Inhibition of the β-barrel assembly machine by a peptide that binds BamD

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Classification: Biological Sciences – Biochemistry

Keywords: β-barrel, outer membrane, protein folding, Bam complex

Abstract

The protein complex that assembles integral membrane β-barrel proteins in the outer membranes of Gram-negative bacteria is an attractive target in the development of new antibiotics. This complex, the β-barrel assembly machine (Bam), contains two essential proteins, BamA and BamD. We have identified a peptide that inhibits the assembly of β-barrel proteins in vitro by characterizing the interaction of BamD with an unfolded substrate protein. This peptide is a fragment of the substrate protein and contains a conserved amino acid sequence. We have demonstrated that mutations of this sequence in the full-length substrate protein impair the protein's assembly, implying that BamD's interaction with this sequence is an important part of the assembly mechanism. Finally, we have found that in vivo expression of a peptide containing this sequence causes growth defects and sensitizes *E. coli* to antibiotics to which they are normally resistant. Therefore, inhibiting the binding of substrates to BamD is a viable strategy for developing new antibiotics directed against Gram-negative bacteria.

Significance Statement

New antibiotics are urgently needed to treat infections caused by Gram-negative bacteria. These bacteria possess an outer membrane, which is impermeable to most currently available antibiotics. We have studied how a protein that is essential for maintaining the integrity of the outer membrane of *E. coli* interacts with its substrates. In so doing, we have identified a peptide that inhibits this protein's function in vitro and accordingly has deleterious effects on growth and membrane permeability in vivo. This study thus provides a starting point for the development of new antibiotics that directly target outer membrane biogenesis.

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Introduction

Membrane proteins with β-barrel structure are found in the outer membranes of Gram-negative bacteria and in the mitochondria and chloroplasts of eukaryotes. These proteins are assembled into their native membranes by conserved protein complexes, which contain a β-barrel protein of the Omp85 family and some number of accessory proteins (1-6). β-barrel assembly is an essential process and, as such, is an attractive target for the development of new antibiotics that could kill Gram-negative pathogens. However, the mechanism by which β-barrel assembly proceeds is only beginning to be elucidated. Structures of the components of the bacterial β-barrel assembly machine (BamA-E) have been determined recently, and several hypotheses about how they facilitate the assembly of its substrates have been proposed (7-17). The β-barrel component, BamA, contains a kinked β-strand, which might allow substrate proteins to be inserted by passing laterally from the lumen of the BamA β-barrel into the hydrophobic membrane (18). BamA also contains a large soluble domain in addition to its integral β-barrel; this region extends into the periplasmic space between the inner and outer membranes and consists of five polypeptide transport-associated (POTRA) domains (19). The POTRA domains bind the other four components of the assembly complex, BamB-E, which are lipoproteins. Structural and biochemical studies have suggested that the POTRA domains, BamB, and BamD may interact with substrate proteins during their assembly (13, 15, 19-22). However, more detailed information is required about the nature of those interactions and whether they are critical in the assembly process in order to develop an integrated mechanistic model.

Only two components of the Bam complex, BamA and BamD, are essential for *E. coli* viability (2, 23). BamD is found in all proteobacteria (24, 25), but it bears no obvious homology to any of the accessory components of the mitochondrial or chloroplastic complexes. It is therefore unclear what its essential function is and whether that function is specific to bacteria. Do the β-barrel assembly complexes in all species use the same mechanism (perhaps with different accessory proteins) or have they evolved different essential components to meet their organism-specific requirements? We have previously demonstrated that BamD binds to two unfolded OMP substrates, OmpA and BamA, and that it directly facilitates the assembly of BamA (21). In this study, we have characterized this interaction to understand its role in the assembly process carried out by the complete Bam complex and to assess whether interfering with the binding of substrates to BamD might be a viable strategy for developing new antibiotics.

Results

A Peptide Derived from BamA Inhibits OMP Assembly In Vitro

As a first step towards developing an inhibitor of the Bam complex, we assessed whether BamD interacts with substrates in a sequence specific manner and whether that interaction is important in the folding mechanism. We previously demonstrated that unfolded BamA binds to and can be co-purified with soluble BamD-His (*i.e.* a BamD construct lacking its N-terminal lipid acylation site) in vitro (21). We performed similar co-purification experiments with unfolded fragments of BamA to determine which region of the BamA sequence BamD recognizes. The BamA protein was divided into two pieces consisting of its soluble POTRA domains and its membrane β-barrel domain (Figure 1A); these were separately expressed, purified, and urea-denatured. Their ability to co-purify with soluble BamD-His was then

determined. BamD bound the β-barrel domain of the unfolded BamA substrate, but not the isolated POTRA domains (Figure 1B). We then further divided the β-barrel of BamA into four pieces and found that BamD specifically bound the C-terminal 96 amino acids of the β-barrel (Figure 1C).

We then determined whether this interaction between the BamA C-terminal fragment and BamD is important in the assembly mechanism. We used our previously described in vitro assembly reactions in which unfolded BamA is assembled by the Bam complex in proteoliposomes (21, 26, 27). In these assays, folding of the substrate protein, BamA, is detected by production of a band of lower apparent molecular weight on a semi-native SDS-PAGE gel; folded β-barrels do not denature under the conditions of these gel assays and consequently migrate faster than their unfolded forms. The C-terminal BamA fragment inhibited the folding of full-length BamA by the BamABCDE complex in a dose-dependent manner, whereas the N-terminal β-barrel fragment was a less effective inhibitor (Figure 1D). The folding inhibition thus mirrored the affinity of BamD for the BamA peptides, suggesting that the C-terminal fragment interferes with the assembly of the full-length substrate by competing for binding to BamD. This interaction is therefore part of the assembly mechanism performed by the complete complex.

Because sequences near or at the C-termini of β-barrels have been previously proposed to act as recognition sequences (28-30), we examined the ability of five smaller peptides derived from the C-terminal region of BamA to inhibit assembly in our in vitro assay. These peptides consist of overlapping sequences of 15 or 16 residues between amino acids 755 and 810 of BamA (Figure 2A). These five peptides were incubated with the Bam proteoliposomes, and full-length BamA substrate was then added. Only peptide 2, containing residues 765-779 of BamA, inhibited the folding of full-length BamA into proteoliposomes containing the complete

BamABCDE complex (Figure 2B, left panel); peptide 2 thus mimics the inhibitory effects of the C-terminal 96-mer. BamA can also assemble into proteoliposomes containing only BamD (21)—albeit less efficiently—and peptide 2 inhibits folding in that case as well (Figure 2B, right panel). Given that the inhibitory peptide is derived from the BamA sequence, one might imagine that it could inhibit the BamABCDE complex by either binding to a substrate binding site or by disrupting the stability of the complex itself by displacing some of the components (*i.e.*, by competing with the substrate BamA or by competing with the assembled BamA). The inhibition observed in the proteoliposomes containing only BamD suggests that the former possibility is more likely. Taken together, these BamABCDE and BamD inhibition experiments indicate that an important step in BamA assembly is the binding of the sequence at 765-779 to BamD. However, peptide 2 also inhibited folding of full-length OmpA by the Bam complex (Figure 2C), implying that other substrates may also bind to the same site in BamD and that peptide 2 could act as a general inhibitor of OMP assembly.

The Inhibitory Peptide Exploits a Conserved Binding Mechanism

Peptide 2 contains a sequence that was previously identified as a "β-signal" in mitochondrial OMPs. Specifically, the essential peripheral membrane component of the mitochondrial β-barrel assembly machine, Sam35, was shown to bind this sequence in a substrate protein (30). The identified sequence contains a polar residue followed by a conserved glycine and two hydrophobic residues with intervening non-conserved residues (*i.e.*, a sequence of the form: ζxGxxΦxΦ) (30). The residues in this putative "β-signal" are conserved in BamA orthologs, including those in clinically important bacteria (Figure 3A), and similar sequences can be found in other *E. coli* OMPs (Figure S1A). We demonstrated that this sequence is necessary

for binding to BamD; we deleted residues 769-776 from the C-terminal 96-mer and observed that the fragment no longer bound to soluble BamD (Figure 3B). Therefore, the inhibitory effect of peptide 2 can be attributed to the eight residues at positions 769-776. We also tested whether similar "β-signal" sequences in another OMP, OmpF, can inhibit BamA assembly in vitro (Figure S1B). A C-terminal fragment derived from OmpF is a less potent inhibitor of BamA assembly, but as predicted, deleting its proposed β-signal sequences reduces its inhibitory ability. This experiment further suggests that the inhibition we observe is not due to a disruption of the Bam complex but rather a competition with substrate, as a fragment of OmpF would not compete with the interactions between the Bam complex components.

Although the putative "β-signal" is conserved, it is relatively non-specific in that only the glycine is invariant; several amino acids of a particular class can appear in the other semi-conserved positions. We made point mutations in this sequence in BamA to determine their effect on binding to BamD and on assembly of the full-length protein. In accord with the results obtained in mitochondria, we observed that most of the residues can be mutated without significantly affecting binding (30). The one exception was the final hydrophobic residue, W776; mutations of this residue to alanine or glutamine reduced its affinity for BamD (Figure 3C), and BamA substrates containing these mutations were less efficiently assembled by the wild-type complex both in vitro and in vivo (Figure 3D). (The effect of the W776A mutation is more modest than that of the W776Q mutation.) A G771A mutation did not affect binding to BamD, but precluded formation of a stable β-barrel even in detergent solution. Again, this is consistent with the results in mitochondria and indicates that although the glycine is required in the sequence, BamD does not specifically recognize it (30). Rather the glycine likely plays a structural role and is required to produce a stable, folded β-barrel. In fact, crystal structures of

the BamA β-barrel indicate that G771 is adjacent to residues F738 and D740, which interact with the highly conserved residue R661 in loop 6 (8, 9). These interactions between loop 6 and residues on the inside of the barrel may be important in stabilizing the barrel structure, and we hypothesize that larger amino acids at position 771 may disrupt these interactions.

It is worth noting that some mutations that do not significantly reduce binding to BamD are assembled less efficiently than wild-type BamA (for example, compare S769A in Figures 3C and 3D). Furthermore, the W776A and W776Q BamA substrates only demonstrated defects in binding to BamD in vitro when they were evaluated in the context of the 96-residue fragments; no significant changes in binding affinity were observed when the full-length BamA proteins were used in the co-purifications (Figure S2). Defects in the assembly of these full-length mutant substrates were nevertheless observed (Figure 3D). Therefore, the assembly of BamA is more sensitive to changes in the sequence at residues 769-776 than the affinity purifications reveal; BamD may be able to bind to other regions of unfolded BamA when there are mutations in the 769-776 sequence, but assembly is most efficient when binding to this sequence is optimal.

The Inhibitory Peptide Interacts with BamD and is Toxic In Vivo

We expressed peptides derived from BamA in the *E. coli* periplasm to determine whether one containing the sequence at residues 769-776 interferes with OMP assembly in vivo. Peptides consisting of approximately 96 amino acids from the N-terminus of BamA (residues 24-119), from the N-terminus of the BamA β-barrel (422-518), and from the C-terminus of BamA (715-810) were fused to the BamA signal sequence followed by a FLAG tag (Figure 4A). The 24-119 and 422-518 peptides were used as controls to account, respectively, for the toxic effects

of secretion of peptides of this length and of any non-specific unfolded protein stress caused by β-peptides. Under conditions of low-level (uninduced) expression, cells expressing peptides derived from the BamA β-barrel exhibited growth defects on plates and demonstrated increased sensitivity to antibiotics that cannot normally penetrate the OM (Figure 4B). Peptides with β-sheet structure might aggregate easily and induce periplasmic stress, which could explain the phenotypes of these strains without invoking any direct effect on the Bam complex (31). However, the C-terminal 715-810 peptide produces a more severe growth defect (*i.e.*, a smaller colony size and fewer colony-forming units) than the 422-518 peptide, and deleting residues 769-776 reverses this increased toxicity. Deleting residues 769-776 also significantly reduces the sensitivity of the cells to vancomycin and rifampicin, suggesting that the OM barrier is not compromised as severely by expression of that peptide (Figure 4B, bottom two panels). The deletion of residues 769-776 in the 715-810 peptide serves as a control, which indicates that the toxicity of the 715-810 peptide cannot be attributed solely to non-specific periplasmic stress. (The 422-518 peptide is included for comparison, but the 769-776 deletion demonstrates the importance of the β-signal sequence.) We therefore conclude that the SAGIALQW sequence, which inhibits BamA assembly in vitro, is also toxic in vivo.

Expression of any of the fragments derived from the BamA β-barrel (422-518, 715-810, and 715-810Δ769-776) results in increased expression of the periplasmic protease, DegP, suggesting that these fragments induce the periplasmic stress response (Figure S3). The stress response changes the transcription of many OMPs, complicating the interpretation of any effects of the expressed fragments on OMP levels in vivo (32). We observe that the 715-810 fragment reduces the cellular level of LptD slightly more than the other fragments (Figure S3). LptD is the essential, β-barrel component of the machine that transports lipopolysaccharide to the outer

leaflet of the OM. The effects on LptD assembly may thus account—at least in part—for the increased toxicity of the 715-810 fragment given that this protein is essential and directly involved in establishing the impermeability of the OM. The expression of the BamA fragments has no obvious effect on the cellular level of full-length BamA, but BamA is also upregulated by the envelope stress response (σ^E) and any inhibition of BamA assembly might be ameliorated in vivo by these transcriptional changes and by the presence of periplasmic chaperones and proteases.

We performed photocrosslinking experiments to establish that the 715-810 peptide interacts with BamD in vivo. *p*-Benzoylphenylalanine was introduced at several positions in or near the "β-signal" sequence in the 715-810 peptide by unnatural amino acid incorporation (33-35). These mutated peptides were expressed at a low (uninduced) level as in the phenotypic experiments described above, and the cells were irradiated with UV light to induce crosslinking to any proteins that interact with the mutated residues. The peptide containing the photocrosslinker at position 776 produced an adduct that reacts with both FLAG and BamD antibodies (Figure 4D). This adduct has a molecular weight of ~35 kDa, which is approximately equal to the sum of the molecular weights of the peptide and BamD. Notably, residue W776 is the one amino acid that also appeared to be important for binding to BamD in vitro (Figure 3C). We have thus demonstrated that a peptide containing a conserved sequence in BamA interacts with BamD in vivo and produces growth, OM permeability, and OMP biogenesis defects.

Discussion

The five components of the Bam complex catalyze the assembly of outer membrane proteins with β-barrel structure; deletions or depletions of any of the components result in less

efficient OMP assembly in vivo (2, 23, 36). Much attention has understandably been paid to BamA because it is the only component for which structural and functional homologs have been identified in Gram-negative bacteria, mitochondria, and chloroplasts (1-6). However, BamD is an essential protein in bacteria and must therefore play an important role (23, 37). We previously demonstrated that BamD interacts with unfolded OMPs (21, 38) and here have shown that interfering with that interaction inhibits β-barrel assembly in vitro and compromises cell growth and OM integrity in vivo. Taken together, these observations suggest that BamD's essential function is to bind unfolded OMP substrates during the assembly process. However, this does not imply that each of the more than 50 different OMPs present in the OM specifically require BamD for their assembly (39). Other components of the Bam complex also interact with substrates (13, 15, 19-22, 38) and may have functions that overlap with that of BamD such that certain OMPs can be assembled in its absence. Rather we suggest that BamD's essentiality in substrate binding reflects a kinetic effect on the overall efficiency of OMP assembly, a specific effect on the assembly of one or a few OMP substrates that are critical for cell growth and OM integrity, or possibly a combination of both kinetic and substrate-specific effects.

We have shown that BamD binds a sequence in unfolded BamA that is the same as the sequence Sam35 binds in mitochondrial OMPs. This implies that aspects of the mechanism of β-barrel assembly are conserved despite the apparent non-conservation of the proteins that carry out those aspects. Sam35 and BamD share no obvious sequence homology but perform the same receptor-like function in binding unfolded substrates at the surface of the membrane into which the substrates are inserted (21, 30); therefore, the conservation of the β-barrel assembly mechanism extends beyond the steps performed by BamA. Previous reports demonstrating that some mitochondrial β-barrels can be assembled in bacteria and that some bacterial OMPs can be

assembled in mitochondria implied that the assembly complexes have similar substrate recognition mechanisms (40-43), and structural studies (13-15, 44-46) suggested that BamD might recognize OMP substrates based on its homology to proteins that bind C-terminal peptides (*e.g.*, a component of the mitochondrial translocase, Tom70, a peroxisomal protein receptor, PEX5, and a factor responsible for organizing Hsp chaperone proteins, Hop). Our results connect these observations and provide the underlying molecular basis for substrate recognition, which explains the reciprocity of β-barrel assembly between mitochondria and bacteria.

The recognition sequence, or "β-signal," is found in BamA approximately 30 amino acids removed from the C-terminus and is found in mitochondrial OMPs near, but often not at the C-terminus (30). However, in other bacterial OMPs, this sequence does sometimes appear at the extreme C-terminus (Figure S1A). The C-terminal residues of some bacterial OMPs have been shown to be important for their assembly (28); this observation is consistent with our results that show that the final residue of the "β-signal" is particularly important for binding to BamD (Figures 3 and 4). Therefore, the positioning of the "β-signal" within OMP sequences may be somewhat variable, and some OMPs may have more than one such sequence (*e.g.*, OmpF). Perhaps the different positions of the "β-signal" reflect differences in the assembly requirements of different OMPs.

It will be critical to understand the relationship between the components of the Bam complex to elucidate how OMP assembly proceeds and what all of their roles are. However, we have identified a step, involving the essential protein, BamD, that provides a strategy for interfering with β-barrel assembly. Because β-barrel assembly is essential in Gram-negative bacteria, compounds that inhibit OMP assembly could have antibiotic utility. The importance of the BamD-substrate binding interaction makes it possible to reduce the problem of how to inhibit

OMP assembly to a simpler one of how to inhibit binding to BamD. We have shown that a small peptide will inhibit BamA and OmpA assembly in vitro and that it has toxic effects in vivo. Molecules that mimic the peptide and bind to BamD in vivo should interfere with OMP assembly, and the lack of sequence conservation between Sam35 and BamD should make it possible to develop antibiotics that selectively target the bacterial complex. Furthermore, as the sequence contained in the inhibitory peptide is conserved across bacterial species, molecules that bind to BamD could be effective against a broad group of Gram-negative organisms. Although we did not see evidence of direct inhibition of BamA assembly in vivo, our in vitro results indicate that BamD inhibitors would reduce the efficiency of BamA assembly. Interfering with BamA assembly should in turn inhibit the assembly of all OMPs, and because those OMPs also have important functions, targeting BamA assembly could have multiple downstream effects.

Materials and Methods

Additional information regarding experimental procedures is provided in the Supporting Information.

Unfolded Substrate Affinity Purifications

FLAG-BamA and all the fragments thereof (P1-5, β-barrel, 422-518, 519-616, 617-714, 715-810, 715-810Δ769-776, and 715-810 point mutants) were urea-denatured and subsequently diluted ten-fold into a solution of soluble BamD-His_6 in TBS (pH 8) and incubated a room temperature for 10 min. In most of these experiments, the final concentrations of the unfolded protein and soluble BamD-His_6 were both 50 μM; however, in the experiment in Figures 1B, the unfolded proteins and BamD-His_6 were mixed in a 1:10 ratio such that their final concentrations

were 10 μM and 100 μM, respectively. A small aliquot of each of these mixtures was removed for use as an "input" sample. The remainder of the mixture was subjected to Ni-NTA affinity purification; after loading the material on the column, it was washed with TBS (pH 8) with 20 mM imidazole and the bound proteins were eluted in TBS (pH 8) with 200 mM imidazole. Proteins in the eluates were precipitated with 10% trichloroacetic acid by incubation on ice for 30 min. The samples were then centrifuged at 16,000 x g for 10 min at 4 ^{o}C, and the pellets were resuspended in a 1:1 mixture of 1 M Tris (pH 8) and 2x SDS sample loading buffer (125 mM Tris, pH 6.8, 4% SDS, 30% glycerol, 0.005% bromophenol blue, 5% β-mercaptoethanol). The proteins in the "input" and these "eluate" samples were separated by SDS-PAGE and stained with Coomassie Blue.

In Vitro Folding Assays

The method for monitoring the in vitro assembly of the β-barrels by the Bam complex has been described previously (21, 26, 27). The particular adaptations of this method for the experiments described in this paper are explained here.

BamA Folding Inhibition in Bam Proteoliposomes

FLAG-tagged BamA fragments 422-518 and 715-810 were mixed with 5 uM full-length FLAG-BamA substrate in 8 M urea and then diluted ten-fold into the empty liposomes and BamABCDE proteoliposomes. The proteoliposomes were also diluted four-fold from their stock concentrations into these reactions. A typical reaction contained 2.5 μL liposomes or proteoliposomes, 6.5 μL TBS (pH 8), and 1 μL of the substrate and fragment mixture such that the final concentrations of the FLAG-BamA substrate and Bam complex were 0.5 μM and ~2.5

μM, respectively, and the fragment concentration was varied from 0-10 μM. The reactions were incubated at room temperature for 60 minutes and then stopped by adding ice-cold 2x SDS sample loading buffer. The quenched samples were immediately applied to SDS-PAGE (4-20% gel), and run at 150 V for 110 min at 4 ^{o}C. The proteins were transferred from the gel to a PVDF membrane by semi-dry transfer in 25 mM Tris-HCl, 192 mM glycine (pH 8.3) at 10 V for one hour. The products of the reaction were detected by immunoblotting with anti-FLAG-HRP antibodies.

The peptides (1-5) containing 15 or 16 amino acids were dissolved at 1 mg/mL in 1:1 TBS (pH 8): DMSO and subsequently diluted with additional TBS (pH 8). These peptides were added to empty liposomes and BamABCDE or BamD proteoliposomes and incubated at room temperature for 10 min. FLAG-BamA substrate (in 8 M urea) was then diluted ten-fold into each reaction and incubated for 60 min or 120 min in the BamABCDE and BamD reactions, respectively. The final concentrations of the reaction components were: 0.5 μM FLAG-BamA substrate, 100 μM peptide, and ~2.5 μM Bam complex. Each reaction contained ~10% residual DMSO from the peptide stock solutions. All reaction products were analyzed by SDS-PAGE and immunoblotting as described above.

OmpA Folding Inhibition in Bam Proteoliposomes

The peptides containing 15 or 16 amino acids were dissolved at 1 mg/mL in 1:1 TBS (pH 8): DMSO and subsequently diluted with 8 M urea to a final concentration of 200 μM in a solution also containing 5 μM full-length FLAG-OmpA substrate in urea. The final concentrations of urea and DMSO in these substrate-peptide mixtures were approximately 4 M and 20%, respectively. A control mixture containing FLAG-OmpA, urea, and DMSO, but

lacking any peptide was also prepared. These mixtures were then diluted 10-fold into reactions containing empty liposomes or BamABCDE proteoliposomes and incubated at room temperature for 60 min. The final concentrations of the reaction components were: 0.5 μM FLAG-OmpA substrate, 20 μM peptide, and ~2.5 μM Bam complex. Each reaction contained ~2% residual DMSO from the peptide stock solutions. All reaction products were analyzed by SDS-PAGE and immunoblotting as described above.

Folding of Mutated FLAG-BamA Substrates in Detergent and Proteoliposomes

All FLAG-tagged substrate proteins were prepared at a concentration of 5 μM in 8 M urea and then diluted ten-fold into a TBS (pH 8) solution containing 0.5% LDAO, empty liposomes, or Bam complex proteoliposomes as described above (with the exception that no inhibitory peptides were included). The folding reactions were incubated at 25 ^{o}C for 60 min and then stopped with ice-cold 2x SDS sample loading buffer. The quenched samples were run on semi-native SDS-PAGE and immunoblotted as described above.

In Vivo Assembly of FLAG-BamA Substrates

BL21(DE3) cells harboring pJW152, pJW153, pJW154, pJW155, pJW156, pJW157 or pJW158 were grown at 37 ^{o}C in LB supplemented with 50 μg/mL carbenicillin and 0.2% glucose to an OD_{600} of approximately 0.3. The cells from a 1 mL sample were collected by centrifugation at 20,000 x g for 5 min and then flash frozen. Once all samples were collected and frozen, they were thawed to room temperature and resuspended in 20 mM Tris (pH 8), 1 mM EDTA, 5 mg/mL lysozyme. The suspensions were subjected to two freeze/thaw cycles by freezing in a CO_2/acetone bath and warming to room temperature in water. The thawed samples

were then treated with lysis solution (20 mM Tris (pH 8), 10 mM EDTA, 4% SDS). The samples were vortexed for 10 seconds and subsequently rocked at room temperature for 30 min. The resulting cell extracts were mixed 1:1 with TBS (pH 8). SDS sample loading buffer was added and the samples were subjected to SDS-PAGE (4-20% gel) at 150 V for 90 min at 4 ^{o}C. The proteins were transferred from the gel to a PVDF membrane via a Pierce G2 fast blotter in 1-step transfer buffer at 25 V for 15 minutes. The folded and unfolded forms of the FLAG-tagged BamA proteins were detected by immunoblotting with anti-FLAG-HRP antibodies.

Growth Analysis of Strains Expressing BamA Peptides

BL21(DE3) cells harboring pJW114, pJW115, pJW144 or pJW146 were grown at 37 ^{o}C in LB supplemented with 50 μg/mL carbenicillin and 0.2% glucose to an OD_{600} of approximately 0.4. The cultures were diluted with LB to an OD_{600} of 0.1 and then serially diluted by a factor of 10. The diluted cultures were then plated in drops on freshly prepared agar containing the indicated antibiotics. The plates were incubated at 37 ^{o}C for 16 hours.

Photocrosslinking

BL21(DE3) cells harboring pSup-BpaRS-6TRN (35) and pJW148, pJW149 or pJW151 were grown at 37 ^{o}C in LB supplemented with 50 μg/mL carbenicillin, 30 μg/mL chloramphenicol and 0.2% glucose. These cultures were used to inoculate 12 mL of LB supplemented with 0.8 mM *p*-benzoylphenylalanine, 50 μg/mL carbenicillin, 30 μg/mL chloramphenicol and 0.2% glucose. These cultures were grown at 37 ^{o}C to an OD_{600} of 0.21 – 0.27. A 540 μL sample of each culture was then removed and incubated on ice for use as a non-irradiated control. In a 96-well clear-bottomed plate, three aliquots of 200 μL of each strain

were incubated on ice and irradiated from above with UV light at 365 nm for 7.5 minutes. 180 μL were then collected from each well (540 μL total for each strain), and all samples were centrifuged at 16,000 x g for 10 minutes. The supernatants were discarded, and the resulting cell pellets were resuspended in SDS sample loading buffer. The samples were boiled for 10 minutes and then applied to SDS-PAGE. Cross-linked proteins were detected by immunoblotting with anti-FLAG-HRP and BamD antibodies.

Acknowledgements

This work is supported by NIH grant AI081059.

Author Contributions

C. L. H. and J. S. W. performed the experiments described here. C. L. H., J. S. W., and D. K. designed the experiments, analyzed the data, and wrote the paper.

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Figure Legends

Figure 1. BamD binds the C-terminal region of unfolded BamA during the assembly of BamA by the Bam complex. **A.** Schematic depicting the domain organization of BamA. The BamA sequence contains a signal sequence (SS) that targets the unfolded polypeptide for secretion into the periplasm, a soluble periplasmic region that consists of five POTRA domains (P1-5), and a β-barrel domain that is integrated into the outer membrane. Red marks depict the artificial division of the β-barrel domain into quarters, which are employed in C and D. **B.** BamD binds to the β-barrel region of unfolded BamA. Full-length FLAG-BamA, the five POTRA domains of BamA, and the β-barrel region of BamA were prepared in 8 M urea, diluted into a detergent-free solution containing a ten-fold excess of soluble BamD-His_6, and then tested for their ability to co-purify with BamD by Ni-NTA chromatography. **C.** BamD binds the C-terminal region of the BamA β-barrel. Urea-denatured BamA fragments each consisting of approximately one quarter of the β-barrel domain were diluted into an equal molar solution of soluble BamD-His_6 and affinity purified. **D.** The C-terminal BamA 715-810 fragment specifically inhibits the assembly of full-length BamA by the Bam complex. The N- and C-terminal fragments of the BamA β-barrel were mixed with full-length FLAG-BamA in 8 M urea and then diluted into reactions containing liposomes or proteoliposomes containing the BamABCDE complex. Folding was stopped after 60 minutes, and the reaction products were run on semi-native SDS-PAGE, which separates the folded and unfolded forms of the FLAG-BamA β-barrel. The products were visualized by immunoblotting with anti-FLAG antibodies. The concentrations of the Bam complex and the FLAG-BamA substrate in each reaction were ~2.5 μM and 0.5 μM,

respectively. The concentration of the added fragment was varied from 0-10 μM, as indicated above the lanes.

Figure 2. A short peptide derived from BamA inhibits OMP folding by the Bam complex in vitro. **A.** Schematic depicting five peptides containing overlapping sequences from the C-terminal region of BamA, which were tested for their ability to inhibit BamA folding in vitro. **B.** Peptide 2 specifically inhibits the assembly of full-length FLAG-BamA by the BamABCDE complex (left) and by BamD alone (right) in proteoliposomes. Each peptide was preincubated with the proteoliposomes before addition of the FLAG-BamA substrate. The final concentrations of the peptides and substrate were 100 μM and 0.5 μM, respectively. The reactions that included the peptides also contained ~10% DMSO, which was used to dissolve the peptides. **C.** Peptide 2 specifically inhibits the assembly of full-length FLAG-OmpA by the BamABCDE complex. Each peptide was mixed with the denatured FLAG-OmpA substrate and then diluted into the proteoliposomes. The final concentrations of the peptides and substrate were 20 μM and 0.5 μM, respectively. The reactions that included the peptides also contained ~2% residual DMSO.

Figure 3. BamD binds a conserved amino acid sequence. **A.** Peptide 2 contains the same sequence identified in mitochondrial β-barrels as a β-signal, and this sequence is conserved in Gram-negative species of pathogenic organisms. The mitochondrial consensus sequence is depicted above the Clustal alignment of five BamA sequences with the residues predicted to be important highlighted in red. **B.** Deleting the β-signal sequence eliminates binding of BamD to the C-terminal region of BamA. The eight amino acids (769-776) containing the β-signal were

deleted from the C-terminal quarter of the β-barrel (715-810). This BamA fragment and the wild-type fragment were denatured in 8 M urea and then diluted into an equal molar solution of soluble BamD-His_6 and affinity purified. **C.** Mutations of W776 to A or Q reduce the affinity of the C-terminal quarter of the BamA β-barrel for BamD, but mutations of the other conserved residues in the β-signal produce no significant change in binding. Denatured BamA 715-810 fragments containing the indicated mutations were diluted into an equimolar amount of BamD-His_6, and co-purified as in the previous experiments. **D.** Certain residues in the β-signal are important in the Bam complex-dependent folding of the BamA β-barrel in vitro and in vivo. In the in vitro experiments (first two panels), full-length BamA substrates containing mutations in the β-signal sequence were denatured and then diluted into solutions containing 0.5% LDAO or BamABCDE proteoliposomes in order to assess, respectively, the effects of the mutations on the stability of the folded β-barrel and on the folding mechanism carried out by the Bam complex. The folding of each mutant β-barrel was analyzed after 60 min of incubation. A mutation of G771 precludes folding of the β-barrel even in detergent solution, indicating that this residue is important for the stability of the folded product. In the in vivo experiments (third panel), FLAG-tagged BamA mutants were ectopically expressed in BL21(DE3) *E. coli* (carrying a wild-type chromosomal copy of BamA). The assembly of the mutant BamA was assessed by semi-native SDS-PAGE and immunoblotting for the FLAG-tag. "Heat" indicates whether the cell lysate was boiled prior to SDS-PAGE analysis. (U: unfolded FLAG-BamA, F: folded FLAG-BamA)

Figure 4. Secreted peptides containing the inhibitory β-signal sequence are toxic and interact with BamD in vivo. **A.** Schematic depicting the fragments of BamA expressed in cells in B. Fragments contained residues 1-23 of BamA (*i.e.*, the signal sequence plus three additional

residues), followed by a FLAG-tag, and finally the sequences designated in the purple boxes below the BamA schematic. Residues 21-23 were included in each fragment to ensure proper and consistent cleavage of the signal sequence by the signal peptidase. **B.** Comparison of the number of colony forming units (CFU) from strains expressing fragments of BamA indicates that secretion of the C-terminal quarter of BamA into the periplasm is toxic and sensitizes *E. coli* to vancomycin and rifampicin. These effects are significantly reduced when the eight amino acid β-signal is deleted from the same fragment. Both fragments derived from the β-barrel domain of BamA (422-518 and 715-810) exhibit deleterious effects, but expression of the C-terminal fragment results in fewer CFU and a smaller colony size in the absence of vancomycin and rifampicin. Carbenicillin is added to the plates to maintain the expression plasmids, and glucose is added to reduce expression of the fragments. **C.** The BamA 715-810 fragment containing a photo-crosslinker at residue 776 interacts with BamD in vivo. Strains expressing the secreted 715-810 fragment with amber mutations encoding the unnatural amino acid, *p*-benzoylphenylalanine, were subjected to UV irradiation to induce crosslinking. The arrows indicate the cross-linked fragment-BamD adduct. This band appears only upon UV irradiation and is detected by both the anti-FLAG (left panel) and anti-BamD (right panel) antibodies.