Membrane integration of an essential β-barrel protein prerequires burial of an extracellular loop

Joseph S. Wzorek[a], James Lee[a,b], David Tomasek[a,b], Christine L. Hagan[a], and Daniel E. Kahne[a,b,c,1]

[a]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138; [b]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138; and [c]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

Edited by Robert T. Sauer, Massachusetts Institute of Technology, Cambridge, MA, and approved February 1, 2017 (received for review October 5, 2016)

The Bam complex assembles β-barrel proteins into the outer membrane (OM) of Gram-negative bacteria. These proteins comprise cylindrical β-sheets with long extracellular loops and create pores to allow passage of nutrients and waste products across the membrane. Despite their functional importance, several questions remain about how these proteins are assembled into the OM after their synthesis in the cytoplasm and secretion across the inner membrane. To understand this process better, we studied the assembly of an essential β-barrel substrate for the Bam complex, BamA. By mutating conserved residues in the β-barrel domain of this protein, we generated three assembly-defective BamA substrates that stall early in the folding process in the periplasm. Two of the three defective substrates, which harbor mutations within β-strands, fail to associate productively with the Bam complex. The third substrate, which harbors mutations in a conserved extracellular loop, accumulates on BamD during assembly, but does not integrate efficiently into the membrane. The assembly of all three substrates can be restored by artificially tethering a region of the substrate, which ultimately becomes an extracellular loop, to the lumen of the forming β-barrel. These results imply that a critical step in the folding process involves the interaction of residues on the interior of the nascent β-barrel wall with residues in one of the extracellular loops. We conclude that a prerequisite for membrane integration of β-barrel proteins is burial of the extracellular loops within the forming β-barrel.

β-barrel | Bam complex | outer membrane | protein folding

Membrane proteins with β-barrel structure can spontaneously assemble into their folded states in detergent solutions and artificial membrane bilayers (1, 2). Nevertheless, in living systems such as Gram-negative bacteria, chloroplasts, and mitochondria, these proteins are assembled into membranes by conserved machines (3–7). Clearly, there must be kinetic barriers to the rapid assembly of these proteins in cells that must be overcome by catalysis. Numerous in vitro biophysical studies on the uncatalyzed assembly pathway for β-barrel proteins into lipid bilayers have led to the conclusion that the slow step in assembly is membrane integration, and that the step involves concerted formation of tertiary structure (2, 8–10). Two limiting possibilities exist for the folding of membrane β-barrels in vivo. The assembly machines could somehow accelerate the uncatalyzed folding mechanism, or they could provide an alternative mechanism to accelerate assembly. It has been suggested that in vivo folding occurs through iterative insertion of β-strands or β-hairpins into the membrane through the assembly machine, which implies that the catalyzed pathway is different from the uncatalyzed pathway (11–13).

One approach to answer the question of how β-barrels are assembled in vivo is to characterize the machines that assemble them. A considerable amount of biochemical, genetic, and structural work has focused on the role of the components of these machines. These experiments have revealed which components perform essential chemistry (5, 14–16), how the components interact with each other (12, 13, 17), and what conformational states may exist during function (18–21). Largely, these studies have emphasized how the machine might alter the membrane structure or how its components might sequentially bind and thereby guide substrates into the membrane. However, much less has been done to understand how structure emerges within a substrate during assembly by these machines. Here, we have taken the approach of characterizing the nascent structure of a substrate as it interacts with the machine to identify the features of the substrate required to permit its proper membrane integration.

We have been studying β-barrel assembly in *Escherichia coli*, which is accelerated by the β-barrel assembly machine (Bam) (7, 22). The Bam complex comprises a central β-barrel protein, BamA, and four lipoproteins, BamBCDE (5, 7, 15, 23, 24). Two of the five components, BamA and BamD, are essential for viability and participate directly in β-barrel assembly (5, 7, 15). The Bam complex has broad substrate scope, in that it can accelerate folding of substrates with as few as 8 or as many as 26 β-strands, substrates with large periplasmic or extracellular domains, and even substrates that encapsulate other proteins (25–27). These diverse substrates may demand somewhat different assembly processes, but key aspects of the β-barrel assembly mechanism are likely shared among all substrates. In an effort to identify common mechanistic features, we have focused on the two essential substrates folded by the Bam complex: LptD, which facilitates lipopolysaccharide transport to the outer membrane (OM), and BamA itself. LptD is a 26-stranded β-barrel, which encapsulates the lipoprotein LptE within the lumen. Here, we study assembly of the smaller 16-stranded β-barrel BamA, which does not encapsulate another protein. Because BamA is both a substrate for the Bam complex and a component of it, it can be

Significance

Membrane proteins must be assembled to adopt a mature functional form. Integral membrane proteins with β-barrel structure are found in the outer membranes of Gram-negative bacteria, mitochondria, and chloroplasts. To understand the mechanism by which these proteins are assembled, intermediate states in the process must be accumulated and characterized. Here, we study assembly-defective mutants of the β-barrel protein BamA. Our findings reveal that a highly conserved, hydrophilic loop is buried within the β-barrel of BamA before membrane integration, suggesting that one barrier to assembly is the translocation of hydrophilic sequences across the membrane. The specific interactions we identify in this early assembly step could potentially be used for design of mechanism-based inhibitors with antibiotic activity.

Author contributions: J.S.W., J.L., D.T., C.L.H., and D.E.K. designed research; J.S.W. and J.L. performed research; J.S.W., J.L., D.T., C.L.H., and D.E.K. analyzed data; and J.S.W., J.L., D.T., C.L.H., and D.E.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. Email: kahne@chemistry.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1616576114/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1616576114

challenging to separate those features of its structure that are important for its folding from those that are important for its function once assembled—in fact, some features of the BamA protein likely have dual roles. The experiments we describe here have been designed to report on how the sequence and structure of the BamA substrate affect its own assembly.

One feature of β-barrel substrates that is necessary for folding is a conserved C-terminal β-signal motif that must be recognized by the Bam complex in order for them to be recruited to the OM and assembled (28, 29). In bacteria, this motif mediates binding of unfolded substrates to BamD (30–33). The β-signal of *E. coli* BamA is slightly removed from the absolute C terminus of the protein; it spans residues 769–776, which are located in what ultimately becomes β-strand 14 of the folded structure (32). Amino acid substitutions at several positions within the β-signal were shown to decrease folding efficiency of BamA substrates in vivo. Most of these BamA variants were defective in binding to BamD in vitro as well; however, replacing a highly conserved glycine in the β-signal, G771, with alanine resulted in assembly defects without decreasing binding to BamD. Therefore, the β-signal must have additional functions beyond its binding to BamD. To define these requirements further, we began to investigate the role of G771 by mutating residues that interact with it in the reported crystal structures of BamA (13, 20, 21, 27, 34). We show here that BamA G771 participates in a network of tertiary interactions between the β-barrel lumen and an extracellular loop (L6); this network must be formed before the protein's insertion into the OM. Our results suggest that hydrophilic extracellular loops must be shielded from the hydrophobic membrane during their translocation across the bilayer and that the Bam complex may facilitate β-barrel assembly by inducing unfolded substrates to form structures that internally bury such loops.

Results

A Change in the BamA β-Signal Creates an Assembly-Defective Substrate that Stalls in the Periplasm. To characterize BamA(G771A) in vivo, we introduced plasmids that constitutively express either WT or mutant *bamA* into a *bamA* depletion strain in which the chromosomal copy of *bamA* is arabinose-inducible (7, 14). The strain expressing BamA(G771A) alone was viable (Fig. 1*B*), but had OM defects as indicated by hypersensitivity to rifampicin (Fig. 1*C*). Rifampicin does not easily penetrate the OM of Gram-negative cells, and therefore we use this antibiotic as a probe for OM permeability (35). To determine whether these OM defects resulted from impaired assembly of BamA(G771A), we expressed plasmid-encoded copies of His-tagged WT or mutant *bamA* in cells also containing WT *bamA* on the chromosome and compared

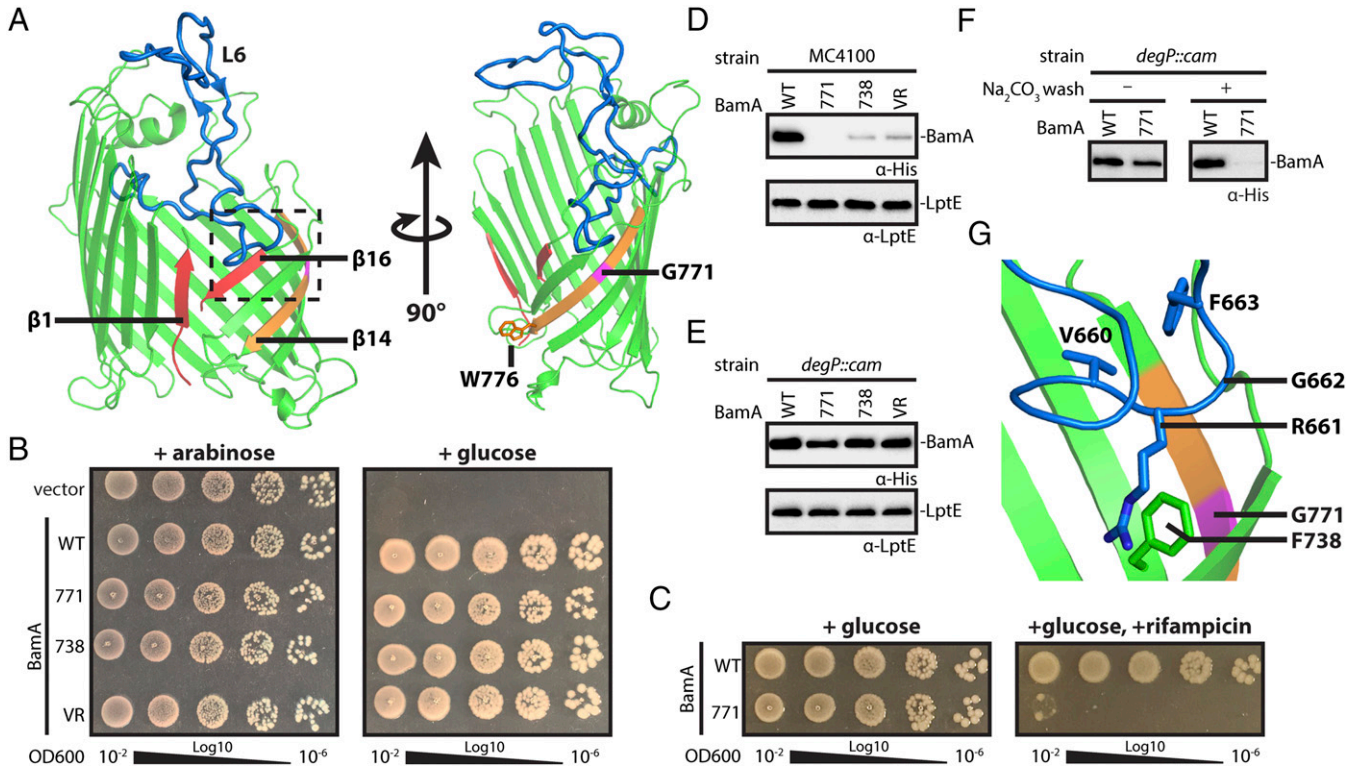

Fig. 1. Disrupting interactions between two conserved features of BamA impairs its assembly at an early stage. (*A*) Structure of the BamA β-barrel domain. The 16-stranded β-barrel BamA contains a weak point of β-barrel closure (β1 and β16; red strands) in addition to a long sixth extracellular loop (L6; blue). Position W776 of BamA resides at the end of β14 (orange) and was previously found to interact with BamD during its assembly as a substrate (32). Crystal structure images were generated from the *E. coli* BamACDE crystal structure [Protein Data Bank (PDB) ID code 5EKQ] (13). (*B*) BamA mutants are functional in vivo. The BamA depletion strain (JCM166) containing an arabinose-inducible WT *bamA* copy was transformed with plasmids that express WT or mutant *bamA* alleles (7, 14). (*B*, *Left*) In the presence of arabinose, all strains were viable. (*B*, *Right*) In the absence of arabinose (i.e., in the absence of chromosomal WT BamA expression), all strains were viable with exception of the vector control. (*C*) The OM of cells expressing BamA(G771A) are leaky to antibiotics. JCM166 cells expressing BamA(G771A) were plated in the presence or absence of 0.75 μg/mL rifampicin under chromosomal WT BamA depleting conditions. (*D* and *E*) BamA mutants are degraded by the periplasmic protease DegP. MC4100 (*D*) or MC4100 *degP::cam* (*E*) cells expressing WT or mutant *bamA* alleles were harvested and analyzed via SDS/PAGE followed by immunoblotting cell lysates. (*F*) BamA(G771A) is not membrane-integrated. Lysates from MC4100 *degP::cam* cells expressing WT BamA or BamA(G771A) were washed with 100 mM Na_2CO_3 and the resulting membranes were extracted and applied to SDS/PAGE followed by immunoblotting. (*G*) The conserved BamA L6 VRGF motif makes contacts to conserved residues on the β-barrel wall. In particular, the side chain of R661 (L6) interacts with F738 in β13. G771 resides beneath the phenylalanine side chain in close proximity to both F738 and R661.

levels of the His-tagged proteins. We were not able to detect BamA(G771A) in these cells (Fig. 1*D*), but we observed partial recovery of BamA(G771A) levels when DegP was genetically deleted (Fig. 1*E*). Because DegP is the principal protease that degrades misfolded OM proteins (OMPs) in the periplasm (36), these results imply that the assembly of BamA(G771A) is impaired at an early stage in the periplasm and, consequently, is degraded by DegP. It is worth noting, in contrast, that we have previously observed that DegP does not degrade an assembly-defective substrate that stalls at a late stage, LptD4213 (Fig. S1*A*). Furthermore, we did not observe discrete degradation fragments of BamA(G771A) at lower molecular weights by SDS/PAGE, suggesting that the substrate is not aberrantly degraded by DegP in its folded form (Fig. S1*B*).

We determined whether the change in the β-signal (G771A mutation) of the BamA substrate affected its ability to integrate into the membrane using a carbonate extraction procedure. This type of experiment is often used to distinguish between membrane-associated vs. membrane-integrated proteins (37). We have previously shown that this procedure also applies to membrane β-barrels (22). Using strains that lack DegP, we observed that BamA(G771A) was not retained in the membrane fraction, suggesting that this substrate is only loosely associated with the OM, rather than fully inserted (Fig. 1*F*). Because changing G771 to alanine makes the substrate more susceptible to proteases in the periplasm and reduces its association with the OM, we conclude that this change causes a folding defect early in the assembly process and that DegP sensitivity is a reasonable proxy for defects in proper membrane integration.

In the crystal structure of *E. coli* BamA, G771 resides in the third-to-last (14th) β-strand of the β-barrel (Fig. 1*A*). On the preceding (13th) β-strand is another highly conserved residue, F738 (Fig. S2). The phenylalanine side chain of this residue is positioned directly above G771 in the lumen of the β-barrel, and we wondered whether this arrangement of residues is functionally important (Fig. 1*G*) (13, 20, 21, 27, 34). Covariance of this glycine-aromatic or "mortise and tenon" motif in the context of autotransporters has been reported (38). We have recently suggested that the β-barrel folding mechanism begins by BamD binding the β-signal containing G771 (32). It seemed possible that unfavorable steric interactions between the side chains of G771A and F738 prevented subsequent steps in the folding process. Therefore, we expressed a plasmid-encoded BamA variant in which F738 was replaced with alanine, BamA(F738A), in the *bamA* depletion strain (Fig. 1*B*). BamA(F738A) exhibited similar phenotypes with respect to viability and DegP susceptibility as BamA(G771A) (Fig. 1 *B*, *D*, and *E*). Because mutation of F738 also results in assembly defects similar to BamA(G771A), we conclude that the interaction of G771 with F738 is important for assembly of BamA as substrate.

Membrane Integration Requires Burial of L6 in the Lumen of the BamA β-Barrel. In the final folded form of BamA, the phenylalanine side chain of F738 not only contacts G771 within the β-signal, but also contacts a conserved motif between β-strands 11 and 12 in extracellular loop 6 (L6), which is folded into the lumen of the β-barrel (Fig. 1*A* and Fig. S2) (13, 20, 21, 27, 34). This motif, consisting of the sequence VRGF, is the most highly conserved segment of BamA and is also found in the much broader Omp85 family of β-barrel proteins (39). It was previously shown that *E. coli* mutants containing two or three changes in the VRGF motif displayed severe defects in assembly of several different OMPs, including BamA. The authors concluded that these mutants impaired substrate BamA assembly, the activity of the mature Bam complex, or both (40).

Given the proximity of F738 to the VRGF motif, we wondered whether the mutations at F738 and G771 affected the long-range L6 VRGF contacts within the β-barrel lumen. To test this hypothesis, we made a double mutant encoding BamA(V660A/R661A), in which the first two residues of the VRGF motif were changed to alanine. When expressed in cells containing WT BamA, levels of BamA(V660A/R661A) were low, similar to BamA(G771A) and BamA(F738A) (Fig. 1*D*). Moreover, protein levels recovered when DegP was deleted (Fig. 1*E*). Therefore, changes in the L6 VRGF motif also result in early assembly defects of the substrate BamA that expose it to periplasmic degradation. Because changes to the conserved residues G771, F738, and V660/R661, which are close to one another in the crystal structure, all result in early assembly defects, we posit that they form a network of interactions important for early folding of BamA in the periplasm. If so, some amount of tertiary structure must be present at an early stage of assembly (see below).

BamA(V660A/R661A) Accumulates on BamD. Because DegP is a general protease that recognizes misfolded β-barrel proteins in the periplasm, it was unclear whether all three assembly-defective BamA variants stall at the same early step during folding. Therefore, we reasoned that we could accumulate folding intermediates in a strain lacking DegP and assess whether cells expressing different BamA variants have different phenotypes. We assessed the rifampicin sensitivity of strains that lack *degP* but express the mutant *bamA* alleles in addition to a chromosomal WT *bamA* copy. We found that strains expressing BamA(G771A) and BamA(F738A) were resistant to rifampicin, but the strain expressing BamA(V660A/R661A) was sensitive to it (Fig. 2*A*). Therefore, expression of BamA(V660A/R661A) increases the leakiness of the OM, even though a WT copy of BamA is present. Expression of the mutant copy produces a dominant antibiotic-sensitivity phenotype, which might suggest that the mutant compromises the function of WT BamA.

BamA(V660A/R661A) is distinct from BamA(G771A) and BamA(F738A) because it has a functional β-signal; therefore, associated phenotypes may result from defects that occur subsequent to engagement with BamD within the Bam complex. If so, this substrate variant should have a longer lifetime of association with the Bam complex, which could be detected by using in vivo cross-linking. Previously, we showed that a C-terminal BamA fragment containing residues 715–810 containing the unnatural amino acid *p*-benzoylphenylalanine (pBPA) (41) at position 776 (Fig. 1*A*) cross-links to BamD in vivo (Fig. 2 *B–D*, construct 1) (32). We interpreted the observed cross-link to reflect binding of this fragment of the unfolded BamA substrate to BamD during assembly. To probe the interaction of full-length BamA substrates with BamD during assembly, we introduced pBPA into position 776 of the full-length proteins, expressed them in cells also containing WT BamA, and irradiated the cells with UV light (Fig. 2 *B–D*, constructs 2–5) (32). The extent of cross-linking was evaluated by Western blotting to determine whether protein intermediates accumulated on BamD during folding. We observed minimal cross-linking with BamA(W776pBPA) (construct 2), BamA(F738A/W776pBPA) (construct 4), or BamA(G771A/W776pBPA) (construct 5), but substantial cross-linking with BamA(V660A/R661A/W776pBPA) (construct 3; Fig. 2 *C* and *D*). Cross-linking cannot reflect interactions between folded BamA(W776pBPA) and BamD (as found in the assembled Bam complex) because the distance between these two features (20 Å) prevents them from interacting (Fig. S3) (13, 20, 21). Because cross-linking efficiency depends on the residence time of the bound substrate, this result implies that the β-signal mutants do not stably associate with BamD, whereas the L6 mutant accumulates on it. Therefore, the V660A/R661A mutation creates an assembly problem that differs from that created by the G771A and F738A mutations; the V660A/R661A mutant remains bound to BamD, but is still unable to fold efficiently.

We wondered whether we could further differentiate the mutants that alter the β-signal (G771A and F738A) from the mutant that alters the L6 VRGF motif (V660A/R661A). We hypothesized that if BamA(V660A/R661A) is stalled on BamD, as the cross-linking suggests, it may exhibit differences in

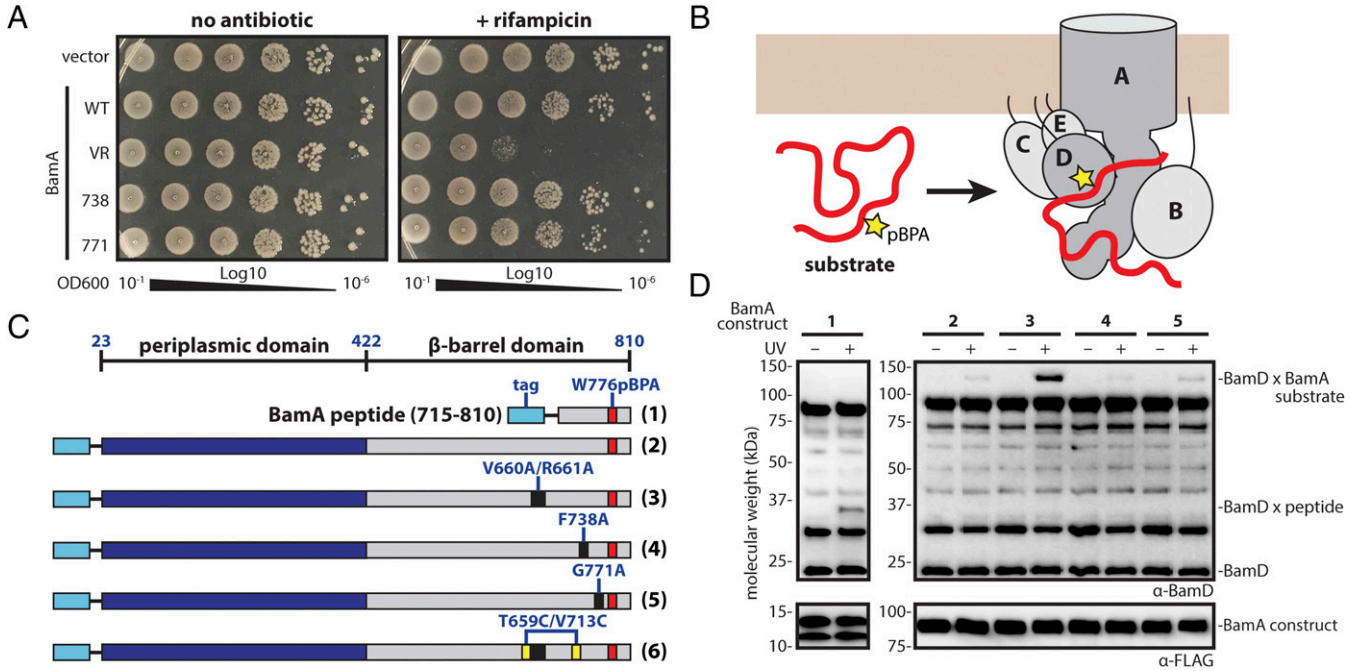

Fig. 2. BamA substrates containing VRGF motif mutations accumulate on BamD. (*A*) Expression of BamA(V660A/R661A) causes a dominant antibiotic sensitivity phenotype. MC4100 *degP::cam* cells expressing WT or mutant *bamA* alleles were plated in the presence or absence of 2.5 μg/mL rifampicin. (*B*) Overview of the in vivo photo-cross-linking assay used to probe interaction of substrate BamA with BamD. The unnatural amino acid pBPA was incorporated into position 776 of BamA, and cross-linking was induced with UV light. (*C*) An overview of the BamA constructs used in the cross-linking experiments. All constructs contain an N-terminal FLAG tag in addition to the W776pBPA mutation. Additional mutations were introduced as depicted in constructs 3–6. Construct 6 lacks the native cysteine residues (C690S and C700S) to ensure that only one disulfide bond can be formed. (*D*) BamA(W776pBPA/V660A/R661A) accumulates on BamD. BL21(DE3) cells expressing the indicated constructs were UV-irradiated or not, and analyzed via SDS/PAGE followed by immunoblotting cell lysates. [Note that the BL21(DE3) strain used in these experiments expresses higher and equal levels of the BamA substrates—unlike the MC4100 strain used in Fig. 1*D*. The higher expression allows observation of the cross-linked species.]

cell-surface exposure compared with the variants that do not bind BamD productively. To test this hypothesis, we assessed cell surface susceptibility of BamA(V660A/R661A) and BamA(G771A) to externally added proteinase K. In intact cells, any protein that has surface-exposed regions is theoretically susceptible to cleavage by an externally added protease, whereas proteins that are not membrane-integrated should be protected (18, 19, 42). We treated cells expressing the mutant BamA proteins (and lacking DegP) with proteinase K and assayed total levels and cleavage products. BamA(G771A) was not susceptible to proteinase K degradation, consistent with an early folding defect that prevents engagement at BamD and subsequent membrane

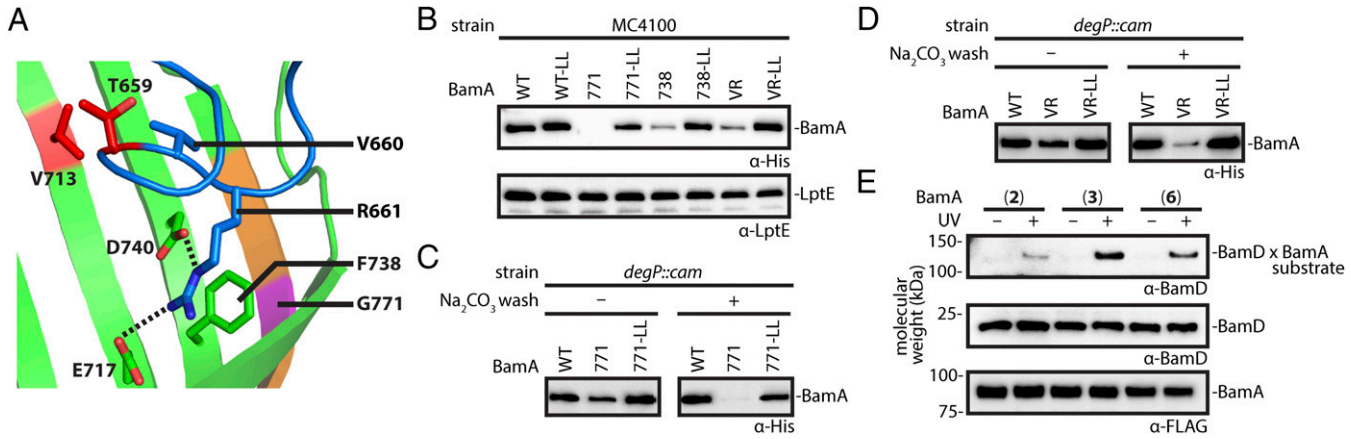

Fig. 3. Efficient membrane integration requires burial of L6 within the lumen of BamA. (*A*) Mutation of residues T659 and V713 to cysteine enables formation of a LL disulfide bond that tethers the L6 VRGF motif to the β-barrel wall (11, 13). (*B*) The LL disulfide rescues assembly of BamA variants. MC4100 cells expressing WT or mutant *bamA* alleles were harvested, and cell lysates were analyzed via SDS/PAGE, followed by immunoblotting. The LL disulfide was introduced into *bamA* alleles lacking the native cysteine residues (C690S and C700S). (*C* and *D*) The LL disulfide promotes membrane integration of BamA(G771A) and BamA(V660A/R661A). Lysates from MC4100 *degP::cam* cells were washed and analyzed in the same manner as described in Fig. 1*F*. Lanes 1 and 2 of each gel in *C* are also shown in Fig. 1*F*. (*E*) BamA(V660A/R661A)-LL has a shorter residence time on BamD. UV Photo-cross-linking was performed in the same manner as described in Fig. 2 *B*–*D*.

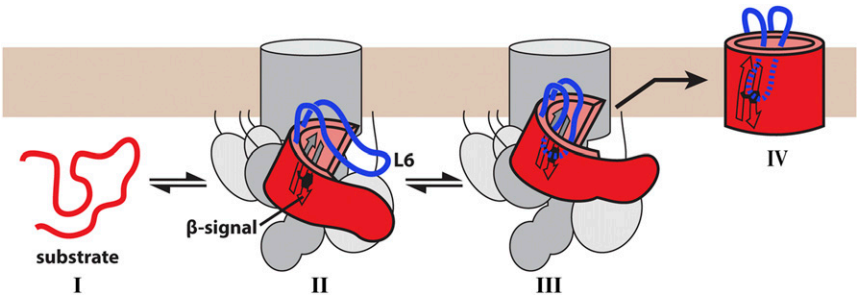

Fig. 4. Model of β-barrel membrane integration catalyzed by internal burial of hydrophilic extracellular loops. BamD initiates the assembly of substrate BamA by binding a sequence near the C terminus of the unfolded protein (i.e., the β-signal). This binding promotes the nucleation of a set of interactions that fold a conserved extracellular loop (L6) into the center of the forming β-barrel. Membrane integration proceeds efficiently after burying this loop, as the barrier to translocating a hydrophilic sequence across the membrane has been removed. We note that we do not mean to imply distinct steps involving intermediates I–IV, but, rather, a continuous folding process.

integration (Fig. S4). In contrast, BamA(V660A/R661A) demonstrated a susceptibility comparable to WT BamA. We conclude that BamA(V660A/R661A) is stalled on the Bam complex in a state that is partially exposed to proteases on both sides of the OM.

Tethering L6 to the β-Barrel Lumen Accelerates Membrane Integration. Given that G771, F738, and the VRGF motif appear to form a network of interactions in the folded BamA structure, we thought it might be possible to rescue the assembly-defective BamA substrates by artificially restoring the interaction of L6 with the β-barrel wall in the lumen. Previously, a loop-to-lumen (LL) disulfide bond was used to tether an extracellular loop to the lumen of PhoE with no apparent effect on assembly; this disulfide bond was found to form in the periplasm before membrane integration (43). More recently, a double cysteine mutant in BamA (T659C and V713C; Fig. 3*A*) was shown to form a disulfide bond without compromising viability (11). We wondered whether this disulfide bond could rescue our assembly-defective *bamA* alleles by restoring L6-to-lumen contact in the periplasm. We replaced residues T659 and V713 with cysteine in WT BamA and in the three assembly-defective mutants, and we expressed all four of these LL tethered proteins in cells also containing WT BamA and DegP. Introduction of the LL disulfide bond increased protein levels of all three assembly-defective BamA mutants (Fig. 3*B*). This rescue of protein levels is specific to the tethering of L6 to the β-barrel wall, because disulfide cross-links between other regions of the BamA β-barrel did not improve assembly (Fig. S5). We conclude that the LL disulfide increases levels of the BamA substrates by preventing degradation in the periplasm by DegP.

Finally, we wanted to establish whether the LL disulfide increases the amount of functional and membrane-integrated (i.e., assembled) protein. Both the BamA(G771A)-LL variant and BamA(V660A/R661A)-LL variant were resistant to membrane extraction by carbonate wash (Fig. 3 *C* and *D*) and were more susceptible to externally added proteinase K (Fig. S4). Increased levels and membrane integration of BamA(G771A)-LL also correlated with improved OM barrier function, because cells expressing this protein were no longer sensitive to rifampicin under depleting conditions in the *bamA* depletion strain (Fig. S6). Moreover, cross-linking of BamA(V660A/R661A)-LL to BamD was reduced, consistent with restored membrane integration and assembly (Fig. 3*E*). Collectively, these experiments suggest that a network of contacts between the VRGF motif and the β-hairpin containing F738 and G771 is required for efficient membrane integration.

Discussion

We have identified three variants of BamA that reveal how the native protein assembles into the OM. Each of these substrates has early folding defects preventing their efficient membrane integration. We showed that assembly defects in two of the substrates, BamA(G771A) and BamA(F738A), which have changes in the β-signal proposed to be important for binding to BamD, were, in fact, due to failure to successfully engage BamD. Although a small amount of these BamA substrates can assemble into the membrane, most are degraded in the periplasm (Fig. 1). The other substrate, BamA(V660A/R661A), which contains amino acid substitutions in the VRGF motif, engaged BamD, but was slow to integrate into the OM (Fig. 3). This substrate displayed a dominant antibiotic susceptibility phenotype, evidently because it stalls on the Bam complex (Fig. 2). We suspect that the susceptibility of these substrates to degradation by DegP is also due to this accumulation, which results in prolonged exposure of nascent protein to periplasmic proteases.

We have also shown that it is possible to promote membrane integration of these slow-folding substrates by forming a disulfide bond that tethers L6 to the β-barrel lumen. This result provides a clue as to how membrane β-barrel proteins solve a crucial problem. These proteins typically contain large extracellular loops that must cross the hydrophobic membrane (26). Our studies show that a network of noncovalent interactions between the β-barrel lumen and the VRGF motif anchor L6 in the lumen so that it is not exposed to the hydrophobic membrane. Disrupting this constellation of interactions slows assembly even when BamD is engaged, but it is possible to restore assembly by covalently tethering the loop to a neighboring β-strand in the lumen. Therefore, these studies allow us to assign a role for the conserved L6 VRGF motif in promoting the assembly of BamA itself. The fact that the disulfide rescues all three BamA substrates, even though they stall at two different stages of assembly, suggests that the identified interactions may form in a single step.

We propose the following model for assembly of BamA. Substrate BamA arrives at the OM in an unfolded state (state I; Fig. 4). The substrate proceeds to state II, in which interactions between F738 and G771 in the β-signal mediate binding to BamD and help organize the binding site for the VRGF motif of L6. Transition from state II to III is mediated by the interaction of L6 with the binding site created in the nascent β-barrel lumen. Burial of L6 within the lumen now allows rapid membrane integration. Membrane integration of substrate BamA requires the formation of conserved tertiary contacts between the hairpin containing the β-signal (β-strands 13 and 14) and the highly conserved L6. Substrates that are able to bind BamD, but cannot form critical tertiary contacts, such as BamA(G771A) (Fig. 1*G*), cannot be membrane-integrated and are therefore susceptible to periplasmic proteases. Introduction of the LL nonnative disulfide bypasses the requirement for a well-defined binding site for the VRGF motif to achieve state III.

There are parallels between folding requirements for BamA and LptD, a large 26-stranded β-barrel that is assembled by Bam around a lipoprotein plug, LptE. Previously, we identified a key interaction between an extracellular loop of LptD with LptE that is required for release from the Bam complex (44). A separately folded protein plug (LptE) is required to scaffold assembly of LptD (25, 45) because its increased size makes folding a more challenging entropic problem; however, the requirement for contacts from a loop to the interior of the β-barrel appears to be conserved. Accordingly, we suggest that luminal scaffolds (LptE

for LptD or the L6 VRGF motif for BamA) are required to interact with the interior surface of β-barrels as they are undergoing folding and membrane integration.

Burial of an extracellular loop against conserved residues in the β-barrel appears to be common in these OMPs. Crystal structures of PhoE, OmpF, and LamB each reveal similar placement of a phenylalanine side chain proximal to both a glycine on an adjacent strand and a large extracellular loop (46–48) (Fig. S7). This interaction was shown to form early in the periplasm during assembly of PhoE without adverse effects (43). It remains to be determined whether other β-barrel substrates use such highly specific contacts that facilitate burial of extracellular loops. Tucking extracellular loops within the nascent β-barrel not only provides a mechanism for translocation of these polar chains across the membrane to the cell surface, but also prevents entrapment of phospholipids within the lumen as it penetrates the membrane. The discovery of specific contacts required for membrane integration of substrate BamA also raises the possibility that these contacts could be targeted with an inhibitor that interferes with the formation of this critical state.

Materials and Methods

Experimental Procedures, Bacterial Strains, and Plasmids. Experimental procedures for in vivo photo-cross-linking, carbonate washing of membranes, proteinase K digestions, and plating are provided in *SI Materials and Methods*. The strains used in this study are described in Table S1. All plasmids are listed in Table S2.

Analysis of Cellular BamA Levels. MC4100 (49) cells harboring pSK476, pJW207, pJW445, pJW261, pJW234, pJW238, pJW463, or pJW453 were grown at 37 °C in LB supplemented with kanamycin to an OD_{600} of ∼1.0 over 8 h. The cells from a 1-mL sample normalized to an OD_{600} of 0.8 were collected by centrifugation at 10,000 × *g* for 10 min. The resulting cell pellets were resuspended in 80 μL of 1× SDS-sample buffer and boiled for 8 min. The samples were applied to SDS/PAGE and analyzed via Western blotting. LptE was detected by immunoblotting with LptE antiserum and secondary anti-rabbit–HRP conjugates. His–BamA was detected by using anti–penta-His–HRP antibodies. The same procedure was followed to analyze protein levels in the MC4100 *degP::cam* strain, with the exception that chloramphenicol was also added to the growth medium.

ACKNOWLEDGMENTS. We thank N. Ruiz and T. Silhavy for helpful discussions. This work was supported by National Institutes of Health Awards F32GM108258 (to J.S.W.), F31GM116210 (to J.L.), and AI081059 (to D.E.K.).

1. Dornmair K, Kiefer H, Jähnig F (1990) Refolding of an integral membrane protein. OmpA of *Escherichia coli*. *J Biol Chem* 265(31):18907–18911.
2. Fleming KG (2014) Energetics of membrane protein folding. *Annu Rev Biophys* 43: 233–255.
3. Reumann S, Davila-Aponte J, Keegstra K (1999) The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: Identification of a cyanobacterial homolog. *Proc Natl Acad Sci USA* 96(2):784–789.
4. Paschen SA, et al. (2003) Evolutionary conservation of biogenesis of β-barrel membrane proteins. *Nature* 426(6968):862–866.
5. Voulhoux R, Bos MP, Geurtsen J, Mols M, Tommassen J (2003) Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* 299(5604): 262–265.
6. Wiedemann N, et al. (2003) Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature* 424(6948):565–571.
7. Wu T, et al. (2005) Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. *Cell* 121(2):235–245.
8. Kleinschmidt JH, den Blaauwen T, Driessen AJ, Tamm LK (1999) Outer membrane protein A of *Escherichia coli* inserts and folds into lipid bilayers by a concerted mechanism. *Biochemistry* 38(16):5006–5016.
9. Kleinschmidt JH, Tamm LK (2002) Secondary and tertiary structure formation of the β-barrel membrane protein OmpA is synchronized and depends on membrane thickness. *J Mol Biol* 324(2):319–330.
10. Gessmann D, et al. (2014) Outer membrane β-barrel protein folding is physically controlled by periplasmic lipid head groups and BamA. *Proc Natl Acad Sci USA* 111(16):5878–5883.
11. Noinaj N, Kuszak AJ, Balusek C, Gumbart JC, Buchanan SK (2014) Lateral opening and exit pore formation are required for BamA function. *Structure* 22(7):1055–1062.
12. O'Neil PK, Rollauer SE, Noinaj N, Buchanan SK (2015) Fitting the pieces of the β-barrel assembly machinery complex. *Biochemistry* 54(41):6303–6311.
13. Bakelar J, Buchanan SK, Noinaj N (2016) The structure of the β-barrel assembly machinery complex. *Science* 351(6269):180–186.
14. Kim S, et al. (2007) Structure and function of an essential component of the outer membrane protein assembly machine. *Science* 317(5840):961–964.
15. Malinverni JC, et al. (2006) YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in *Escherichia coli*. *Mol Microbiol* 61(1):151–164.
16. Misra R, Stikeleather R, Gabriele R (2015) In vivo roles of BamA, BamB and BamD in the biogenesis of BamA, a core protein of the β-barrel assembly machine of *Escherichia coli*. *J Mol Biol* 427(5):1061–1074.
17. Kim KH, Aulakh S, Paetzel M (2011) Crystal structure of β-barrel assembly machinery BamCD protein complex. *J Biol Chem* 286(45):39116–39121.
18. Rigel NW, Schwalm J, Ricci DP, Silhavy TJ (2012) BamE modulates the *Escherichia coli* β-barrel assembly machine component BamA. *J Bacteriol* 194(5):1002–1008.
19. Rigel NW, Ricci DP, Silhavy TJ (2013) Conformation-specific labeling of BamA and suppressor analysis suggest a cyclic mechanism for β-barrel assembly in *Escherichia coli*. *Proc Natl Acad Sci USA* 110(13):5151–5156.
20. Gu Y, et al. (2016) Structural basis of outer membrane protein insertion by the BAM complex. *Nature* 531(7592):64–69.
21. Han L, et al. (2016) Structure of the BAM complex and its implications for biogenesis of outer-membrane proteins. *Nat Struct Mol Biol* 23(3):192–196.
22. Hagan CL, Kim S, Kahne D (2010) Reconstitution of outer membrane protein assembly from purified components. *Science* 328(5980):890–892.
23. Eggert US, et al. (2001) Genetic basis for activity differences between vancomycin and glycolipid derivatives of vancomycin. *Science* 294(5541):361–364.
24. Sklar JG, et al. (2007) Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. *Proc Natl Acad Sci USA* 104(15): 6400–6405.
25. Freinkman E, Chng S-S, Kahne D (2011) The complex that inserts lipopolysaccharide into the bacterial outer membrane forms a two-protein plug-and-barrel. *Proc Natl Acad Sci USA* 108(6):2486–2491.
26. Hagan CL, Silhavy TJ, Kahne D (2011) β-Barrel membrane protein assembly by the Bam complex. *Annu Rev Biochem* 80:189–210.
27. Noinaj N, et al. (2013) Structural insight into the biogenesis of β-barrel membrane proteins. *Nature* 501(7467):385–390.
28. Kutik S, et al. (2008) Dissecting membrane insertion of mitochondrial β-barrel proteins. *Cell* 132(6):1011–1024.
29. Struyvé M, Moons M, Tommassen J (1991) Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. *J Mol Biol* 218(1): 141–148.
30. Sandoval CM, Baker SL, Jansen K, Metzner SI, Sousa MC (2011) Crystal structure of BamD: An essential component of the β-Barrel assembly machinery of gram-negative bacteria. *J Mol Biol* 409(3):348–357.
31. Hagan CL, Westwood DB, Kahne D (2013) Bam lipoproteins assemble BamA in vitro. *Biochemistry* 52(35):6108–6113.
32. Hagan CL, Wzorek JS, Kahne D (2015) Inhibition of the β-barrel assembly machine by a peptide that binds BamD. *Proc Natl Acad Sci USA* 112(7):2011–2016.
33. Albrecht R, Zeth K (2011) Structural basis of outer membrane protein biogenesis in bacteria. *J Biol Chem* 286(31):27792–27803.
34. Albrecht R, et al. (2014) Structure of BamA, an essential factor in outer membrane protein biogenesis. *Acta Crystallogr D Biol Crystallogr* 70(Pt 6):1779–1789.
35. Ruiz N, Falcone B, Kahne D, Silhavy TJ (2005) Chemical conditionality: A genetic strategy to probe organelle assembly. *Cell* 121(2):307–317.
36. Sklar JG, Wu T, Kahne D, Silhavy TJ (2007) Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. *Genes Dev* 21(19):2473–2484.
37. Molloy MP (2008) Isolation of bacterial cell membranes proteins using carbonate extraction. *Methods Mol Biol* 424:397–401.
38. Leyton DL, et al. (2014) A mortise-tenon joint in the transmembrane domain modulates autotransporter assembly into bacterial outer membranes. *Nat Commun* 5:4239.
39. Moslavac S, et al. (2005) Conserved pore-forming regions in polypeptide-transporting proteins. *FEBS J* 272(6):1367–1378.
40. Leonard-Rivera M, Misra R (2012) Conserved residues of the putative L6 loop of *Escherichia coli* BamA play a critical role in the assembly of β-barrel outer membrane proteins, including that of BamA itself. *J Bacteriol* 194(17):4662–4668.
41. Ryu Y, Schultz PG (2006) Efficient incorporation of unnatural amino acids into proteins in *Escherichia coli*. *Nat Methods* 3(4):263–265.
42. Lenhart TR, Akins DR (2010) *Borrelia burgdorferi* locus BB0795 encodes a BamA orthologue required for growth and efficient localization of outer membrane proteins. *Mol Microbiol* 75(3):692–709.
43. Eppens EF, Nouwen N, Tommassen J (1997) Folding of a bacterial outer membrane protein during passage through the periplasm. *EMBO J* 16(14):4295–4301.
44. Lee J, et al. (2016) Characterization of a stalled complex on the β-barrel assembly machine. *Proc Natl Acad Sci USA* 113(31):8717–8722.
45. Chimalakonda G, et al. (2011) Lipoprotein LptE is required for the assembly of LptD by the β-barrel assembly machine in the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci USA* 108(6):2492–2497.
46. Cowan SW, et al. (1992) Crystal structures explain functional properties of two *E. coli* porins. *Nature* 358(6389):727–733.
47. Schirmer T, Keller TA, Wang YF, Rosenbusch JP (1995) Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. *Science* 267(5197): 512–514.
48. Ziervogel BK, Roux B (2013) The binding of antibiotics in OmpF porin. *Structure* 21(1): 76–87.
49. Casadaban MJ (1976) Regulation of the regulatory gene for the arabinose pathway, araC. *J Mol Biol* 104(3):557–566.