HHS Public Access

Author manuscript

EcoSal Plus. Author manuscript; available in PMC 2019 September 01.

Published in final edited form as:

EcoSal Plus. 2019 March ; 8(2): . doi:10.1128/ecosalplus.ESP-0035-2018.

Outer membrane protein insertion by the β-barrel assembly machine

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SUMMARY

Like all outer membrane (OM) constituents, integral OM β-barrel proteins in Gram-negative bacteria are synthesized in the cytoplasm and trafficked to the OM where they are locally assembled into the growing OM by the ubiquitous β-barrel assembly machine (Bam). While the identities and structures of all essential and accessory Bam components have been determined, the basic mechanism of Bam-assisted OM protein integration remains elusive. Here, we review mechanistic analyses of OM β-barrel protein folding and Bam dynamics and summarize recent insights that inform a general model for OM protein recognition and assembly by the Bam complex.

Introduction

The presence in Gram-negative bacteria of an extracytoplasmic outer membrane (OM), which is distinct from the inner membrane (IM) both in constitution and in function, presents a complex topological problem, as all proteinaceous and lipidic OM components are synthesized cytoplasmically (1). In order to reach their destination in the growing OM, these components must translocate across the IM and traverse the aqueous, crowded periplasmic space. This problem is solved through a series of semi-independent and highly conserved transport pathways that coordinate the efficient delivery and integration of all OM constituents.

OM-specific lipopolysaccharide (LPS) is trafficked via a multicomponent trans-envelope protein bridge (2, 3), the LPS transport (Lpt) pathway, which terminates at an OM-integral translocase (LptDE) that incorporates free LPS into the outer leaflet of the OM (4). A transport system that enables retrograde (OM to IM) phospholipid transport has been described (5–10), but the mechanism of anterograde (IM to OM) transport is mysterious and represents an area of active investigation. Periplasmic lipoproteins, which can be anchored to either membrane via N-terminal lipid moieties, are sorted in a sequence-dependent manner to the OM via the Lol system, which extracts IM-associated lipoproteins and shuttles them to the OM via a soluble periplasmic carrier (11). Finally, integral OM β-barrel proteins (OMPs) are translocated in an unfolded form across the IM, ferried to the OM in a non-

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native but folding-competent state via a diverse network of periplasmic chaperones, and integrated into the OM in a manner dependent on a ubiquitous, essential multiprotein complex known as the β-barrel assembly machine (Bam).

Despite the functional and structural heterogeneity observed across OMP families, the *in vivo* folding and membrane integration of all OM β-barrel proteins require Bam, an OM-associated heteromeric complex composed of BamA (itself a β-barrel protein) and a variable number of OM lipoproteins (BamB-F) that bind to and act in concert with BamA to drive the OMP assembly process (1, 4, 12, 13). At present, definitive roles cannot yet be unambiguously assigned to the individual components, and the general mechanism of BamA-dependent OMP assembly remains elusive and controversial. Here, we offer a compendious review of recent inquiry into the mechanism of OM β-barrel folding and its catalysis by the Bam complex.

Bam complex constituents

BamA, the central component of the Bam complex, is composed of a C-terminal β-barrel domain and an N-terminal periplasmic domain that serves as the physical hub of a functional network that includes substrates (14–18), accessory lipoproteins (19–26), periplasmic chaperones (27–29), and proteases (30). This fishhook-shaped domain is typically subdivided into five structurally homologous POTRA domains (31, 32) that may nucleate the early formation of OMP secondary structure through β-strand augmentation (26, 32, 33), and which, together with the Bam lipoproteins, form a cavernous periplasmic ring that circumscribes the vestibule of the BamA β-barrel lumen (24, 34). The OM-integral BamA β-barrel domain is atypical and can be distinguished from canonical membrane β-barrels in three fundamental ways: 1) the interstrand hydrogen bond network that seals β1 and β16 (to complete the barrel) is metastable, allowing transient destabilization of this seam and reversible lateral opening of the barrel (35–38); 2) the β-barrel forms an unusually narrow protein-lipid interface adjacent to the seam that is thought to physically alter the local properties of the OM (37, 39–41); 3) a conserved, essential latching loop (L6) internally braces the BamA barrel interior and globally stabilizes the otherwise thermolabile β-barrel domain (42–46), likely compensating for the metastability at the β1-β16 seam (Fig. 1). The mechanistic implications of these features are discussed in an ensuing section.

BamA is the central catalyst of OMP insertion, but the partner lipoprotein BamD also plays an apparently essential role in the process (13, 14, 16, 17, 20, 47–51). BamD is a solenoid protein thought to serve as a generic receptor for substrate OMPs through recognition of the β-signal, a semi-degenerate C-terminal peptide motif common to all prokaryotic and eukaryotic OMPs (14, 52–60). BamD plays a critical role in the binding and OM localization of OMPs (including BamA), although BamB, a β-propeller protein, has been proposed to perform an overlapping function for some Bam substrates (17, 26, 47). Additionally, commensurate with its role as an OMP receptor, BamD has been implicated in regulation of the conformational dynamics and activity of BamA during the OMP assembly cycle, tentatively linking the recognition and binding of nascent OMP C-termini to conformational changes in BamA that enable OM insertion (14, 48, 61, 62).

The remaining Bam lipoproteins (BamB, BamC, BamE, and the BamC-like α-proteobacteria-specific protein BamF) are variably conserved, are not central to the mechanism of Bam-catalyzed OMP assembly, and instead serve accessory roles that enhance the efficiency of the process for at least a subset of OMPs (13, 63). BamCE associate indirectly with BamA via BamD and play an adjunctive role in the regulation of BamA dynamics and function, potentially by stabilizing the interaction between BamA and BamD following each round of OMP assembly (20, 21, 47, 49, 61, 64–67). BamB interacts with BamA in a BamD-independent manner through direct contacts with multiple POTRA domains (20–22, 25, 26) and has been shown to contribute significantly to the efficiency of substrate assembly for certain OMPs (17, 47, 68–75). We have proposed that BamB (together with the OMP chaperone SurA) influences substrate flux to ensure streamlined assembly of both high-abundance targets (e.g. OmpA, porins) and low-abundance, high-priority targets (e.g. LptDE) (28). Intriguing recent work has also uncovered a role for BamB in the sequestration of Bam complexes into tightly-clustered “assembly precincts” that are proposed to accelerate the assembly and multimerization of abundant OMP species (75).

Bam as a folding chaperone and catalyst of OM protein insertion

A wide variety of OM β-barrel proteins can autonomously fold in hydrophobic environments in accordance with Anfinsen’s dogma. Decades of *in vitro* studies using model OMPs have revealed the following key observations (Fig. 2):

1. In the appropriate hydrophobic context, OMPs spontaneously and rapidly fold into extremely stable membrane integral species (76–89);
2. Native OM phospholipids impose a kinetic barrier to OMP assembly (a critical phenomenon that prevents the lethal assembly of OM β-barrels into the IM) (40, 41, 90–92);
3. The kinetics of OMP assembly can be dramatically accelerated by altering physical properties of the membrane so as to induce local defects (e.g. bilayer thinning or increased curvature) (39, 54, 93, 94);
4. OMP folding is a concerted process in which barrel formation and insertion happen concurrently, and in which all β-strands integrate simultaneously rather than sequentially (79, 95–99).

The fact that β-barrel folding and insertion occurs spontaneously implies a role for Bam in accelerating the intrinsic folding kinetics of OMPs, akin to classical folding chaperones. However, alternative pathways for *in vivo* folding have been surmised that involve the formation of transient chimeric BamA:OMP barrels (37, 100) or elongated substrate β-sheets nucleated at BamA β1 (101) as necessary intermediates. These models have explanatory power but are problematic from a thermodynamic perspective and require unnecessary invention of a distinct and strictly BamA-dependent folding pathway for β-barrel proteins (102). A recent biochemical analysis of mitochondrial β-barrel insertion by the BamA homolog Sam50 yielded observations consistent with a β-strand exchange model (100), however the use of non-native, truncated substrates complicates interpretation, and the available evidence does not rule out more parsimonious alternatives. We argue that existing

evidence better comports with a view of Bam as an OM-adjacent Anfinsen cage that positions client proteins for OM insertion, prevents aggregation, degradation, and off-pathway misfolding, and accelerates the native OMP folding reaction. Numerous studies have established the role of lipid bilayer defects in the acceleration of β-barrel folding kinetics, and BamA has been shown to give rise to such defects (36, 37, 39–41, 103). These observations inform a simple model in which Bam complexes effectively localize client proteins to OM "entry points" generated by the local defects imposed by BamA itself, removing the primary barrier to rapid OMP folding and enabling OM biogenesis on physiologically relevant time scales (15, 36, 90, 104).

The importance of the BamA lateral gate is a matter of ongoing debate. Artificial locking of this seam is lethal *in vivo* (34, 105) and moderately impairs OmpT folding kinetics into proteoliposomes (38), but it has no effect on the *in vitro* assembly of OmpA or OmpX, both small OMPs with minimal loop structure (36, 41). This incongruity might be reconciled if certain Bam substrates do not require opening of the gate whereas others do, such as those with large barrels or extensive hydrophilic extracellular loops (45, 106), which could avoid the entropic penalty associated with membrane translocation by traversing the OM through the hydrophilic lumen of the open BamA barrel, with the gate serving as a transient "slit" allowing passage of loops attached to transmembrane β-strands that are integrating into the lipid phase. This bears some resemblance to the mechanism of polytopic inner membrane (IM) protein assembly by the SecYEG insertase/translocase, where transmembrane segments diffuse into the membrane adjacent to a lateral gate and periplasmic loops are translocated through the activated, ungated SecYEG pore (107–109). However, it is also clear that specific loops from certain substrates are buried within the lumen of the barrel during folding, potentially scaffolding barrel formation and driving the maturation process, likely rendering an assisted loop translocation process dispensable.

The requirement for both rapid OM growth *and* a reliably impermeable OM represents an intriguing paradox and raises the possibility that local defects induced by BamA form only when OMP insertion is imminent, as a constitutively "open" complex would likely generate membrane instability and allow indiscriminate diffusion across the OM. This paradox implies tight regulation of Bam activity and a concerted mechanism for Bam activation that is linked to substrate recognition (14, 16, 48, 62). Consistent with this notion, there is mounting evidence that the BamA barrel exists in equilibrium between two conformations: a "closed" state in which extracellular loops form a dome that stabilizes a thermostable and complete β-barrel, and an "open" state in which extracellular loops reorient and the N-terminal β-strands of the barrel are dramatically wrenched outward, begetting a thermolabile, incomplete β-barrel with an exposed aqueous pore (21, 34, 37). This equilibrium can apparently be altered through mutations in L6 that prevent loop latching and destabilize the barrel (42, 43, 110), or through mutations in the BamCDE subcomplex that are presumed to slow the restoration of the closed conformation following a round of OMP assembly (61). Together with observations linking barrel-proximal POTRA 5, lipoproteins, and L6 to conformational dynamics within the BamA barrel (14, 38, 44, 61, 62, 64, 65, 111, 112), these findings extend the mechanistic model to include a role for nascent OMPs as homotropic allosteric activators of Bam that initiate an activation cascade resulting in transient opening of the BamA barrel and concomitant OM integration of folding OMPs.

A model for BamA-assisted β-barrel folding

In light of the emerging mechanistic details reviewed above, the *in vivo* β-barrel assembly process can be summarized as follows (Fig. 3). Unfolded OMPs are transferred from periplasmic chaperones to Bam, where they are scaffolded through direct interaction with the POTRAs and one or more Bam lipoproteins (18, 23, 24, 26). β-strands of substrate OMPs may be organized circumferentially according to their relative positions in the final folded β-barrel structure (Fig. 2b) (18, 113–115). Recognition of the β-signal by BamD triggers a conformational rearrangement in the complex that includes rotation of the POTRAs away from the BamA barrel lumen and/or adoption of an open barrel conformation (14, 21, 34). Local perturbation of the OM, induced by BamA and exacerbated by conformational dynamics within the activated β-barrel domain, allows rapid, spontaneous folding and simultaneous OM insertion of OMP substrates along the native folding pathway, with long, hydrophilic loops potentially threaded through the BamA barrel lumen during OMP folding via the lateral slit. Although the precise translocation path of BamA substrates remains undefined, the orientation of the periplasmic ring-like Bam apparatus relative to the OM raises the possibility that substrates are funneled through a proteinaceous aperture adjacent to the BamA barrel that could serve as both a substrate exit channel and a site of OM insertion (24). Following substrate release, the closed conformation of the BamA barrel is restored through conformational dynamics among the Bam lipoproteins and BamA, priming the complex for an ensuing round of assembly (61, 116). We anticipate that the availability of *in vitro* reconstitution systems, comprehensive panels of informative mutants, high-resolution imaging techniques, sensitive biophysical assays and models, and an abundance of structural data will enable a detailed analysis of the Bam catalytic mechanism and the potential variations on the generic theme presented here for diverse OMP substrates.

Acknowledgments

The authors thank fellow lab members for suggestions and critical reading of the manuscript. TJS was supported by a grant from NIGMS (R35GM118024).

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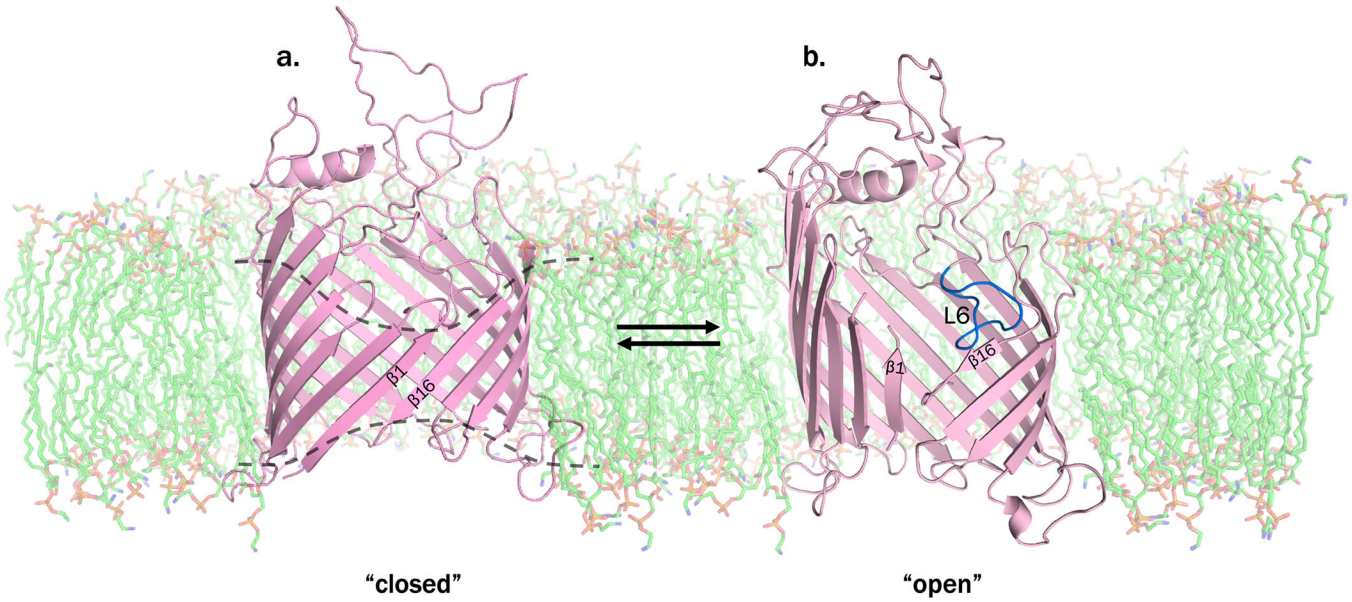

Fig. 1. Unique features of the BamA β-barrel domain.

(**a**) the BamA β-barrel (pink) is asymmetric, with one face forming a narrow protein-lipid interface (approximated by the dashed line) that is thought to physically alter the local properties of the bilayer (green). (**b**) The activated BamA β-barrel undergoes a dramatic conformational rearrangement that disrupts the continuous β-barrel structure, separates the β-strands comprising the lateral gate (β1 and β16), and exposes an aqueous channel that spans the membrane. Additionally, a highly conserved extracellular loop (L6, blue) internally braces and globally stabilizes the β-barrel domain and compensates for the instability introduced by the conformational dynamics. Image generated using PDB structures 4K3B (left) and 5EKQ (right).

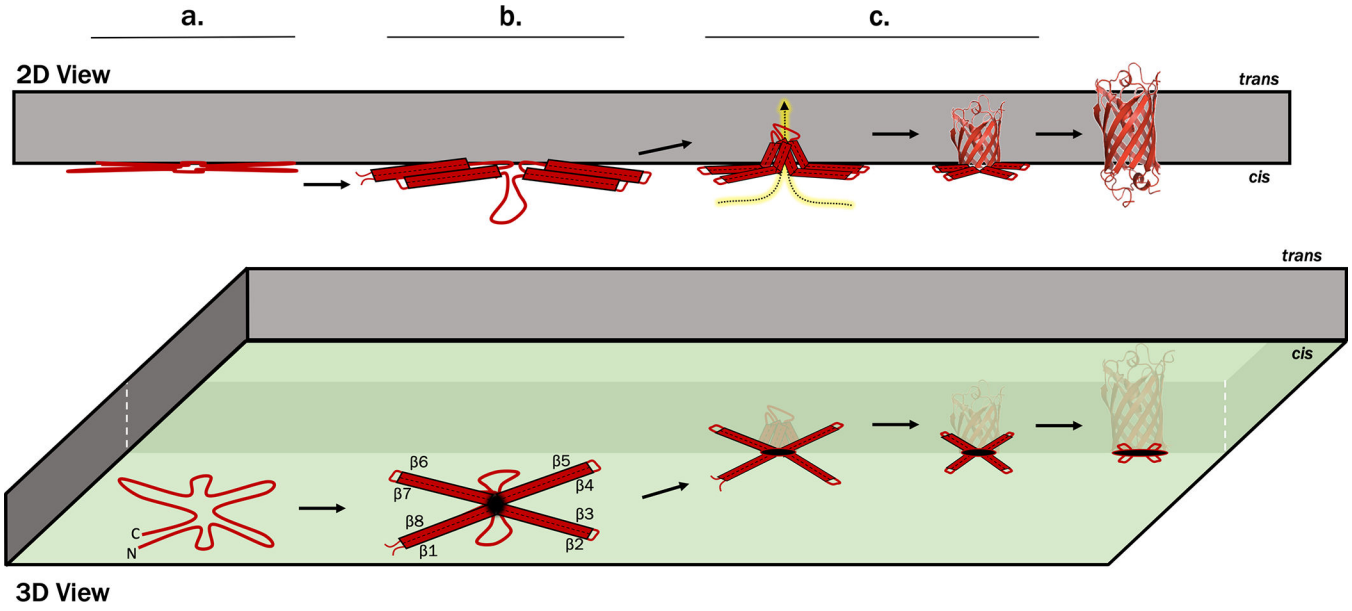

Fig. 2. Proposed unassisted folding model for a membrane β-barrel protein.

(**a**) A nascent eight-stranded OMP rapidly adsorb to the *cis* surface (green) of the lipid bilayer, where hydrophobic lipid-facing side chains begin to penetrate into the membrane and β-strands assume a cloverleaf-like circular arrangement according to their relative position in the folded protein. (b) β-hairpins begin to form as the trans ends of the TM β-strands, oriented toward the center of the cloverleaf, plunge into the lipid phase. (c) Hydrogen bonds form between neighboring β-hairpins as they enter the membrane, stabilizing the native fold in concert with membrane insertion. The highlighted dashed line indicates the proposed path of the leading (*trans*) ends of the β-strands.

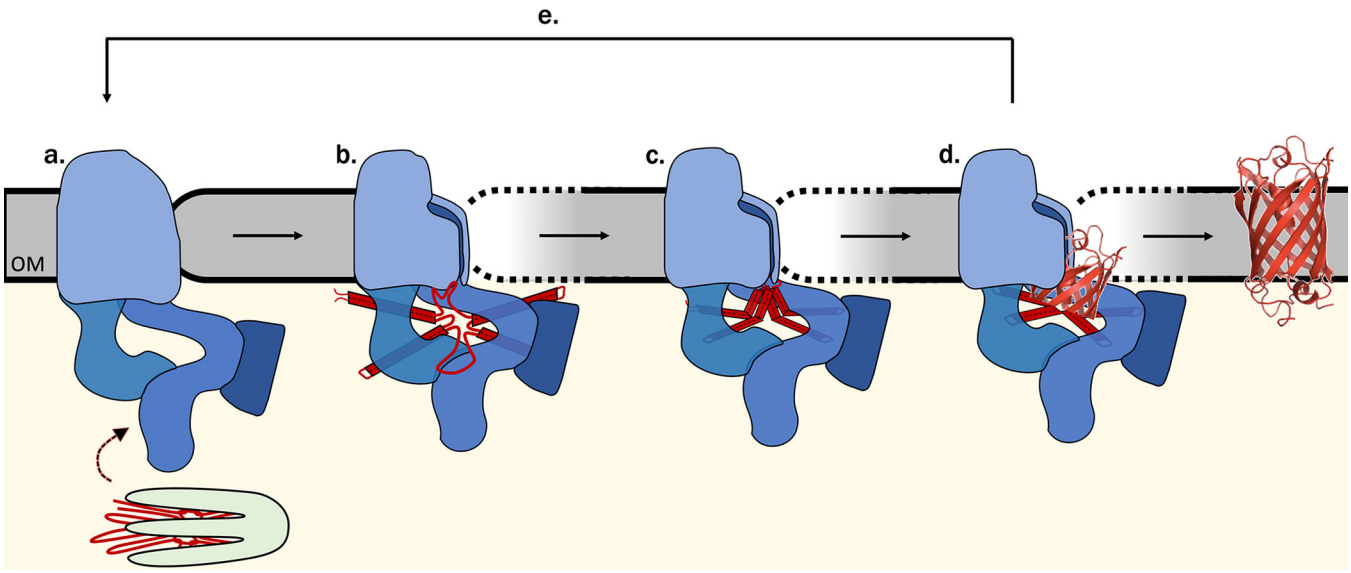

Fig. 3. Proposed Bam-assisted folding model.

(**a**) Nascent OMPs (red), maintained in a folding-competent state by periplasmic chaperones (green), are transferred to the Bam complex (blue). (**b**) Client proteins associate with multiple epitopes on Bam, potentially stimulating formation of early β-structure and orienting circularly-arranged β-strands/hairpins toward the presumptive substrate exit pore. Recognition of conserved OMP motifs triggers a conformational change in BamA that exposes the barrel lumen and destabilizes the lateral gate, further perturbing the local membrane environment and generating an OM integration path for OMP substrates. (**c**) The Bam complex prevents aggregation, protects substrates from proteolysis, and lowers the kinetic barrier to OM integration to enable rapid OMP folding along the native pathway. (**d**) OMPs spontaneous fold into the locally destabilized membrane, with the exposed BamA lumen potentially accommodating the folding barrel and/or secreted extracellular domains of client proteins. (**e**) Release of substrate from the complex prompts restoration of the closed, inert state of the complex to enable an ensuing round of assembly.