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The β -barrel membrane protein insertase machinery from Gram-negative bacteria

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Abstract

The outer membranes (OM) of Gram-negative bacteria contain a host of β -barrel outer membrane proteins (OMPs) which serve many functions for cell survival and virulence. The biogenesis of these OMPs is mediated by the β -barrel assembly machinery (BAM) complex which is composed of five components including the essential core component called BamA that mediates the insertase function within the OM. The crystal structure of BamA has recently been reported from three different species, including a full-length structure from *Neisseria gonorrhoeae*. Mutagenesis and functional studies identified several conformational changes within BamA that are required for function, providing a significant advancement towards unraveling exactly how BamA and the BAM complex are able to fold and insert new OMPs in the OM.

β -barrel membrane proteins in Gram-negative bacteria

Gram-negative bacteria contain an inner and outer membrane (OM) with the OM serving as a hub for β -barrel outer membrane proteins (OMPs), which serve many functions within the cell including nutrient import, cell signaling, and adhesion. In pathogenic strains, OMPs can also serve important roles as virulence factors during the invasion and infection of hosts. Despite the vital roles these OMPs serve in cell survival and virulence, exactly how they are folded and inserted into the OM has remained unknown.

The β -barrel assembly machinery

OMPs are first synthesized in the cytoplasm with an N-terminal signal sequence that facilitates Sec-mediated transport across the inner membrane into the periplasm [1] (Figure

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Conflict of interest statement

Nothing declared.

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1). Chaperones (SurA and Skp) then bind the nascent OMPs and escort them across the periplasm to the inner face of the OM where the β -barrel assembly machinery (BAM) complex then folds and inserts the nascent OMPs directly into the OM [2–4]. The BAM complex consists of five components called BamA, B, C, D, and E. BamA, a member of the Omp85 superfamily, is an OMP itself and BamB-E are lipoproteins anchored to the inner leaflet of the OM. BamA and BamD form the core of the complex essential for cell viability, with BamA containing the central membrane domain important for the insertase function of the BAM complex [5].

Until recently, only structures of the periplasmic domains of BamA and BamB-E had been reported [6–18]. And while these structures provided clues to how each lipoprotein may interact with substrate OMPs, they offered little information about the insertase function of the BAM complex in folding and inserting new OMPs into the OM. The structures of these Bam components will not be reviewed here but for further reference, Kim et al. have thoroughly reviewed the crystal structures of these periplasmic domains [6]. Interestingly, recent *in vitro* and later *in vivo* studies have shown that BamB and BamD assist in the biogenesis of BamA, thereby promoting the formation of the fully assembled BAM complex, which then catalyzes the biogenesis of additional BamA as well as substrate OMPs [19,20]. These studies helped address ‘the chicken or the egg’ question about the formation of BamA since BamA was assumed to be needed for its own biogenesis.

The structure of BamA provides functional clues

Until recently the only structural information available for BamA was based on the soluble N-terminal polypeptide transport associated (POTRA) domains, which had been studied by X-ray crystallography, NMR and SAXS [7–9,21]. These structures provided insight into how the β -barrel substrate may be transferred towards the membrane, through a putative β -augmentation mechanism (e.g. new β -strands of the substrate form using the exposed edge of strands found within the POTRA domains of BamA) aided by the flexibility of the POTRA domains. These studies did not, however, reveal how BamA carries out the membrane insertion of substrate OMPs. Within recent months though, four crystal structures of BamA containing the C-terminal β -barrel domain have now become available, providing new information into how this molecular machine may carry out its function.

The first structures were obtained from one construct of full-length BamA from *Neisseria gonorrhoeae* (NgBamA) and from another lacking the first three POTRA domains from *Haemophilus ducreyi* (HdBamA) [22]. Subsequently, two structures were obtained from *E. coli* BamA (EcBamA) with one containing the β -barrel domain linked to POTRA 5 [23] and the other one containing only the β -barrel domain [24]. Despite the differences in expression and purification methods (refolding of the EcBamA constructs versus membrane expression of NgBamA and HdBamA) and differences in the crystallization methods (detergent for the EcBamA constructs versus bicelles for NgBamA and HdBamA) the overall structure of the β -barrel domains from all three species is very similar and confirms a common fold for BamA family members.

Each crystal structure of BamA showed a 16-stranded β -barrel (Figure 2A) which is closed from the extracellular side by a large capping dome consisting of extended inter-sheet loops (loops 4, 6, and 7) (Figure 2B, *top*). The tight-knit cap on BamA may act to prevent the free diffusion of molecules across the OM, and thus maintain the membrane integrity during OMP insertion. The interior of the β -barrel domain is empty, forming a cylindrical space approximately 21 Å long and 23 Å across with a volume of $\sim 13,000$ Å³ (Figure 2B, *bottom*) [22–25]. The inside of the β -barrel domain of BamA is too small to house a fully folded substrate OMP but is large enough to accommodate one or a few β -hairpins, which may suggest a holding chamber for the folding of substrate OMPs prior to membrane insertion.

Access to this chamber differs in each of the three structures containing POTRA 5. In *NgBamA*, POTRA 5 sits closely underneath the β -barrel domain and appears to be held in place by interactions with four periplasmic loops [22]. However, in the *HdBamA* and *EcBamA* crystal structures, POTRA 5 is positioned away from the β -barrel domain to differing extents [22,23] (Figure 2C). Subsequent mutagenesis and functional assays indicate that a stabilized interaction of POTRA 5 with the periplasmic loops is not required for function, which is consistent with the variability seen in the crystal structures [7–9,22,23,26]. Therefore, the observed alternate conformations may represent snapshots of a dynamic region of the structure that suggests movements of the POTRA domains as a possible mechanism to regulate substrate OMP access to the holding chamber of the β -barrel domain. However, a recent solid-state NMR study probing the flexibility of reconstituted *EcBamA* into liposomes concluded the angle between the barrel and POTRA 5 is rigid [27]. Exactly what effect sample preparation for solid-state NMR or the reconstitution into liposomes may have had on the conformation of the POTRA domains of BamA is not clear, therefore, exactly how the POTRA domains move in the context of the whole BAM complex or what role this may play is unknown.

A striking feature observed in all four crystal structures of BamA is the mismatch between the predicted membrane thickness on the front and back faces of the β -barrel domain [15,23,24,26]. Inspection of the usual markers of membrane position, both surface electrostatics and the position of membrane anchoring residues comprising the aromatic belt, suggests a reduced surface area of membrane interaction on the back face of BamA at the junction of strands $\beta 1$ and $\beta 16$, which interact to seal the β -barrel domain (Figure 2D). Molecular dynamic simulations of BamA in lipid bilayers suggests the reduced width of the aromatic belt causes the tails of adjacent lipids to become significantly disordered and the membrane to drastically decrease in thickness [22,26] (Figure 2E). Consistent with these experiments, membrane thinning has been directly observed experimentally by electron microscopy on BamA reconstituted into liposomes [27]. A destabilized, thinner membrane would decrease the energetic requirement for substrate protein insertion into an otherwise densely packed, lipid-headgroup imposed barrier [26,28,29]. This membrane distortion is not seen in bilayer simulations of the related transporter FhaC, suggesting that this may be a unique function of BamA that is directly related to its role as a membrane insertase.

Structural comparison of BamA, TamA, and FhaC

Also recently reported was the crystal structure of TamA from *E. coli* (*Ec*TamA), a close homolog of BamA, that is important for the biogenesis of select autotransporters into the OM [25]. Much like BamA, TamA has a large C-terminal 16-stranded β -barrel domain, yet only three POTRA domains. Despite the conformational differences between the POTRA domains, both BamA and TamA are structurally similar, particularly throughout the β -barrel domain (Figure 3A). Interestingly, much like that observed for the *Ng*BamA structure and the *Ec*BamA structure from Albrecht et al., TamA was also found with its C-terminal strand (β 16) unpaired from strand β 1 and sitting tucked inside the β -barrel domain, possibly representing a general characteristic of OMP insertases (Figure 3B) [22,25,26]. Despite lacking the α -helix in extracellular loop 4 and an extended loop 7, the β -barrel domain of TamA superimposes well with that of BamA, with the primary difference found in the C-terminal strand where a shift in hydrogen bonding interactions with strand β 1 produces a different tilt in the membrane (shear number; $S=22$ for BamA, $S=20$ for TamA). Interestingly, TamA shares its shear number with FhaC ($S=20$), however what significance this may play functionally remains to be determined.

A shared conformation for loop 6 among the Omp85 superfamily

Omp85 superfamily members (e.g. BamA, TamA, and FhaC) contain a conserved extracellular loop 6 and (V/I)RG(F/Y) motif. Based on the first reported crystal structure of FhaC, loop 6 and the (V/I)RG(F/Y) motif are positioned deep inside the β -barrel domain in an extended conformation stretching towards the periplasm (Figure 3C) [30]. However, in all BamA and TamA crystal structures solved to date, the conformation of loop 6 is conserved, with the (V/I)RG(F/Y) motif positioned near the top of the β -barrel domain facilitating interactions with the opposite barrel wall. The observed conformational differences between FhaC and BamA/TamA loop 6 led to the hypothesis that it may cycle between multiple conformations [22,31]. In BamA and TamA, the arginine within the (V/I)RG(F/Y) motif was found to be significantly stabilized by highly conserved salt bridges with neighboring aspartate and glutamate residues, raising doubt whether loop 6 is as dynamic as initially predicted. This mystery was recently resolved when the coordinates for an improved, higher resolution structure of FhaC were released into the Protein Data Bank. The new structure of FhaC (PDB code 4QKY) now shows loop 6 in the same conformation as observed for BamA and TamA, with the conserved (V/I)RG(F/Y) motif interacting with the opposite wall of the β -barrel domain (Figure 3C). These new structural insights align well with recent biophysical and crosslinking studies for FhaC and BamA which have both shown that a conformational change in loop 6 is not required for function [26,32,33]. While the new FhaC structure helps solidify the idea that loop 6 and the (V/I)RG(F/Y) do *not* cycle between two differing conformations as first thought, exactly what role this highly conserved motif serves remains elusive.

Lateral opening in BamA

Despite the similar conformation of loop 6 observed in the FhaC, TamA and BamA structures, differences in the overall shape of the barrel domains result in loop 6 ‘capping’

the barrel domain to different extents. In the case of FhaC, the conformation of loop 6 narrows the extracellular opening but fails to fully occlude the barrel domain. In addition, the other extracellular loops in FhaC have an open conformation; the structure of FhaC is well suited to mediate secretion given the less restricted passage through the β -barrel domain to the surface once the H1 helix in the pore has been ejected. (Figure 3A) [30,32,33]. However, BamA and TamA both differ significantly in that loop 6 is able to fully cap their β -barrel domain, sealing the membrane and occluding a direct path from the periplasm to the outside of the cell (Figures 2A and 3A) [22,25]. And unlike TamA, BamA has two additional loops (loop 4 and 7) that fold over the top of the cap, possibly serving to further stabilize the conformation of loop 6 (Figure 3A). These observations led to the hypothesis that unlike FhaC, which secretes its substrate across the OM to outside the cell, BamA may 'secrete' its substrate OMPs directly into the membrane. Crystal structures and MD simulations suggested that the BamA β -barrel could open laterally, providing direct access to the lipid bilayer (Figure 4A) [22,26]. To confirm this hypothesis, it was recently shown experimentally that lateral opening of the β -barrel domain of BamA is required for function. Five separate pairs of disulfide crosslinks were engineered between strands 1 and 16 to prevent the lateral opening, in each case resulting in ablation of BamA function which could be fully recovered by pre-treatment with reductant (Figure 4A). The BamA and TamA crystal structures represent the first β -barrel membrane protein structures reported to exhibit a significant unpairing of the first and last strands, which likely represents an initial step in lateral opening of the β -barrel domain, and appears to be a common theme for these insertases [26]. The lateral opening has been hypothesized to be responsible for direct membrane insertion of the β -strands of the substrate OMP, possibly even involving a complex integration into the β -barrel domain of BamA itself. An exit pore has also been observed which may be important for the formation of surface exposed loops in newly formed OMPs (Figure 4B) [24,26].

Conclusions

The structural and functional work reported recently identifies likely mechanisms for the role of BamA in folding and inserting substrate OMPs into the membrane, with two possible working models. The first model represents a 'BamA-assisted model' in which BamA serves to prime the local membrane and would serve as a receptor for recruiting substrate OMPs into proximity of the primed OM for spontaneous insertion (Figure 4C) [22,28,29]. Localization would be mediated by specific recognition of the conserved β -signal (e.g. typically the last strand of the β -barrel domain) of substrate OMPs by BamA, possibly by β -signal exchange (e.g. the substrate β -signal exchanges with BamA's own β -signal) and transient integration into the β -barrel domain of BamA following the lateral opening. To finalize insertion, the β -signal of the new OMP would be released from BamA and pair with its complementary strand β 1. The second model represents a 'BamA-budding model,' where in addition to priming the localized membrane for substrate OMP insertion, a complex series of conformational changes within BamA facilitate the threading of substrate OMPs through the β -barrel domain and directly into the membrane via lateral opening (Figure 4D) [6,22,26]. Given its species-specific conservation and importance in folding kinetics, it seems most plausible that the β -signal of substrate OMPs may serve as the initial step

triggering insertase function by BamA. The exit pore would then mediate the formation of surface loops. To account for the amphipathic nature of the β -strands of the substrate OMPs as they are formed, direct integration into the β -barrel domain of BamA could occur, necessitating eventual ‘budding’ of the new OMP β -barrel domain to prevent the formation of a ‘super-pore’ within the OM. Termination would then be initiated by the presence of the first β -strand of the substrate OMP, which would have a higher affinity for its own β -signal than does BamA, leading to β -strand exchange and subsequent diffusion into the membrane.

The two models discussed above are BamA-centric, ignoring contributions from other Bam components. However, it is known that the other Bam components play important roles within the BAM complex, with BamD being essential for function [5]. With crystal structures now available for all the Bam components and numerous functional assays being utilized, it is anticipated that the next few years will be critical in determining exactly how the BAM complex functions as a fully assembled molecular machine capable of folding and inserting substrate OMPs into the OM of Gram-negative bacteria.

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Highlights

1. The structure of BamA contains a unique 16-stranded C-terminal β -barrel domain
2. The C-terminal strand of BamA plays an important role for its insertase function
3. The β -barrel domain of BamA primes the membrane for substrate OMP insertion
4. A single conformation for loop 6 is shared among the Omp85 family members
5. Disulfide crosslinking reveals that lateral opening is required for BamA function

Figure 1. The biogenesis of outer membrane proteins in Gram-negative bacteria

The biogenesis of outer membrane proteins (OMPs) requires a complex network of interactions since the nascent OMPs must cross the inner membrane (IM), periplasm (PP), and peptidoglycan (PG) in order to arrive at the outer membrane (OM). Synthesis begins in the cytoplasm as a pre-protein that contains an N-terminal signal sequence that directs the nascent OMPs to the Sec translocon for transport across the IM. Chaperones (SurA and Skp) further escort the nascent OMPs across the periplasm and PG (not shown) to the inner leaflet of the OM. The BAM complex accepts the nascent OMPs from the chaperones and folds and inserts them into the OM.

Figure 2. The structure of BamA primes the outer membrane for substrate OMP insertion
A. The structure of *HdBamA* showing POTRA 4 (P4), POTRA 5 (P5), and the β -barrel domain in orange, purple, and green, respectively. The conserved loop 6 is shown in blue. **B.** A surface representation of the barrel domain of BamA showing a side view (*top*) and a periplasmic view (*bottom*). The conserved loop 6 is shown in blue. The cap structure is indicated by the dotted lines. **C.** Comparison of POTRA domain conformations in all reported BamA crystal structures aligned along the β -barrel domain (shown in gray). *HdBamA* is shown in orange (P4) and dark purple (P5), *NgBamA* is shown in gold (P4) and light purple (P5), and *EcBamA* is shown in violet (P5). Conformational shifts between *HdBamA* and *NgBamA* are indicated by dashed arrows. **D.** An electrostatic surface potential representation of BamA illustrates the differences in hydrophobic thickness between the front and back sides of the β -barrel domain. The hydrophobic thinning of the back side represents an accompanied thinning of localized membrane (dashed lines). **E.** Molecular dynamic (MD) simulations have provided further support that the β -barrel domain of BamA thins the localized membrane along the back side, possibly by as much as 16 Å. Shown here are results from MD simulations representing the thickness of the membrane by color coded spheres where blue represents the most stable (thickness ~49 Å) and red represents the most

destabilized (thickness ~ 26 Å). The *left* panel depicts the membrane view while the *right* shows a view from the surface.

Figure 3. Structural comparison of the Omp85 family members BamA, TamA, and FhaC
A. The structures of BamA (green), TamA (gold), and FhaC (pink) are shown in surface representations with the conserved loop 6 (L6) shown in blue. Loop 4 (L4) and loop 7 (L7) of BamA are highlighted in cyan and orange, respectively. The top row shows a membrane view, the second row shows a surface view, the third row shows a surface view where L6 has been removed, and the last row shows a cutaway periplasmic view. While removed for our comparison of the β -barrel domains, the location of the H1 helix in FhaC is indicated by the red dashed circle. **B.** There have now been four crystal structures solved for BamA and two for TamA. Two of the BamA structures (PDB codes 4K3B and 4C4V) and both TamA structures (PDB codes 4C00 and 4N74) were found with C-terminal strands ‘tucked’ towards the inside of the β -barrel domain. However, the other two BamA structures (PDB codes 4K3C and 4N75) as well as FhaC have C-terminal strands that are fully ‘zipped’ with strand β 1. For reference, the C-terminal strand in each is indicated by a black arrow. **C.** The FhaC crystal structure (PDB code 2QDZ) was the first from the Omp85 family which showed L6 in an extended conformation towards the periplasm within the β -barrel domain. A newly solved structure of FhaC (PDB code 4QKY) with higher resolution has now corrected this to show L6 in a conformation nearly identical to that observed in all BamA and TamA crystal structures. For reference, the tip of L6 for each structure is indicated by a color-matching arrow.

Figure 4. The role of BamA as an insertase within the BAM complex

A. Lateral opening has been shown to be essential for function in BamA. Depicted here are surface representations of the ‘closed’ (gold, X-ray crystallography) and ‘open’ (green, MD simulations) conformations of the lateral gate (dashed ellipse). Crosslinking strands $\beta 1$ (blue arrow) and $\beta 16$ (black arrow) (blue and green spheres) were used to trap the lateral gate in a ‘locked’ conformation (gold ribbon) which fully inactivated BamA function, however, this could be reversed by treatment with a reducing agent. **B.** Exit pore formation has also been shown to be important for function in BamA. A surface representation from MD simulations is shown depicting the site of the exit pore (dashed circle) along the junction of loop 1 (L1) and loop 6 (L6). Crosslinking L1 to L6 (purple spheres) was used to trap the exit pore in a ‘locked’ conformation (gold ribbon) which fully inactivated BamA function, however this could be reversed by treatment with a reducing agent. **C.** The ‘BamA-assisted’ model is depicted where (i) nascent OMPs are transported across the inner membrane (IM) by the Sec translocon into the periplasm and interact with SurA, (ii) SurA then escorts the nascent OMPs to the BAM complex where the β -signal directly interacts with BamA by strand exchange following lateral opening, and then (iii) the OMP undergoes spontaneous folding/insertion into the locally ‘BamA-primed’ membrane. **D.** The ‘BamA-budding’ model is depicted where (i) nascent OMPs are transported across the inner membrane (IM) by the Sec translocon into the periplasm and interact with SurA (exactly as in panel C), (ii) SurA then escorts the nascent OMPs to the BAM complex where the β -signal directly interacts with BamA by strand exchange following lateral opening and subsequent interaction with the POTRA domains (and possibly other Bam components) initiates threading of the substrate OMPs through the β -barrel domain of BamA, (iii) as the OMPs are threaded through the β -barrel domain, β -strand templating occurs producing a BamA:OMP hybrid barrel where the

OMP β -barrel would continue to grow and eventually ‘bud’ off from the BamA β -barrel, and (iv) biogenesis would be finalized by strand exchange and pairing of the first and last strands of the substrate OMP.

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