



Substrate binding to BamD triggers a conformational change in BamA to control membrane insertion

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The β -barrel assembly machine (Bam) complex folds and inserts integral membrane proteins into the outer membrane of Gram-negative bacteria. The two essential components of the complex, BamA and BamD, both interact with substrates, but how the two coordinate with each other during assembly is not clear. To elucidate aspects of this process we slowed the assembly of an essential β -barrel substrate of the Bam complex, LptD, by changing a conserved residue near the C terminus. This defective substrate is recruited to the Bam complex via BamD but is unable to integrate into the membrane efficiently. Changes in the extracellular loops of BamA partially restore assembly kinetics, implying that BamA fails to engage this defective substrate. We conclude that substrate binding to BamD activates BamA by regulating extracellular loop interactions for folding and membrane integration.

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

The outer membranes (OMs) of Gram-negative bacteria, mitochondria, and chloroplasts all contain integral membrane β -barrel proteins (1, 2). In *Escherichia coli* these OM proteins (OMPs) are folded and membrane-integrated by the β -barrel assembly machine (Bam) (3–5). Some β -barrels facilitate passage of nutrients through the OM, while others play structural roles in the maintenance of cell morphology. This functional diversity demands significant structural diversity (2). The Bam complex assembles a wide range of β -barrel sizes (2), from 8 to 26 β -strands, and β -barrels with complex topologies. Given this structural diversity, the mechanism by which the Bam complex assembles substrates has to account both for substrates that can assemble rapidly and efficiently and those that may require more coordinated and ordered assembly processes.

The Bam complex is composed of five components, BamABCDE (3–6). BamA, a β -barrel itself, serves as a scaffold to which the lipoproteins BamBCDE bind (7). BamA contains a large integral membrane β -barrel domain that plays an essential role in substrate membrane insertion. BamD is required to localize substrates to the membrane by binding to a β -signal embedded in the C-terminal portion of the substrate (8–10). BamB and BamD independently interact with BamA through its soluble periplasmic domain (3, 4), while BamC and BamE interact indirectly with BamA through BamD (4, 5).

Biochemical studies of the Bam complex suggest that it is functionally modular. Subcomplexes consisting of BamAB and BamCDE can be separately overexpressed and combined to reconstruct the Bam complex (11). Genetic and biochemical data suggest that these BamAB and BamCDE subcomplexes can each recognize substrates independently of one another (11–13). Despite this seemingly functional redundancy, each subcomplex contains an essential component and the five components are believed to exist as one complex in the cell. How these two modules cooperate is not well understood because few intermediate states in the catalytic cycle of the Bam complex have been defined.

To probe how the Bam complex accelerates folding and insertion we studied the assembly of a slow-folding β -barrel substrate, LptD. Together with the lipoprotein LptE, LptD forms the translocon that exports lipopolysaccharide across the OM (14). We identified a mutation in the C terminus of LptD that further slows its maturation. This substrate still binds to BamD but fails to integrate into the membrane efficiently. A change within the β -barrel of BamA can restore substrate assembly kinetics of this LptD mutant. Our results suggest a mechanism in which substrate recruitment by BamD in the periplasm regulates extracellular loop interactions to activate BamA for folding and insertion.

Results

A Change in the C Terminus of LptD Causes Early Assembly Defects in the Periplasm. We are interested in characterizing mutations that slow the folding of substrates during assembly by the Bam complex and have identified a C-terminal recognition sequence within an unfolded substrate that is required for substrate recruitment and folding. The positioning of this sequence is somewhat variable, with some positioned at the very C terminus (8, 15) and others positioned ~30 residues removed from the C terminus (16). In substrate BamA a recognition sequence resides within the third-to-last strand of the eventual β -barrel, and

Significance

The outer membrane of Gram-negative bacteria, mitochondria, and chloroplasts contains proteins that adopt β -barrel structures. To maintain the integrity of this structure, the β -barrel assembly machine (Bam) folds and inserts integral membrane proteins into the outer membranes of Gram-negative bacteria. By studying the assembly of an essential β -barrel substrate we found that the two essential components of the complex, BamA and BamD, coordinate with each other upon substrate binding to facilitate folding and insertion. These results could enable the design of strategies to combat Gram-negative pathogens.

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changes within this sequence cause assembly defects (8, 17). We wondered whether a similar sequence within LptD might reside in a similar location near the C terminus of the substrate. We made amino acid substitutions in a region between positions 716 and 726 of LptD, which encompasses the third-to-last strand of the β -barrel domain (Fig. 1A and Fig. S1). In cells expressing a single copy of LptD we observed that a substitution at a conserved tyrosine at position 721 (LptD_{Y721D}) conferred increased sensitivity to antibiotics, such as vancomycin (Fig. 1B) and bacitracin (Fig. S24), that typically do not penetrate the Gram-negative OM. However, in the presence of a WT copy of LptD, the integrity of the OM is restored, suggesting that this change is a loss-of-function mutation. Since LptD/E forms the translocon that delivers lipopolysaccharide to the cell surface, we conclude that the Y721D mutation must impair LptD assembly, function, or both.

To ascertain whether the OM defects in haploid strains expressing *lptD*_{Y721D} are caused by improper assembly of the LptD variant we compared LptD levels from cells expressing either WT LptD or LptD_{Y721D} and observed decreased levels of LptD_{Y721D} compared with those of WT LptD (Fig. 1C, lanes 1–4). We previously showed that the native LptD substrate undergoes oxidative rearrangement to form two disulfide bonds between nonconsecutive cysteines ([1,3][2,4]-LptD) that migrates at a higher molecular weight than the reduced form by

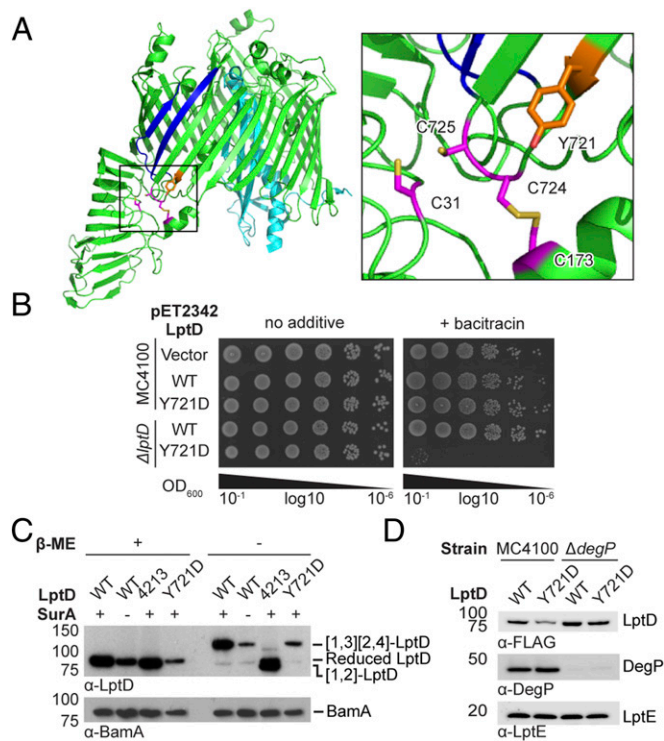


Fig. 1. A change in the C terminus of LptD causes early assembly defects. (A) LptD_{Y721} (orange) is a conserved residue in β 24 that is proximal to two conserved cysteines (magenta). The first and last strands are depicted in blue. Images were generated from the structure of LptD/E from *Shigella flexneri* [Protein Data Bank (PDB) ID code 4Q35] using PyMOL. (B) LptD_{Y721D} exhibits defects in OM permeability. Efficiency of plating assays of strains carrying a plasmid-borne variant of LptD with or without a chromosomal copy of LptD on LB were supplemented with 625 mg/L bacitracin. (C) LptD_{Y721D} exhibits a periplasmic folding defect. Whole-cell lysates were subject to immunoblot analyses in MC4100, *surA*, *lptD4213*, and *lptD*_{Y721D} strains. (D) LptD_{Y721D} is degraded by the periplasmic protease DegP during assembly. Whole-cell lysates were subjected to immunoblot analyses in MC4100 and *degP* strains expressing FLAG-tagged LptD.

SDS/PAGE (18, 19). However, we found that LptD_{Y721D} still adopts the mature, properly oxidized form. Therefore, we conclude that LptD_{Y721D} is folding-competent.

Although LptD_{Y721D} can assemble into the mature oxidized form, it was unclear whether the decreased levels were a result of a defect during folding or instability of the mature form after membrane insertion. To reach the OM, unfolded LptD is escorted by the chaperone SurA in the periplasm (20–22). Defects in SurA lead to decreased assembly efficiency of LptD (20, 22). Substrates that fall off-pathway are cleared by periplasmic proteases such as DegP (20, 23, 24). Cells which lack SurA show proper maturation of LptD but have decreased protein levels comparable to that of LptD_{Y721D} (Fig. 1C). Consistent with an early assembly defect, chromosomal deletion of *degP* restores LptD_{Y721D} levels (Fig. 1D). These results are in contrast with another LptD variant that we have previously characterized, LptD4213. LptD4213 accumulates on the Bam complex as a late-stage assembly intermediate (25) which does not exhibit significant degradation and adopts an intermediate state containing consecutive disulfide bonds ([1,2]-LptD) (Fig. 1C and Fig. S2). Therefore, we conclude that LptD_{Y721D} exhibits an early folding defect in the periplasm.

The Mutant LptD Substrate Interacts with BamD but Is Slow to Membrane-Integrate. Since BamD binds the C terminus of unfolded substrates and LptD_{Y721D} harbors a mutation near its C terminus we wondered whether this change would alter the ability of LptD_{Y721D} to interact with BamD. We assessed the affinity between soluble BamD and β -barrel-derived peptides of LptD in vitro by microscale thermophoresis (MST). This technology monitors changes in the movement of fluorescently labeled molecules along a temperature gradient depending on their interaction with other biomolecules. In this assay, fluorescently labeled peptide fragments of LptD were mixed with soluble BamD-His, and we found that the peptide comprising the C-terminal 96 aa of the β -barrel (residues 689–784) bound to BamD with micromolar affinity (Fig. 2A, black). A 96-residue-long peptide was selected empirically because of difficulties in the expression and solubility of shorter peptides. Deletion of residues 719–726 within this peptide bound BamD with significantly lower affinity (Fig. 2A, blue). The peptide containing the Y721D mutation demonstrated only slight differences in binding affinity (Fig. 2A, green). Taken together these results suggest that the region consisting of 719–726 in the C terminus of LptD is important for the recognition of substrate LptD by BamD, but a single point mutation, Y721D, does not appear to significantly alter affinity.

To test whether this specific LptD C-terminal sequence interacts with BamD in vivo we employed a site-specific photocross-linking strategy. We introduced the unnatural amino acid p-benzoylphenylalanine (pBPA) into multiple positions at or proximal to Y721 in His-tagged LptD (Fig. 2B). Previously, we used this strategy to show that an LptD variant, LptD4213, accumulates on the Bam complex and remains bound to BamD throughout folding and membrane integration (25). Since prolonged residency time on the Bam complex allows for more efficient cross-linking, we used the stalled LptD4213 substrate to determine if this recognition sequence in LptD is the region that interacts with BamD. Following UV irradiation, we observed that substitution of pBPA at residues L717 and Y726 in the substrate produced a UV-dependent cross-link to BamD (Fig. 2B and C). All observable cross-links in the WT substrate were enriched in the LptD4213 substrate and no additional cross-links were detected (Fig. 2C). Similar photocross-linking experiments with LptD_{Y721D} were unsuccessful owing to the instability of the mutant protein.

Because LptD_{Y721D} is not defective in its recruitment to the Bam complex via BamD, the susceptibility of LptD_{Y721D} to periplasmic proteases may be a result of a failure to insert into the membrane. To assess the degree of substrate membrane integration

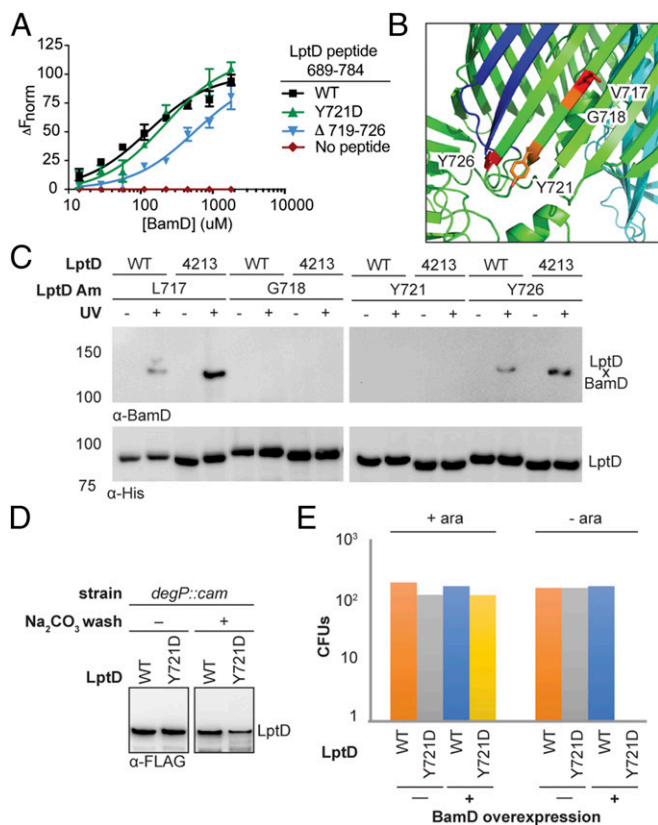


Fig. 2. The mutant LptD substrate interacts with BamD but is slow to membrane-integrate. (A) MST analysis of fluorescently labeled LptD peptides mixed with soluble BamD. Labeled peptide (5 nM) was titrated with increasing concentrations of soluble BamD up to 1.8 mM. Experiments were performed in duplicate and results are shown as mean \pm SD. (B) Specific sites in LptD cross-link to BamD. In His-tagged LptD four residues (orange) in LptD were mutated to incorporate pBPA and two (red) were found to cross-link to BamD. (C) In vivo photocross-linking of LptD to BamD. MC4100 strains both harboring the amber suppression system and expressing a His-tagged LptD/LptD4213 pBPA derivative were either left untreated or irradiated with UV. Adducts were identified after affinity purification and immunoblot analyses under reducing conditions. (D) LptD_{Y721D} fails to membrane-insert. Lysates from *degP* strains expressing FLAG-tagged LptD or LptD_{Y721D} were washed with 100 mM Na₂CO₃ and the resulting membranes were extracted and analyzed by immunoblotting. (E) Overexpression of BamD is synthetic lethal with LptD_{Y721D}. The *lptD* depletion strain containing an arabinose-inducible WT LptD copy (HC329) was transformed with a plasmid expressing WT LptD or LptD_{Y721D} and a plasmid with or without overexpression of BamD.

we subjected cells expressing WT LptD or LptD_{Y721D} to a carbonate extraction procedure. In this assay, proteins that have fully inserted into the membrane such as WT LptD are resistant to washing by sodium carbonate, while those that are only peripherally associated are not retained within the membrane fraction (27). Because strains that lack DegP normalize total LptD levels, we wondered if this correlated with improved membrane integration. Cell lysates from strains lacking DegP and expressing WT LptD or LptD_{Y721D} were incubated with sodium carbonate solution and the resulting membranes were detergent-solubilized (Fig. 2D). LptD_{Y721D} was more easily washed than WT LptD, suggesting that DegP degrades LptD_{Y721D} substrates that fail to membrane-integrate.

If LptD_{Y721D} is stalled on BamD, but cannot membrane-integrate, we reasoned that the substrate could be titrated away from a functional Bam complex by varying the expression levels of BamD. We found that we were unable to overexpress

BamD in cells expressing LptD_{Y721D} and conclude that this produces a synthetic lethal phenotype (Fig. 2E). Accumulation on BamD causes prolonged exposure of the substrate to periplasmic proteases, which results in the observed degradation. Free BamD, which is not in complex with BamA since it is overexpressed, can compete with BamD from a mature complex for the LptD_{Y721D} substrate, which sequesters it from the effective available substrate pool. In contrast, excess free BamD is unable to titrate away the native substrate from complexed BamD because its biogenesis is efficient enough so that it does not have a prolonged residence time on the Bam complex. Consistent with an accumulation of the LptD_{Y721D} substrate on BamD, expression of a second WT copy of LptD suppresses the BamD overexpression synthetic lethality in these strains (Fig. 2E). Therefore, we conclude that substrate recruitment by the Bam complex involves initial interaction of the C terminus with BamD, followed by subsequent substrate engagement with BamA. These results provide further evidence that substrates can be recruited to BamD independently of BamA (8, 12).

Substrate Engagement by BamD Modulates BamA Activity. We identified suppressors of the OM defects conferred by LptD_{Y721D} (Fig. 3A) by selecting resistance to bacitracin. We isolated one resistant allele that mapped to the *bamA* locus and results in a phenylalanine-to-leucine substitution at residue 494 (designated as BamA_{F494L}). Residue F494 is located near an extracellular loop in the β -barrel region of BamA (Fig. 3B) (28–30) and has previously been implicated in the assembly and function of BamA (31, 32). Cells expressing BamA_{F494L} in the presence of LptD_{Y721D} also exhibited decreased antibiotic sensitivity (Fig. 3A and Fig. S2A and S3A) and were no longer sensitive to BamD overexpression (Fig. 3C). Expression of BamA_{F494L} in otherwise WT cells did not produce significant phenotypes with the exception of enhanced sensitivity to antibiotics under nutrient-depleting conditions (Fig. S4). We assessed total levels of LptD_{Y721D} via whole-cell blotting in cells expressing either WT BamA or BamA_{F494L}. Consistent with improved barrier function, BamA_{F494L} also improves the levels of mature oxidized LptD (Fig. 3D and Fig. S3B). We wondered whether BamA_{F494L} improves LptD_{Y721D} assembly by altering substrate interactions with BamD. We probed whether BamA_{F494L} changes the interaction between substrate LptD and BamD by using the photocross-linking approach employed above (Fig. 2C). When pBPA was substituted at residue L717 in substrate WT LptD and LptD4213 we observed the appearance of a UV-dependent cross-link to BamD in both cells expressing WT BamA and BamA_{F494L} (Fig. 3E). Therefore, BamA_{F494L} does not influence the ability of BamD to interact with substrate.

BamA_{F494L} was previously isolated as a suppressor for a synthetic lethal double BamB/BamE deletion (32). In that study, the authors identified multiple point mutations in the BamA β -barrel, with F494L being the most frequently isolated. All of the other mutations mapped to the sixth extracellular loop (L6) (32). We tested whether these mutations also suppress LptD_{Y721D}-associated defects and found that all of them do to varying degrees (Fig. S5A). BamA_{G669A} was the best of these suppressors, restoring growth on vancomycin to near WT levels. The fact that defects in the region of the substrate that interacts with BamD can be suppressed by changes in the extracellular loops of BamA suggests that substrate binding to BamD can influence BamA function.

To assess whether BamD and the BamA suppressor mutation (F494L) influence the conformation of the BamA extracellular loops we used a biochemical assay that monitors changes in the surface exposure of BamA L6 (33). BamA contains two cysteine residues in L6, which can be selectively labeled with a membrane-impermeable methoxypolyethylene glycol-maleimide (Mal-PEG) reagent following DTT reduction. We have previously shown

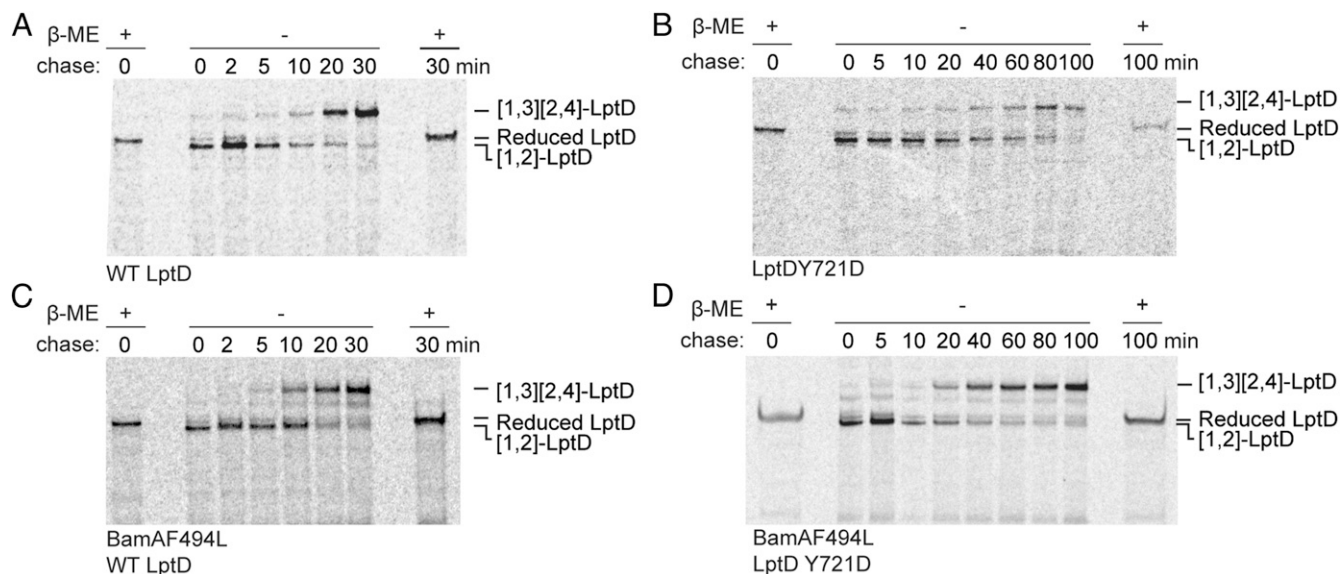


Fig. 4. LptD_{Y721D} is a slow-folding substrate. (A–D) LptD_{Y721D} exhibits slower folding kinetics (B) compared with WT LptD (A). BamA_{F494L} restores assembly kinetics of LptD_{Y721D} (D) but does not improve the assembly of the (C) WT LptD. MC4100 or *bamA*_{F494L} cells expressing FLAG-tagged LptD(WT/721D) were pulsed with [³⁵S]methionine and chased with cold methionine. Samples were subsequently immunoprecipitated and analyzed by SDS/PAGE/autoradiography.

rescues an early assembly defect in this substrate as well. These observations suggest that in cells lacking SurA substrates such as LamB cannot be properly maintained in a folding-competent state in the periplasm and subsequently cannot productively engage the Bam complex via BamD in a manner similar to LptD_{Y721D}. The observation that BamA_{F494L} can partially bypass the requirement for BamD suggests a general mechanism of suppression for defects in BamD function.

The suppressor mutations likely operate by altering the conformation of the extracellular loops of BamA. Normally, this conformational change would be caused by unfolded OMP substrates bound properly to BamD either between direct interaction between the subunits of the Bam complex or by BamA interacting with the BamD-bound OMPs. Residue F494 is located in extracellular loop three (L3) in close contact with L6. L6 contains the most conserved sequence within the greater Omp85 family of proteins and its proper positioning has been proposed to be important in the assembly and function of BamA itself (17, 32, 33, 40). Based on the crystal structures, BamA has been proposed to cycle between open and closed conformations of the β -barrel (28–30, 41, 42), and this appears to be partially mediated by L3 and L6 (Fig. 5). Additionally, we have previously shown that BamDE can modulate the conformation of the β -barrel (34) and the surface exposure of L6 (33). The altered binding interaction with LptD_{Y721D} prevents BamD from performing its normal function, which is to induce the conformational change in BamA that facilitates integration. Understanding the structural changes in BamA and BamD that occur during substrate binding and the assembly reaction may provide important clues in constructing a physical picture of the complete catalytic cycle of OMP assembly.

Materials and Methods

Strains and Growth Conditions. Strains and plasmids are provided in Tables S1 and S2, respectively. Unless otherwise noted, cultures were grown at 37 °C and supplemented with the appropriate antibiotics and amino acids.

Analysis of Antibiotic Sensitivities. Plating of the strains was performed as previously described (17). All strains were grown at 37 °C to an OD₆₀₀ of ~0.8. Cells were normalized to an OD₆₀₀ of 0.1 and then subject to five serial 10-fold dilutions. Five microliters of the dilution series were plated on agar plates containing the indicated additive and incubated at 37 °C for 18–20 h.

Analysis of Cellular Protein Levels. All strains were grown at 37 °C to an OD₆₀₀ of ~0.8 over the course of 8 h. The cells from a 1-mL sample normalized to an OD₆₀₀ of 0.3 were collected by centrifugation at 10,000 \times g for 10 min. The resulting cell pellets were resuspended in 80 μ L of 1 \times SDS sample buffer (+ β -mercaptoethanol, β -ME) and boiled for 8 min. Samples were not boiled under seminative conditions. The samples were applied to SDS/PAGE and analyzed via Western blotting.

MST. MST experiments were performed using a NanoTemper Monolith NT.115 (NanoTemper Technologies GmbH). Peptides were fluorescently labeled according to the manufacturer's recommendations with a Monolith NT Protein Labeling kit RED-NHS (NanoTemper Technologies GmbH) in 8 M urea at a 1:1 molar ratio of peptide to dye. Increasing concentrations of BamD were titrated against 5 nM labeled peptide in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 0.05% Tween, and 0.8 M urea. Samples were incubated for 5 min and loaded into premium coated capillaries (NanoTemper Technologies GmbH). MST measurements were performed using 5%

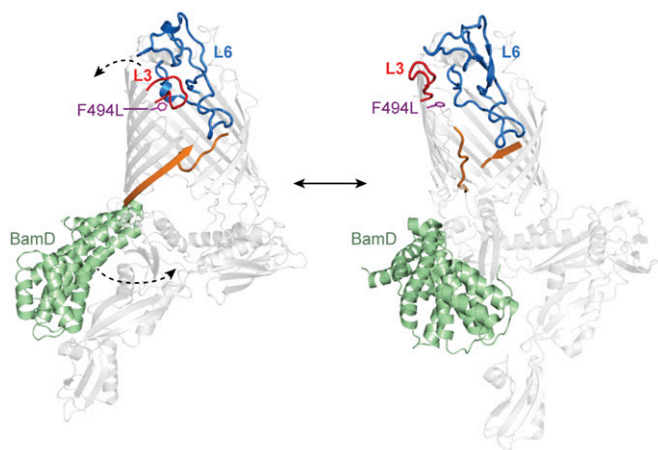


Fig. 5. Extracellular loops mediate opening of the BamA barrel. Interactions between extracellular loops L3 and L6 stabilize the closed state of the BamA barrel. Images were generated from the published structures of the BamABCDE complex (PDB ID code 5D00) and the BamACDE complex (PDB ID code 5D0Q) from *E. coli* (29) using PyMOL.

excitation power and medium MST power. Datasets were processed with the MO.Affinity Analysis software (NanoTemper Technologies GmbH).

Site-Specific in Vivo Photocross-linking. Photocross-linking experiments are based on techniques as previously described (43), with modifications. A detailed description is provided in *SI Materials and Methods*.

Membrane Extraction with Sodium Carbonate. Membrane extraction was performed in a manner similar to that described by Molloy (27). A detailed description is provided in *SI Materials and Methods*.

Pulse-Chase Analysis. Pulse-chase experiments were performed as previously described (19). A detailed description is provided in *SI Materials and Methods*.

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