complex produce synthetic phenotypes, and a strain containing a quadruple deletion of SurA, FkpA, and two other periplasmic PPIases, PpiA and PpiD, exhibits diminished growth and increased antibiotic sensitivity relative to the single deletions (39, 40). FkpA thus clearly plays a supporting role under normal growth conditions but may be more important under stress conditions when efficient OMP assembly is critical. Therefore, it may be useful to understand the role of this protein in relation to the major OMP chaperones and to determine whether it can deliver unfolded substrates to the assembly machinery in the OM.

Skp has received considerable attention as an OMP chaperone, but its precise role is also not clear, and several hypotheses about its function have been generated. Skp (seventeen kilodalton protein) is a trimer in its functional form and has been shown to bind denatured OMPs (41, 42). Each Skp monomer contributes an α-helical "tentacle" domain to the "jellyfish" trimeric structure to produce a large hydrophobic cavity that can accommodate molten globule states of OMPs (43). Recent studies have demonstrated that the β-barrel domain of OmpA is bound in an unfolded state within the Skp cavity while the periplasmic domain of OmpA remains outside the cavity and can fold independently (44, 45). The fact that Skp can be cross-linked to the Sec machine in vivo has led to the hypothesis that it acts early in the biogenesis of OMPs and then hands them to SurA for delivery to the OM (46, 47). However, a direct interaction between Skp and SurA has not been observed, and this hypothesis is difficult to reconcile with the genetic evidence that these proteins function in separate parallel pathways. Either protein can be deleted individually, but the double deletion is lethal (26).

Conversely, others have suggested that Skp may act late in the assembly pathway because it contains a putative LPS-binding site, and LPS, in concert with Skp, has been shown to improve the efficiency of OmpA assembly into lipid bilayers (41, 43). OmpA folding was also shown to depend on the pH of the solution and the negative charge of lipids in the membrane (48). It is difficult to evaluate whether the LPS-facilitated assembly in this in vitro system is relevant in the in vivo system where the Bam complex participates in OMP assembly. However, the possible involvement of LPS in Skp function may suggest that Skp could be more important under conditions when the cell experiences stress. Such stress might also result in defects in LPS biogenesis that would allow LPS to interact with Skp; under normal conditions, LPS is only found in the outer leaflet of the OM and thus would not be expected to encounter Skp (49). The possibility that Skp primarily sequesters substrates that have fallen off the more efficient SurA folding pathway is attractive given that Skp is believed to function in the same pathway as the OMP degrading machine, DegP (26).

The accumulation of misfolded or aggregated proteins in the periplasm would be toxic; therefore, cells have developed intricate mechanisms for recognizing the presence of such species and activating stress responses, which produce proteins that sequester and degrade the misfolded proteins. All of the periplasmic chaperones and the Bam complex in the OM are regulated by σ^E cell envelope stress response (40, 50–53). The expression of the periplasmic protease, DegP, is tightly controlled by the σ^E and Cpx regulons (54), and its activity is further regulated by the oligomeric state of the protein.

The resting state of the protein is hexameric, but upon interaction with an unfolded OMP, it can convert to large cage-like structures containing either 12 or 24 monomers (55–57). The protease is inhibited in the hexameric state by a loop in a PDZ domain from a neighboring monomer; in the 12- and 24-mer structures, this loop is displaced from the active site. The cage-like structures degrade misfolded proteins, and formation of the active protease increases with DegP concentration (55, 57). A cryo-electron microscopy structure of the 12-mer revealed a folded OMP monomer contained within the cage. This observation and the finding that the 24-mer associates with lipid membranes have led to the hypothesis that DegP acts as a chaperone and may be able to directly insert folded OMPs into the OM (56). However, this hypothesis needs to be tested and shown to be relevant in vivo. If these folded, caged species are physiologically relevant, they are likely part of a minor assembly pathway, because DegP is not an essential protein, but the Bam complex is essential.

The cell has evolved similar proteins to handle unfolded and misfolded proteins in the cytoplasm and periplasm. SurA is structurally similar to TF, and the large cage-like DegP structures are reminiscent of the cytoplasmic GroEL chaperone and proteasome degradation machineries (14, 57). Clearly, there are conserved mechanisms that maintain proteins in folding-competent states and remove those that fall off the folding pathway, but the details of how the unfolded OMPs in these two pathways are managed still need to be clarified. It has recently been demonstrated that SurA can deliver unfolded substrates to the Bam complex (58), but the ability of other chaperones (including the minor ones) to function in this manner needs to be determined. The stoichiometry of the binding of SurA to its substrates could reveal how it maintains their folding-competent state and whether it could actively participate in folding. It also remains unclear how Skp and DegP function together to handle proteins that fall off the efficient folding pathway and whether those proteins can reenter the folding pathway. Finally, it will be interesting to determine if the features of the unfolded substrate that are recognized by the chaperones are also important for recognition by the Bam machinery.

OUTER MEMBRANE RECOGNITION AND ASSEMBLY

The assembly steps, which occur once OMPs have reached the OM, are the least well understood, but in the past 10 years, significant progress in this area has been made as the multiprotein complex responsible for facilitating these steps has been identified. Studies of the structure and function of this complex are beginning to address the fundamental questions of how unfolded OMPs are recognized as β-barrels at the OM and are subsequently assembled into their folded structures and inserted into the membrane.

Identification of the Assembly Machine in the Outer Membrane

Identifying the factors involved in OMP assembly at the OM had been very difficult. Before a large number of bacterial genome sequences were available, OMPs could only be identified by fractionation—that is, by separating the OM from other cellular components and isolating the proteins. This classic approach was able to identify a few highly abundant OMPs, including Braun's lipoprotein, OmpA, OmpC, and OmpF (59–61), but not the low-abundance proteins, which are responsible for assembling the OM.

It has long been assumed that the machinery for assembling proteins in the OM must be essential because the OM is essential. However, when work began on the identification and characterization of the β-barrel assembly machinery, only two OMPs had been shown to be essential, LptD (formerly Imp) and LolB (62–64). LolB is an OM lipoprotein that interacts with a periplasmic chaperone, LolA, which delivers newly synthesized lipoproteins to the OM. LolB thus determines where newly synthesized lipoproteins are inserted. LptD is an

integral OMP identified in genetic selections for altered OM permeability. LptD was the first essential OM β-barrel protein discovered in *E. coli*, and depletion analysis of LptD indicates a role in LPS assembly (63, 65, 66). Certain *E. coli lptD* alleles that encode proteins containing deletions compromise the function of LptD, leading to increased membrane permeability and giving LptD its former name (Imp) (62). The knowledge of these two proteins implied that there should be essential machinery in the OM for assembling integral OMPs, but additional techniques were still required to discover the components of that machine.

The availability of large numbers of bacterial genome sequences enabled the discovery of the central component of the assembly machine, BamA (or originally Omp85), in *Neisseria meningitidis* by searching for sequence conservation and similarity to the chloroplast homolog (67). This protein was then clearly shown to be involved in β-barrel assembly (67). However, the other members of the Bam complex are less well conserved and not all are essential, so it was not possible to identify them from genome sequences. Traditional genetic selections were also not successful in identifying these proteins because the cell typically responds to gross defects in OM permeability by producing mutations in many different genes, which were difficult to deconvolute.

This problem was overcome by a different approach whereby specific chemical conditions provided a specific selection pressure to which the cell responded with specific mutations. This "chemical conditionality" approach has its origins in Pardee, Jacob, and Monod's seminal studies on the *lac* operon (68, 69). They used galactoside analogs to investigate the regulation of gene expression, but the general principles can be expanded to large numbers of different chemicals, which challenge cells in a multitude of different, specialized ways. Because membrane permeability is dictated by membrane composition and structure, this approach potentially could be used to identify factors involved in any aspect of making a membrane, from the beginning (synthesis of components) to the end (assembly) and any of the intermediate steps (targeting).

Specifically, a chemical genetic screen in an *E. coli* strain with a leaky OM identified the second component of the assembly complex, BamB (formerly YfgL) (70). The screen made use of a strain that contains a mutant *lptD* allele, *lptD4213* (formerly *imp4213*), that confers OM permeability defects; this strain was treated with a set of toxic molecules that kill the cells if they can reach their targets in the periplasm. The selection produced suppressor mutations in BamB that specifically decrease the permeability of the OM to the toxic molecule. It is remarkable that these mutations restore the barrier function of the OM such that the toxic molecule used in the selection is excluded but other toxic molecules are not excluded (71, 72). Different small molecules select for mutations in different components of the machinery involved in membrane biogenesis, presumably because each component contributes differently to membrane integrity, and the small molecules reveal those differences. The cell thus tunes the OM permeability to the specific chemical condition used and thereby reveals more subtle effects of OMP assembly factors.

Although the chemical genetic screen identified BamB, it was not trivial to determine its function. It was simple to determine that it is an OM lipoprotein, but it is not an essential protein, and there were no known homologs. Affinity chromatography experiments demonstrated that it stably associates with BamA (formerly YaeT) and three other lipoproteins BamC, -D, and -E (formerly NlpB, YfiO, and SmpA, respectively) and is thus part of an important assembly complex (71, 73, 74). The interactions between the complex components are stable and specific; any member of the complex can be His tagged and used to pull down all of the other components. Moreover, the isolated complex runs as a single band on blue native gel electrophoresis—a more stringent measure of the stability of the interaction of these proteins (58, 75). More recently, Omp85 in *N. meningitidis* was also shown to be part of a complex containing homologs of BamC, -D, and -E and

POTRA domain: polypeptide transport-associated domain

a fourth protein, RmpM (76). Increasingly, it seems clear that many of the components of the complex are found across bacterial species (77–79).

Two of the components of the complex, BamA and BamD, are essential (73, 80). BamA is a predicted integral β-barrel protein with a large periplasmic domain and is discussed in detail in the next section. BamD is an OM lipoprotein that has no known structural homologs but is predicted to contain five or six tetratricopeptide repeats. Tetratricopeptide repeats commonly mediate protein-protein interactions and therefore could be important in interactions among the complex members or with substrate proteins (78). BamD has been shown to mediate the interaction of BamC and -E with BamA (75, 80). OMP assembly is undoubtedly a multistep process that involves binding unfolded substrates, folding them, and inserting them into the membrane. It is an interesting question whether the two essential proteins of the Bam complex function in concert throughout the process or are responsible for completing separate steps.

BamB, -C, and -E are also lipoproteins and are not essential. However, deletions of any of these lipoproteins also produce defects in OMP biogenesis as indicated by lower levels of folded OMPs in the OM, induction of the σ^E stress response, and increased sensitivity to bile salts and antibiotics (40, 73, 74, 80). Deletions of BamB and other proteins in the OMP biogenesis pathway (including the chaperones SurA, DegP, and FkpA) produce synthetic phenotypes and indicate that BamB may play a role in determining the efficiency of assembly (40, 81, 82). BamC and -E are important for the stability of the complex but are less well conserved among bacterial species, and deletions of these proteins produce less severe phenotypes (74, 78). BamA and BamD therefore play the more important roles and likely participate directly in the assembly process of folding and inserting OMPs. BamB, -C, and -E presumably must modulate the activity of the essential proteins or coordinate OMP assembly with other cellular processes.

Structure and Function of BamA

BamA, the central component of the assembly complex, is conserved across all Gram-negative species, and there are orthologs of it in the mitochondria and chloroplasts of eukaryotes. The soluble domain of all of the proteins in this superfamily contains one or more polypeptide transport-associated (POTRA) domains. All BamA orthologs in Gram-negative bacteria contain five POTRA domains, whereas those in chloroplasts and mitochondria contain three and one, respectively (83–86). In bacteria, these POTRA domains are found in the periplasm and receive substrates translocated across the inner membrane. The mitochondrial β-barrel proteins are synthesized outside the mitochondria (in the cytoplasm) but are not inserted directly in the mitochondrial OM from the outside. Instead they are translocated into the intermembrane space before being assembled by Sam50 (the mitochondrial ortholog of BamA) from the inside. The fact that assembly in mitochondria occurs from the same face of the membrane as in bacteria suggests that the process of β-barrel assembly has retained features of the prokaryotic systems from which these organelles originated (10, 85, 87, 88). Given that the structure and function of this protein has been conserved from bacteria to humans, studies of how BamA assembles OMPs could reveal general principles about how all living cells assemble integral membrane β-barrels.

The POTRA domains of BamA have been the subject of crystallographic and NMR structural studies that have shed light on how the domains scaffold the lipoprotein components of the complex, on the importance of certain POTRA domains in the function of the complex, and on how unfolded substrate polypeptides might interact with these domains (75, 89, 90). Each domain consists of approximately 75 residues arranged in two antiparallel α-helices folded on top of a three-stranded β-sheet. The domains have low sequence but high structural similarity to each other; the conserved residues are found primarily in the hydrophobic core of each domain (75, 91). The structures show that

the domains are modular, suggesting that each domain can fold independently; they are connected by linkers and do not appear to make significant contacts with each other. The structures also defined where each domain starts and ends, which made it possible to construct individual deletions of the domains.

The POTRA domains are located in the periplasm and are responsible for binding the four lipoprotein components of the complex. Affinity chromatography experiments using the POTRA deletion constructs indicate that BamC, -D, and -E bind to POTRA domain 5 (P5) and that the association of BamB is affected by the deletion of any of the P2–5 domains (75). Therefore, BamB associates with BamA separately from BamC, -D, and -E. This observation was used to develop methods to overexpress and purify large quantities of the five-protein complex (58). BamA and -B were overexpressed in one strain and BamC, -D, and -E in a second, and the five-protein Bam complex was then reconstructed in vitro (58). The fact that the two essential proteins, BamA and -D, can be expressed separately without interfering with the function of the native complete Bam complex may indicate that these proteins have separate functions, which must be coordinated.

The P3–5 domains are essential in *E. coli* (75). Because P5 is responsible for scaffolding BamD, which is an essential protein, it is not surprising that it is also essential; it is required for assembling a functional Bam machine. The fact that P3 and P4 are essential but do not scaffold an essential component of the machine implies that they play a direct role in the assembly of OMP substrates (see below). The P5 deletion produces an interesting phenotype even in cells simultaneously expressing wild-type BamA; it is toxic (75). Therefore, the P5 deletion can compete with the wild-type BamA in some way that directs OMPs off pathway. One possible explanation for this dominant-negative phenotype is that the deletion allows substrates to start but not complete folding. In this model, the essential function of BamD would occur after the initiation of OMP assembly on BamA.

Because the Bam complex assembles all OMPs, it must assemble BamA as well. Some of the POTRA domains are clearly required for proper BamA assembly. Remarkably, in the absence of full-length BamA, BamAΔP2 is able to assemble itself in the OM and exhibits growth like the wild-type control (75). BamAΔP3 and ΔP4 were stably expressed in the presence of the full-length copy, but they were not detected by Western blot analysis in its absence, suggesting that these variants could not properly assemble themselves and were consequently degraded (75). This observation is consistent with the essentiality of these domains. The deletion of the P1 domain had more moderate effects on viability and OMP levels, and a recent study on the effects of smaller deletions in P1 demonstrated that this domain also affects the assembly of BamA (92). An interesting question is whether this domain participates intramolecularly in the assembly of its own β-barrel and thereby functions as a self-chaperone or affects the assembly of other BamA molecules once it is assembled in the OM.

The essentiality of the POTRA domains in other organisms is still an open question. In mitochondria, the BamA ortholog contains only one POTRA domain, and its essentiality has been debated (93, 94). In the *N. meningitidis* ortholog, sequential truncations of the POTRA domains indicated that only P5 is essential for viability in this organism (95). Interestingly, LPS is not essential in *N. meningitidis*, and this organism also lacks a homolog of BamB (76). The fact that suppressor mutations in BamB were the response to a defect in the machinery that assembles LPS suggests that BamB may help coordinate LPS assembly or the assembly of LptD, which is responsible for LPS assembly (70, 71, 73). Furthermore, it has been suggested that SurA chaperones LptD and that the functions of SurA and BamB are related (34, 35, 40, 71). Thus, the differences in the essentiality of the POTRA domains may reflect the different OM assembly requirements in these two bacterial species. The number of POTRA domains found in the mitochondrial,

chloroplastic, and bacterial orthologs differ but have been conserved within their respective kingdoms; therefore, the structural differences among these proteins must reflect the different requirements of the membranes in which they exist.

Structures of the POTRA domains have provided evidence for a possible mechanism by which they could interact with unfolded OMP substrates and facilitate OMP assembly, and these structures may thus explain the essentiality of P3 (**Figure 2**). Two crystal structures of a construct containing P1–4 and a fragment of P5 were independently determined; in both structures, the fragment of P5 was bound to the β2-strand of P3 in a second monomer (75, 89). The fragment extended the β-sheet of P3 by an additional strand—a binding mechanism that has been termed β-strand augmentation (96). The additional strand was bound in a parallel orientation in one structure (75) and in an antiparallel orientation in the other (89). An NMR study has also shown that OMP-derived peptides can interact with the β1-strand of P1 and β2-strand of P2 and that an α-helical peptide with a sequence derived from the periplasmic maltose binding protein did not interact (90). This binding mechanism appears to be specific for peptides that adopt secondary structures composed of β-strands rather than for a particular amino acid sequence, and therefore it is an attractive mechanism by which OMP substrates could bind to and be recognized by BamA. In fact, the machinery required to sense misfolded OMPs in the periplasm also binds β-strands by β-augmentation (97–99). Because the Bam complex handles a large number of different substrates, it is an interesting question whether it recognizes its substrates through a general motif—like the propensity to form β-structure—or through a specific signal sequence.

The two POTRA domain crystal structures demonstrate that the β-augmentation can occur through a parallel or antiparallel interaction. If the hypothesis is correct that BamA binds incoming OMP substrates by this mechanism, the two orientations may suggest that, during the assembly of an OMP substrate, the POTRA domains bind segments of the OMP in alternating orientations. Furthermore, the two different conformations of the POTRA domains in the determined crystal structures—one fishhook and one more linear—indicate that there is flexibility between P2 and P3 (75, 89). Whether this "hinge" is functionally

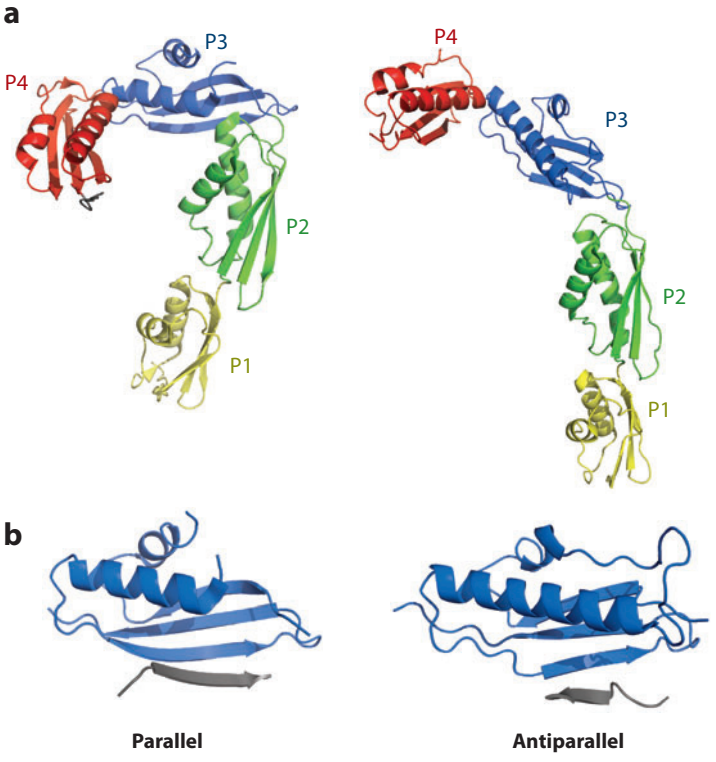

Figure 2

Crystal structures of the polypeptide transport–associated (POTRA) domains of the central component of the β-barrel assembly machine (BamA). (*a*) Very similar fragments of BamA containing the first four POTRA domains and a short peptide from POTRA domain 5 (P5) crystallized in different conformations. The fishhook [Protein Data Bank (PDB): 2qdf] and more linear (PDB: 3efc) structures suggest that there may be a flexible hinge between P2 and P3, which perhaps could help generate β-hairpins in the outer membrane protein substrates of BamA. (*b*) β-strand augmentation interactions observed in both crystal structures. The POTRA domains crystallized as a dimer in both structures in which the short peptide from P5 (*gray*) bound to the β2-strand of P3 (*blue*) of a second monomer extending the β-sheet by an additional strand. P3 bound the peptide in parallel (*left*) and antiparallel (*right*) orientations.

relevant remains to be determined, but if the POTRA domains actively participate in assembling substrates, one would expect them to move during that process. The β-augmentation may initially facilitate the formation of β-structure in the substrate, but the β-strands would still need to be assembled into β-hairpins and ultimately into a closed barrel. This tertiary structure formation would seem to require conformational changes in the assembly machine to facilitate the formation of hydrogen bonds between distal residues along the two edges of the β-sheet that come together to close the β-barrel.

The POTRA domains are in the periplasm and may start the assembly process, but the OMP substrates must ultimately be inserted into the membrane. It seems reasonable to assume that the β-barrel domain of BamA plays a role in these later steps in the membrane. The BamA β-barrel has not been crystallized but is predicted to contain 12 or 16 β-strands (86, 100). The structure of FhaC, a member of the Omp85-TpsB superfamily, has been determined and provides useful hypotheses about how the BamA β-barrel could function (101). FhaC is a component of the two-partner secretion system that secretes the β-helical protein, filamentous hemagglutinin (FHA), in *Bordetella pertussis*. It contains two POTRA domains that are required for secretion of FHA and a 16-stranded β-barrel. A long loop (L6), between strands 11 and 12 of the barrel, is folded back inside the barrel extending to its periplasmic face; this loop is required for secretion and is believed to undergo conformational changes that expose it to the extracellular environment during secretion (101, 102). The working model of FhaC-mediated secretion involves recognition of the unfolded FHA by the POTRA domains, which direct the protein into the β-barrel, where it interacts with loop L6; this interaction causes L6 to flip out of the barrel and drag the FHA protein with it. BamA is also predicted to contain a long loop between two conserved regions of the β-barrel (100, 103), and it would be useful to investigate whether this loop is required for OMP assembly.

Mechanistic Studies of the Bam Complex

It seems reasonable to assume that β-barrel assembly is a multistep process. In order to understand the mechanism of OMP assembly, it is necessary to break the process into steps and determine how the Bam complex facilitates each step. Therefore, it would be very useful to observe substrates binding to the Bam complex at intermediate stages of folding and inserting into the membrane. It is challenging to detect such intermediates or to disrupt the process and observe partially assembled OMPs because assembly is a highly efficient and essential process. Furthermore, mutations in the proteins in the assembly pathway often result in pleiotropic phenotypes characteristic of defective OMs (e.g., lower levels of OMPs in the OM, sensitivity to antibiotics and bile salts, induction of the σ^E stress response), but their specific effects on the recognition, folding, or insertion of OMPs have been difficult to identify. Nevertheless, several recent in vivo and in vitro studies have begun to elucidate aspects of the assembly mechanism; not surprisingly, these studies have provided more information about the early steps of substrate recognition and binding to the Bam complex than the later folding and insertion steps, which are inherently more difficult to understand.

Substrate recognition. The Bam complex must distinguish between its substrates and soluble proteins in the periplasm. Proteins are directed to the IM Sec machine by an N-terminal signal sequence, and studies have addressed whether OMPs are directed to Bam by an analogous mechanism in which features of the substrate's sequence determine its targeting. Those features could relate to the substrate's ability to form β-strands with hydrophobic periodicity (via β-augmentation), could be a specific "signal" sequence of amino acids, or could be combined with features of the periplasmic chaperones, which deliver OMPs.

It has been suggested that a specific amino acid sequence at the C terminus of β-barrels

could be important for recognition by BamA (104, 105). BamA was reconstituted into planar lipid bilayers and shown to exhibit channel activity that could be altered by the addition of denatured PhoE or LamB. Deletion of the C-terminal phenylalanine of PhoE abolished the channel opening activity, and peptides containing the last 12 or 11 residues of PhoE mimicked the activity of the full-length and mutant proteins, respectively (105). Similarly, the Sam complex exhibited channel activity in planar lipid bilayers, and that activity was altered by the addition of a peptide containing a sequence that is present in the C-terminal strand of mitochondrial OMPs (94). The structure of the C terminus of β-barrels is undoubtedly important in their assembly and stability as it is required to close the barrel. In fact, many β-strands in a β-barrel end in aromatic residues such that the assembled barrel contains aromatic "girdles" at the membrane surfaces (106, 107). The channel activity of BamA is clearly affected by aromatic residues, but whether that activity correlates with OMP assembly is unclear. Because proteins are translocated across the IM from their N to C terminus, OMPs would need to be completely translocated across the IM before the Bam complex could recognize them. Perhaps it is worth noting that the five POTRA domains on BamA are of sufficient length to span a significant portion of the periplasm and thus might interact with substrates as they emerge from the Sec machine (89). It remains an open question whether translocation could be coupled to assembly in the OM, but the answer would have clear implications for how OMP substrates are first recognized by the Bam complex.

Chaperones facilitate the transit of substrates across the periplasm, but it is not clear whether they interact directly with the Bam complex or simply maintain substrates in a folding-competent state. SurA has been shown to cross-link to the Bam complex in vivo, and cross-linking to BamA does not appear to depend on the presence of BamB (26, 81). Similar amounts of SurA were cross-linked to BamA in the presence or absence of BamB, and a region of the first POTRA domain of BamA near residue R64 was shown to cross-link to SurA (81, 92). Interestingly, Skp has never been shown to cross-link to the Bam complex in vivo, which is consistent with its less central role in the assembly process (26). These studies suggest that SurA can be in close proximity to BamA but do not reveal whether there is specific recognition of the chaperone or if the chaperone is associated with the Bam complex during folding of a substrate and influences the folding process.

Genetic interactions and kinetic studies support the idea that SurA is involved in the assembly steps that occur at the OM. The synthetic, severely defective phenotype of a strain lacking both SurA and BamB suggests that these proteins play related functions (40, 71). Individual deletions of these proteins also produce identical defects in the kinetics of assembly of LamB—specifically affecting the transformation of unfolded monomers to folded monomers (35). These studies implicate BamB in the initial steps of OMP assembly at the OM; BamB may not be required to recognize and bind SurA-bound substrates (as it is not an essential protein and is not required for cross-linking) but may facilitate their interaction with BamA. Consistent with this hypothesized role, an in vitro reconstitution of OMP assembly indicated that BamB is required for efficient assembly of an OMP delivered by SurA (58). Therefore, OMPs can interact with BamA and can be assembled in the absence of BamB, but this protein dramatically improves the efficiency of the process.

Several pieces of evidence suggest that multiple SurA molecules aid in folding substrates on the Bam complex. The in vitro reconstitution of the Bam complex demonstrated that multiple SurA molecules increased the yield of assembled OmpT, an OMP with protease activity. The amount of active OmpT produced demonstrated a sigmoidal dependency on the concentration of SurA, implying a cooperative effect of the chaperone (58). Furthermore, a crystal structure shows that SurA can bind a model peptide as a dimer, and it has been pointed out that OMPs, including OmpT, contain multiple

sites for SurA binding (108, 109). The involvement of multiple SurA molecules in OMP assembly evokes an attractive model in which sequential dissociation of SurA molecules allows parts of the substrate to begin folding while others remain protected. Consistent with this hypothesis, an autotransporter, EspP, containing a mutation that stalls the secretion of its passenger domain was shown to cross-link to the Bam complex and to the chaperones SurA and Skp; residues in the β-barrel domain cross-linked to BamA, and residues in the passenger domain cross-linked to the chaperones (110). These data are remarkable in that they suggest that this autotransporter is actually assembled by the β-barrel biogenesis pathway and that assembly is a sequential process throughout which the substrate is protected by the Bam complex and chaperones.

Substrate folding. Although most OMPs can fold spontaneously in vitro, different OMPs fold with varying degrees of efficiency depending on the pH, temperature, and lipids used in these systems (111). The Bam complex facilitates the assembly of all OMPs and therefore must overcome these intrinsic differences. As previously discussed, the problem of β-barrel assembly suggests that folding and insertion likely occur in a concerted process. Consequently, it is incredibly challenging to observe and interpret the structures of substrates at different stages. Clearly, the work that has revealed the most about partially assembled states has been that of the assembly of a β-barrel in isolated mitochondria. Using a radiolabeled substrate, Tom40, and blue native gel electrophoresis, it was shown that the substrate first associates with the Sam complex, is then inserted into the membrane, and finally assembles into the multiprotein complex in which it functions. N-terminally truncated variants of Tom40 could associate with Sam but were not subsequently inserted and assembled in the membrane, and deletions in the last strand of the β-barrel impaired the formation of the Sam-bound intermediate (94). A more recent study indicated that other components of the Tom complex (into which Tom40 ultimately assembles) are associated with Tom40 while it is still on Sam (112, 113). Therefore, these studies have established that intermediates exist but also that the insertion and assembly steps are not entirely distinct.

The in vitro reconstitution of the Bam complex activity provides hope that intermediates like those observed in the mitochondrial system can be identified and that the involvement of the components of the Bam complex in the different steps of assembly can be dissected. In this reconstitution, purified Bam complexes were incorporated into liposomes, and as mentioned above, SurA was used to deliver denatured OmpT to the complex. A real-time assay based on the enzymatic activity of folded OmpT demonstrated that in the first few minutes of the experiment the rate of assembly was orders of magnitude faster in the presence of the Bam complex than in liposomes alone or in the presence of subcomplexes lacking BamB or BamCDE (58). This timescale for folding is consistent with that observed in the cell (30 s to several minutes) (35, 114). The yield of folded OmpT produced by the Bam complex is also significantly higher than that produced by the subcomplexes or empty liposomes. Therefore, the reconstitution recapitulates elements of the cellular process and demonstrates that the functions of the Bam proteins are coordinated. The Bam machine provides a facilitated pathway for OmpT assembly that does not require an input of energy and is different from the spontaneous folding of OMPs into liposomes.

The yields of folded protein in the in vitro reconstitution are less than the amount of Bam complex present. Therefore, it is not clear whether the purified Bam complex in this context can perform multiple turnovers—i.e., whether a single Bam complex can fold multiple OmpT molecules. It is difficult to determine whether the observed activity reflects the function of a few very active Bam complexes in the liposomes or whether most Bam complexes are active but each folds only one OmpT molecule. If each complex can only fold one substrate molecule, the assembly process

must inactivate the complex in some way because the complex must function catalytically in the cell. It is important to identify whether the complex is inhibited by its product (i.e., whether folded OmpT is not released from the complex), whether the complex becomes unstable and dissociates as a result of assembly, or whether a conformational change occurs during assembly that must be reset. Any of these possibilities will provide insight into the mechanism of assembly.

Substrate insertion. We have highlighted how β-barrels are intrinsically different from α-helical proteins, but an interesting question is whether there are any general principles that guide the assembly of both classes of proteins. Our understanding of the last step of the assembly of β-barrels, membrane insertion, is perhaps the weakest. It is not clear whether insertion could occur through the β-barrel of a BamA monomer or within a hydrophobic cavity created either by the lipoproteins or by the association of multiple Bam complexes. This question has been similarly debated for the Sec machine (22, 23, 115–118). There is evidence that BamA alone forms aggregates; BamA, overexpressed and refolded from inclusion bodies, was shown to form oligomers (up to tetramers) in vitro by size-exclusion chromatography and blue native electrophoresis. It was consequently suggested that OMPs could be assembled between the BamA monomers and then released into the membrane by dissociation of the monomers (105). This model provides a proteinaceous environment in which to fold OMPs and a mechanism for insertion that does not require breaking any bonds within the BamA β-barrel. However, it is not clear whether BamA assembles into oligomeric structures in vivo; when the five-component Bam complex is isolated from cells, its apparent molecular weight on a blue native gel is consistent with a monomeric complex (58, 75). Size-exclusion chromatography and light-scattering analysis of the overexpressed and purified five-protein complex also indicated that it contains only one copy of BamA (58). The oligomerization of BamA in vitro could be an artifact of the lack of the associated lipoproteins, or perhaps the five-protein complex could oligomerize in vivo.

If the complex functions as a monomer in vivo, insertion would presumably be coordinated by the lipoproteins and occur adjacent to the BamA β-barrel or would require local, transient opening of the BamA β-barrel to allow lateral diffusion in a manner analogous to the Sec machine. Breaking hydrogen bonds between strands of the β-barrel within the membrane to allow lateral diffusion from the lumen of the barrel would at first seem impossible. However, other OMPs that handle substrates that must diffuse in and out of the membrane contain regions in which the β-strands are not completely hydrogen bonded. PagP modifies LPS molecules in the OM, and FadL imports fatty acids from the environment. Both proteins have crenellated β-barrels in which two adjacent strands are not completely hydrogen bonded near the extracellular edge. The substrates are proposed to pass through this lateral opening in and out of the membrane (119–121). In fact, in vitro thermal denaturation experiments have suggested that the BamA β-barrel is significantly less stable than other OMPs, which may be consistent with an incomplete barrel structure (111). It is more difficult to imagine how an OMP could be released through an opening in the BamA β-barrel, but this possibility cannot be ruled out and illustrates the importance of obtaining structural information about this region of the protein.

A MODEL OF β-BARREL ASSEMBLY

The Bam complex facilitates OMP assembly by providing a pathway to folding with a lower energy barrier; the complex dramatically alters the kinetics of OmpT folding in vitro (58). The two essential proteins in the complex must be responsible for the chemistry that occurs, and the three nonessential proteins presumably modulate their activity. The barriers to OMP assembly include removing the chaperones that solvate the unfolded protein in the periplasm,

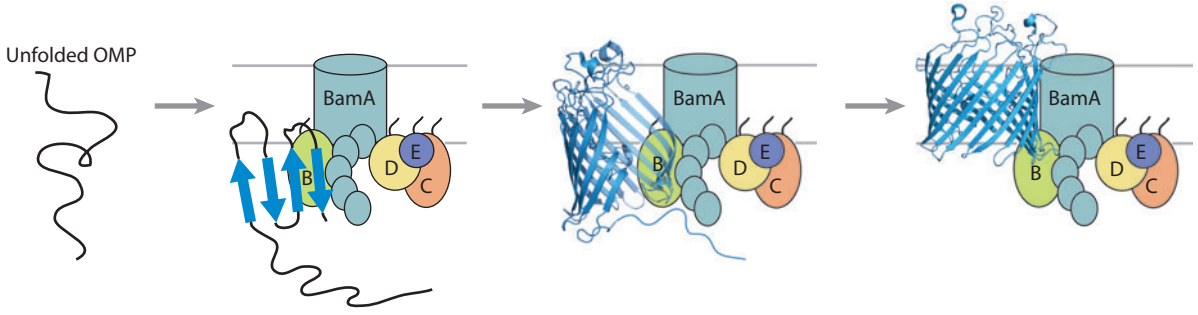

Figure 3

A model of how β-strand augmentation could provide a template for β-barrel folding. Binding of an unfolded outer membrane protein (OMP) to the polypeptide transport–associated (POTRA) domains of BamA by β-strand augmentation initiates β-structure formation. The POTRA domain thereby satisfies the hydrogen bonds on one edge of the β-sheet as folding proceeds. Ultimately, the two edges of the sheet are brought together, and the barrel is inserted into the membrane. BamA, central β-barrel component of the β-barrel assembly machine; BamB, -C, -D, and –E, lipoprotein components of the assembly machine.

forming β-hairpins, bringing the two termini together into a closed conformation, and rearranging lipids in the membrane to insert the folded protein. BamB appears to facilitate the interaction of substrates with BamA perhaps by inducing a more favorable conformation in BamA for SurA to bind (35, 40, 58). The cell can survive without BamB, but OMP assembly is clearly less efficient. β-strand augmentation could then explain how the Bam complex lowers the barrier to structure formation (75, 89, 90). By binding segments of the unfolded substrate, the POTRA domains of BamA initiate the formation of β-strands, thereby paying part of the entropic cost of forming β-structure. If multiple POTRA domains are able to bind β-strands and move relative to one another (as has been suggested) (89), they might bring consecutive strands together to form β-hairpins or bind the first and last strands of the barrel and thereby satisfy the exposed edges of the β-sheet until it is closed into a cylinder and inserted (**Figure 3**).

Although very little is known about the essential function of BamD, it is tempting to speculate that it is responsible for the later steps of insertion into the membrane and possibly for the assembly of oligomeric OMPs into their final states. The fact that BamA and BamD can be expressed separately and recombined into a functional five-protein complex may suggest that these proteins do have separate functions that need to be coordinated (58). Perhaps BamD helps to dissociate a folded OMP from BamA and release it into the membrane so that the substrate ultimately satisfies all of its hydrogen bonds internally rather than continuing to make use of those in BamA. Because BamC and E stabilize the BamA-D interaction, they may thereby help to tightly couple their functions.

We have not addressed how the orientation of OMPs in the membrane is determined or how oligomeric OMPs are assembled. As stated above, OMPs are asymmetric and contain long hydrophilic loops on the outside of the membrane and short turns on the inside. Their orientation could be determined by the direction in which they are delivered to the Bam complex; the N and C termini of OMPs are always found on the inside of the membrane. Thus, if BamA recognizes substrates and initiates folding from the termini, their orientation could be simultaneously established. Alternatively, perhaps the long extracellular loops are specifically directed through the BamA β-barrel in a manner similar to the way FhaC secretes its substrate using a flexible loop in its own β-barrel domain (101). This possibility would allow the hydrophilic loops to reach their final destination without needing to pass through the hydrophobic membrane.

It is not clear whether the Bam complex participates in the assembly of folded OMP monomers into their oligomeric forms or whether folded monomers diffuse within the membrane until they encounter and associate with another monomer. The latter possibility does not require that the Bam complex consecutively fold multiple monomers of the same protein, which would require coordination in the periplasm. Furthermore, there is evidence for the existence of folded monomers of proteins that go on to become trimeric in their native states (35). By contrast, it appears that no process in the cell is unregulated, and more work needs to be done to understand whether the Bam complex coordinates this process or not.

Our understanding of the mechanism of assembly of β-barrel proteins is clearly not at the level of that of α-helical membrane proteins. Structural studies of the Sec machine significantly advanced the study of IM protein assembly, and similar structural information about how the Bam proteins interact with each other and with substrates may be required to discern how the Bam complex functions. Biochemical studies of how substrates interact with the Bam machine and of the kinetics of assembly may help identify steps in the process and the involvement of different components in those steps. Each of those interactions or steps could then become points at which we could interfere with the process and thereby disrupt OMP assembly. The dearth of antibiotics available to treat Gram-negative infections and the essentiality and surface location of the Bam complex make it an attractive new target and underscore the necessity of learning its mechanism.

SUMMARY POINTS

1. β-barrel membrane protein assembly presents inherently different challenges from α-helical protein assembly because the basic secondary structural element, the β-hairpin, contains edges at which the backbone hydrogen bonds are not internally satisfied.
2. β-barrel proteins are exposed to two aqueous compartments during their biogenesis and therefore require chaperones to keep them in a folding-competent state.
3. The central component of the Bam complex, BamA, has been conserved from bacteria to humans.
4. The polypeptide transport–associated domains of BamA are responsible for binding the lipoprotein components of the complex and have an essential function likely related to binding unfolded OMPs by β-strand augmentation.
5. The functions of the Bam proteins are coordinated such that they provide a facilitated pathway for OMP assembly that is different from the spontaneous folding process.

FUTURE ISSUES

1. What are the individual functions of the Bam proteins—especially those of the β-barrel of BamA and the essential lipoprotein BamD?
2. Which periplasmic chaperones deliver substrates to the Bam complex, and how do they do so?
3. How is the Bam complex assembled, and does it function as a monomer or oligomer?
4. How does the Bam complex facilitate the membrane insertion of OMPs?

5. Are other components of the OM (e.g., lipopolysaccharides and other proteins) important for OMP assembly?
6. How is OMP assembly coordinated with LPS and lipoprotein assembly?
7. Could OMP assembly be coupled to an energy-driven process at the inner membrane?
8. Is the Bam complex involved in the secretion of extracellular proteins?

DISCLOSURE STATEMENT

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