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Bam Lipoproteins Assemble BamA *in Vitro*

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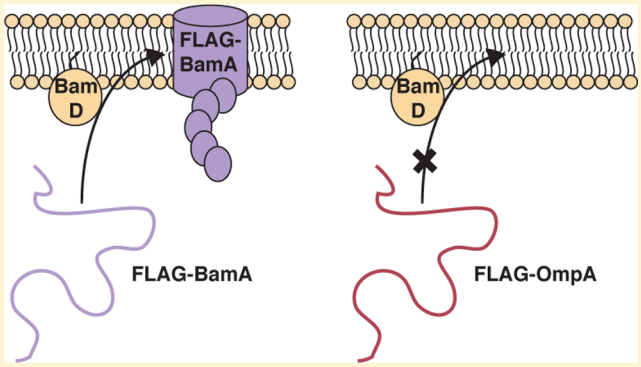
Supporting Information

ABSTRACT: The Bam machine assembles β-barrel membrane proteins into the outer membranes of Gram-negative bacteria. The central component of the Bam complex, BamA, is a β-barrel that is conserved in prokaryotes and eukaryotes. We have previously reported an *in vitro* assay for studying the assembly of β-barrel proteins by the Bam complex and now apply this assay to identify the specific components that are required for BamA assembly. We establish that BamB and BamD, two lipoprotein components of the complex, bind to the unfolded BamA substrate and are sufficient to accelerate its assembly into the membrane.



The outer membranes (OMs) of Gram-negative bacteria contain transmembrane proteins with β-barrel structure. These proteins are synthesized in the cytoplasm with N-terminal signal sequences, which target them for secretion across the inner membrane (IM) via the Sec machine.[1−3] They are then transported in complex with chaperones across the aqueous periplasmic compartment between the membranes and are finally assembled in the OM by the β-barrel assembly machine (Bam).[4] The *Escherichia coli* Bam complex contains two proteins that are essential for cell viability: an integral membrane β-barrel, BamA, and an OM lipoprotein, BamD, which is anchored to the membrane by an N-terminal lipid and which binds to the soluble region of BamA that extends into the periplasm.[5−7] Three other lipoproteins, BamB, -C, and -E, associate with these two proteins but are not essential.[5,8−10] The mechanism of β-barrel assembly is believed to be highly conserved because orthologs of BamA are found in all organisms that contain β-barrels.[11−16] However, very little is known about how that mechanism proceeds; it is thought to involve multiple steps, including substrate recognition, folding, and membrane insertion, but it is not clear how the components of the Bam complex accomplish those steps.

We have reconstituted the process of β-barrel assembly *in vitro* from purified components and now make use of this system to dissect the Bam complex and observe the effects of its individual components.[17,18] We chose to study the assembly of BamA because it is an essential outer membrane protein (OMP), and given that its function is to assemble other OMPs, we hypothesized that its assembly mechanism might reveal or reflect aspects of how it functions in the more general OMP assembly process. Through this analysis, we have determined that BamB and BamD bind to unfolded substrates.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. The methods used to express and purify the proteins used in this study are described in the Supporting Information.

Proteoliposome Preparation. Proteoliposomes containing the Bam complex and Bam subcomplexes were prepared by the detergent dilution methods described previously.[18] Briefly, *E. coli* phospholipids (40 μL of a 20 mg/mL sonicated aqueous suspension) were added to the purified Bam complexes (200 μL of 10 μM solutions) in TBS (pH 8), 0.03% DDM, and 1 mM TCEP and incubated on ice for 5 min. These phospholipid/detergent/protein complex mixtures were then diluted with 8 mL of TBS (pH 8) and incubated on ice for 30 min. They were then ultracentrifuged at 300000*g* for 2 h at 4 °C. The pelleted proteoliposomes were resuspended in 200 μL of TBS (pH 8). Empty liposomes were prepared in parallel with these proteoliposomes by the same detergent dilution method, simply omitting the Bam proteins. Liposomes and proteoliposomes that were not used immediately were flash-frozen in liquid nitrogen and stored at −80 °C.

Folding Assays. *Folding into Bam Proteoliposomes.* The unfolded FLAG-BamA or FLAG-OmpA substrate was prepared at a concentration of 5 μM in 8 M urea and then diluted 10-fold into solutions containing empty liposomes or the Bam proteoliposomes. The proteoliposomes were also diluted 4-fold from their stock concentrations into these reaction mixtures. A typical reaction mixture contained 2.5 μL of liposomes or proteoliposomes, 6.5 μL of TBS (pH 8), and 1 μL

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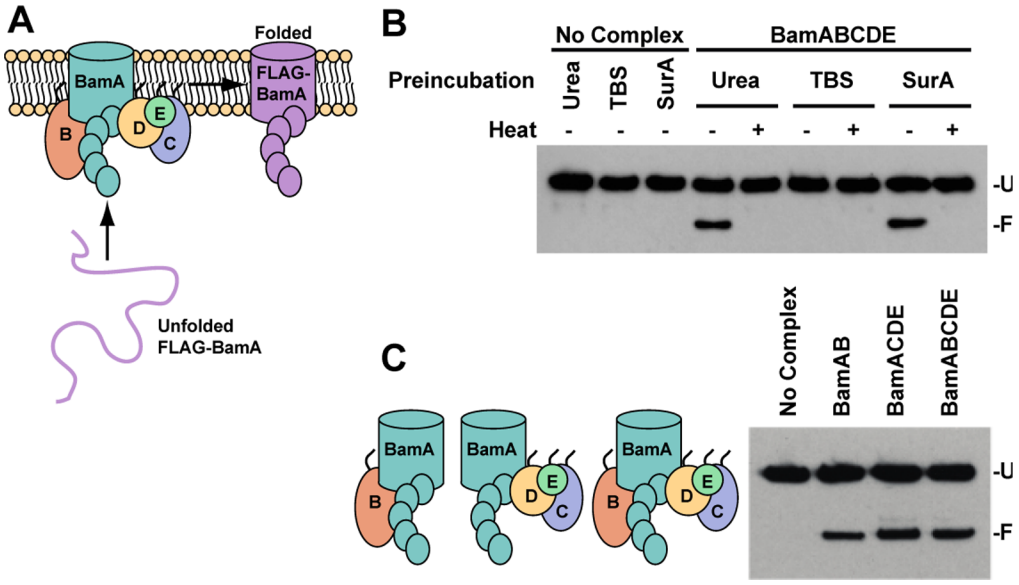

Figure 1. BamA can be folded by a minimal set of OMP assembly components. (A) Schematic of the experimental design. The purified Bam complex is incorporated into liposomes composed of *E. coli* phospholipids, and unfolded FLAG-tagged BamA is added to these proteoliposomes with or without a chaperone. (B) Urea and SurA maintain the folding competence of BamA equally well. FLAG-BamA was prepared in 8 M urea and then diluted directly into empty liposomes or proteoliposomes containing the Bam complex, or the denatured substrate was first incubated in solutions of Tris-buffered saline (TBS) or SurA and then added. The final concentrations of the substrate and SurA were 0.5 and 5 μM, respectively. The reactions were stopped after 60 min, and the products were analyzed by SDS−PAGE and immunoblotting with anti-FLAG antibodies. (C) Bam subcomplexes lacking the lipoproteins demonstrate activity similar to that of the complete complex in assembling full-length FLAG-BamA (U, unfolded FLAG-BamA; F, folded FLAG-BamA).

of 5 μM substrate such that the final concentrations of the substrate and Bam complex were 0.5 and ~2.5 μM, respectively. If the experiment included a preincubation, the substrate was first diluted 10-fold from a 50 μM solution in 8 M urea to a solution of TBS (pH 8) or purified SurA in TBS (pH 8) and incubated at 25 °C for 10 min. These preincubated solutions were then diluted 10-fold into the proteoliposomes. Unless noted otherwise in the figures, the concentrations of SurA and the substrates were 50 and 5 μM, respectively, in the preincubation and 5 and 0.5 μM, respectively, in the final reaction mixtures. Reactions were stopped after incubation at 25 °C for 60 min (unless noted otherwise in the figures) by adding ice-cold 2× SDS sample loading buffer [125 mM Tris (pH 6.8), 4% SDS, 30% glycerol, 0.005% bromophenol blue, and 5% β-mercaptoethanol]. For the time course experiments, aliquots of the reaction mixtures were removed at the indicated time points, quenched by the same method, and kept on ice. All quenched samples were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (4 to 20% gel) at 150 V for 110 min at 4 °C. The proteins were transferred from the gel to a PVDF membrane by semidry transfer in 25 mM Tris-HCl and 192 mM glycine (pH 8.3) at 10 V for 1 h. The products of the reaction were detected by immunoblotting with FLAG-HRP antibodies (used at a dilution of 1:200000). The blot images were scanned, and ImageQuant TL was used to calculate the densities of the observed bands. The percent yields of folded protein were determined by comparing the densities of the unfolded and folded bands in each lane.

Folding in Detergent. FLAG-tagged substrate proteins were prepared at a concentration of 5 μM in 8 M urea. They were then diluted 10-fold into a solution of TBS (pH 8) and 0.5% LDAO and incubated at 25 °C for 1 h. The folding reactions were stopped with 2× SDS sample loading buffer. The quenched samples were subjected to SDS−PAGE and immunoblotted as described in the previous section.

Folded Chimeric BamA Affinity Purification. FLAG-tagged wild-type and mutant BamA substrates were prepared at a concentration of 100 μM in 8 M urea. These substrates were then diluted 10-fold into TBS (pH 8) and 0.5% LDAO and incubated at 25 °C for 60 min to allow their β-barrels to fold. Concentrated, purified BamCDE-His$_6$ complex was then added to each of the folded substrates to a final concentration of 10 μM. Aliquots of these mixtures were removed and used as "input" samples for SDS−PAGE analysis. The remainder of each mixture was subjected to Ni-NTA affinity chromatography in TBS (pH 8) and 0.05% DDM. Proteins in the eluates were precipitated with 10% trichloroacetic acid and incubated on ice for 30 min. These samples were then centrifuged at 18000*g* for 10 min at 4 °C, and the pellets were resuspended in 1 M Tris (pH 8) and 2× SDS sample loading buffer. The input and these "eluate" samples were subjected to SDS−PAGE on a 4 to 20% gradient gel at 200 V for 45 min. The proteins were then detected by staining with Coomassie blue.

Unfolded Substrate Affinity Purifications. Urea-denatured FLAG-BamA and FLAG-OmpA were prepared at a concentration of 100 μM and subsequently diluted 10-fold into a solution of soluble BamB-His$_6$, BamD-His$_6$, or BamE-His$_6$ in TBS (pH 8) and incubated at room temperature for 10 min. The final concentrations of the unfolded OMP and the soluble Bam proteins were 10 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 the material had been loaded 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 and resuspended in

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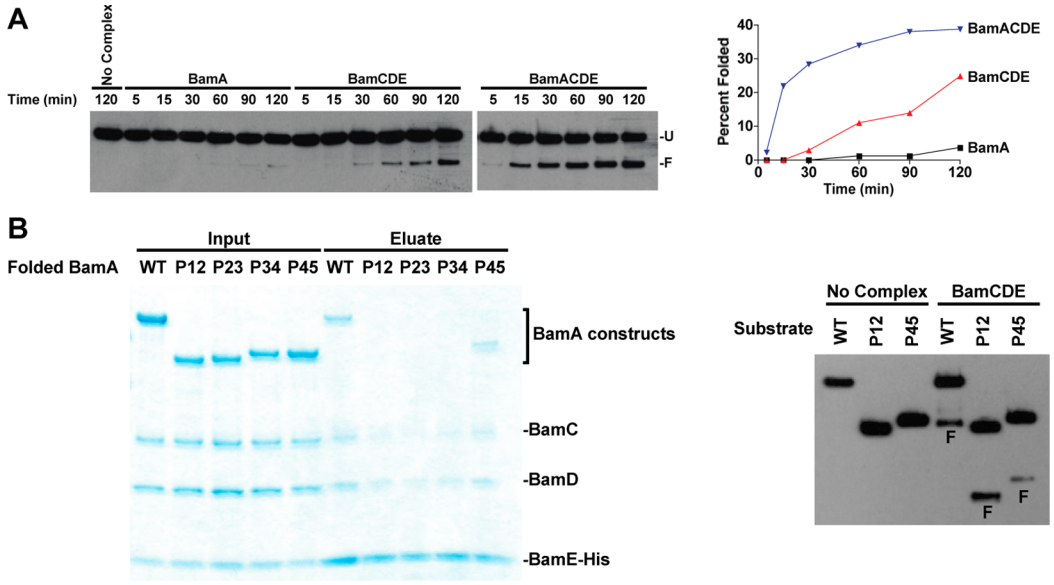

Figure 2. Bam lipoproteins are sufficient to facilitate BamA assembly. (A) The BamCDE lipoproteins facilitate FLAG-BamA assembly more effectively than BamA alone, but BamA and BamCDE function most effectively as a complex. The assembly of FLAG-BamA into proteoliposomes containing BamA, BamCDE, or BamACDE was monitored over the course of 2 h. Reactions were stopped at the indicated time points. Folding yields were determined by comparing the densities of the folded and unfolded bands and plotted. (B) The chimeric $BamA_{P12}$ substrate does not bind to BamCDE after it is folded (left), but these lipoproteins do facilitate its assembly (right). The FLAG-tagged wild-type, chimeric (P12, P23, and P34), and truncated (P45) BamA substrates were folded in detergent (0.5% LDAO) for 60 min; purified BamCDE-His_6 lipoproteins were then added, and the complexes were isolated by Ni-NTA affinity chromatography (left). Unfolded FLAG-tagged BamA substrates were added to empty liposomes or BamCDE proteoliposomes, and the folding reactions were stopped after 120 min (right) (F, folded FLAG-BamA construct).

1 M Tris (pH 8) and 2× SDS sample loading buffer as described in the previous section. The proteins in the input and these eluate samples were separated by SDS−PAGE and stained with Coomassie blue.

RESULTS

We began by identifying the minimal set of components that are required to assemble a BamA substrate *in vitro*. The fact that the OMP chaperones and some of the Bam proteins are nonessential suggests that they are not required for the assembly of all OMPs. We found that BamA can be assembled *in vitro* without a chaperone (Figure 1A,B). We diluted urea-denatured, FLAG-tagged BamA with or without SurA, a major periplasmic chaperone,[19−21] into proteoliposomes containing the Bam complex.[17,18] The reaction products were separated via seminative SDS−PAGE, and the folded and unfolded forms of the proteins were then visualized by immunoblotting with anti-FLAG antibodies. (β-Barrels do not unfold in SDS unless they are boiled; therefore, their folded and unfolded forms have different mobilities on SDS−PAGE. The FLAG tag on the substrate BamA distinguishes it from the untagged BamA in the complex.) The substrate did become less foldable in the absence of solubilizing factors (i.e., in Tris-buffered saline), and SurA maintained its folding-competent state. However, SurA could be functionally replaced by urea (Figure S1 of the Supporting Information). These results are consistent with *in vivo* measurements demonstrating that BamA levels do not decrease when *surA* is deleted.[22−25] Therefore, the BamA substrate can be delivered to the Bam complex in different ways without affecting its assembly on the machine. Accordingly, we proceeded with our *in vitro* analysis of the direct effects of the Bam complex components on this substrate in the absence of a chaperone.

We found that no specific Bam lipoprotein is required to assemble BamA *in vitro*; BamAB and BamACDE subcomplexes both assembled BamA into proteoliposomes (Figure 1C and Figure S2A of the Supporting Information).[17] The BamAB subcomplex is less effective, but it appears that BamB can at least partially substitute for BamCDE. Clearly, BamD is essential *in vivo*, while BamB is not; therefore, these proteins must have additional nonredundant functions that may relate to the assembly of other OMP substrates. Given that BamA is the only common component in the subcomplexes, we examined whether the functions of the lipoproteins are critical in the assembly mechanism or whether BamA alone is capable of assembling more BamA. We compared the activities of proteoliposomes containing just BamA or BamCDE to that of BamACDE proteoliposomes to determine if BamA functions cooperatively with the lipoproteins (Figure 2A and Figure S2B of the Supporting Information). Surprisingly, the unfolded BamA substrate assembled into proteoliposomes containing only the BamCDE lipoproteins more efficiently than into proteoliposomes containing only BamA. Therefore, the assembly of an unfolded BamA molecule does not require a preassembled BamA molecule in the membrane.

We considered an alternate explanation for the observed folding in the BamCDE proteoliposomes in which the folded BamA product might form a complex with BamCDE and thereby produce a more active assembly machine. We discounted this hypothesis because a BamA substrate that cannot bind to the lipoproteins after it is folded is assembled equally well by them (Figure 2B and Figure S3 of the Supporting Information). The periplasmic region of BamA contains five polypeptide transport-associated (POTRA) domains. The most C-terminal of these, P5, is adjacent to the β-barrel and known to bind to BamCDE.[7] We generated chimeric and truncated BamA substrates containing two

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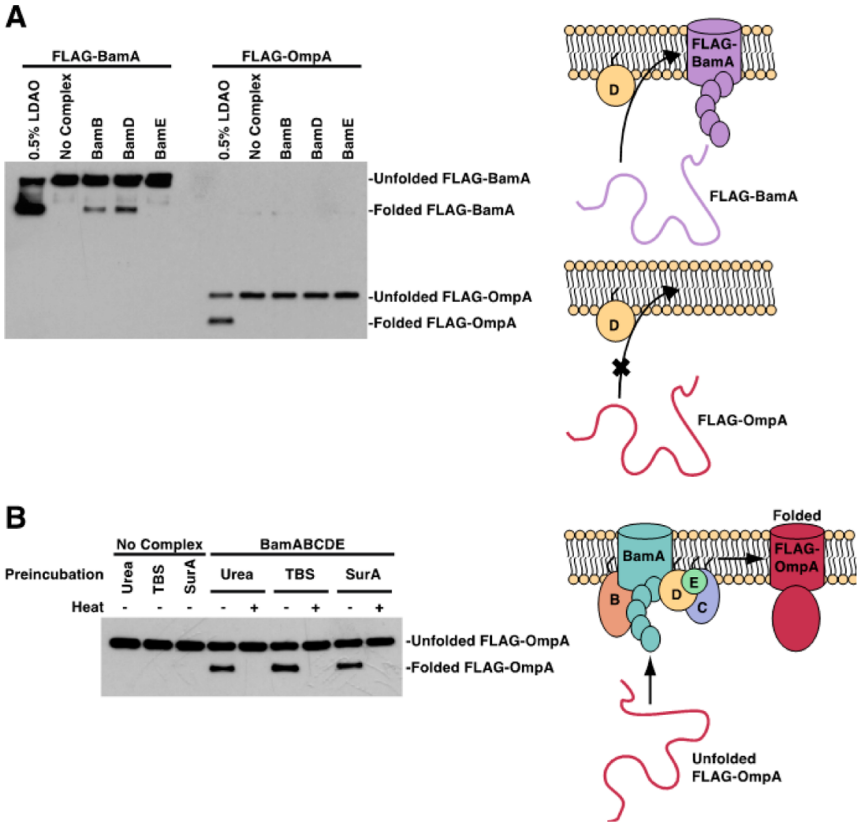

Figure 3. BamB and BamD are individually sufficient to facilitate BamA's self-assembly but are not sufficient to assemble OmpA. (A) Unfolded FLAG-BamA or FLAG-OmpA was added to a detergent solution or to proteoliposomes containing BamB, BamD, or BamE. Reactions were stopped after 120 min. (B) OmpA is assembled by the complete Bam complex without a chaperone. FLAG-OmpA was added directly to proteoliposomes or preincubated in TBS or a 10-fold excess of SurA as described for Figure 1B.

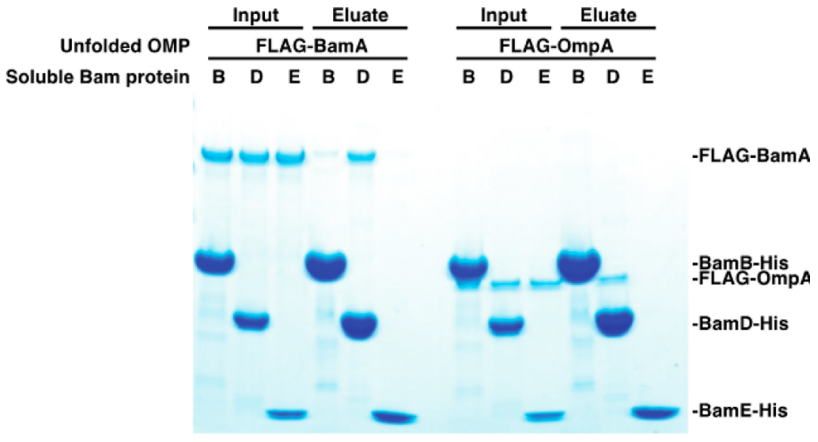

Figure 4. Certain Bam lipoproteins bind unfolded BamA and OmpA. Urea-denatured FLAG-BamA or FLAG-OmpA was diluted into a detergent-free solution containing a 10-fold molar excess of the indicated soluble, His-tagged Bam protein (lacking its N-terminal lipid anchor). Binding of the unfolded OMP to the Bam protein was then assessed by its ability to copurify by Ni-NTA affinity chromatography.

POTRA domains and the β-barrel domain. When the truncated substrate containing POTRA domains 4 and 5 is folded in detergent and then mixed with the BamCDE lipoproteins, it can be copurified with the lipoproteins; the chimeric BamA proteins containing other pairs of POTRA domains (P12, P23, or P34) do not copurify with the lipoproteins. Nevertheless, the BamCDE lipoproteins facilitate the assembly of the chimeric $BamA_{P12}$. We therefore favor a model in which the lipoproteins directly and independently facilitate the assembly of the unfolded BamA substrate (*vide infra*).

In vivo experiments have indicated that BamA assembly is facilitated by the Bam complex.[7,24] Our results do not contradict those studies; the fact that the BamACDE complex was more active than BamCDE clearly demonstrates that when BamA is preassembled in a complex with the lipoproteins it facilitates the assembly of more BamA. Given the difference in the kinetics of the BamABCDE- and BamCDE-catalyzed processes, the latter is unlikely to occur in a wild-type cell. However, by dissecting the Bam complex *in vitro*, we were able to observe the independent function of the BamCDE

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lipoproteins. It seemed possible that the lipoproteins might perform this same function in the context of the complete complex, and we therefore began to characterize how they assemble BamA to understand their mechanistic role.

We examined whether other OMPs can also be assembled by the lipoproteins alone or if BamA is unusual in this regard. We compared how the individual Bam lipoproteins affect the folding of BamA and OmpA, an abundant but nonessential OMP (Figure 3). Individually, BamB and BamD both facilitated BamA assembly *in vitro*, but BamE did not. (BamC could not be purified individually in a stable form and consequently was not examined.) Clearly, the lipoproteins must have an important, direct effect because BamA does not assemble efficiently into empty liposomes or into BamE proteoliposomes. BamB and BamD must share a common function that has a specific effect on the BamA substrate. In contrast, none of the lipoproteins was sufficient to fold OmpA, but this substrate was assembled if the Bam complex was present in the membrane (Figure 3 and Figure S4 of the Supporting Information).[26] Therefore, OmpA is not inherently unstable in the membranes used here, and we attribute the difference in the ability of BamA and OmpA to assemble to a difference in the properties of the substrates. BamB and BamD have a clear function in the folding process, but it is not sufficient to assemble OmpA.

We hypothesized that the common function of BamB and BamD relates to binding substrates at the OM as has been suggested by some crystal structures and cross-linking experiments.[27−33] We examined whether the lipoproteins interact directly with unfolded OMPs; urea-denatured BamA or OmpA was mixed with an excess of soluble, His-tagged BamB, -D, or -E and then affinity-purified. Soluble constructs of BamB, -D, and -E, which lack their N-terminal lipids, were used so that detergents could be omitted from the experiment to prevent folding of the substrates. BamA copurified with BamD-His, to a lesser extent with BamB-His, and not at all with BamE-His (Figure 4). Therefore, the lipoproteins likely facilitate the assembly of the BamA substrate by binding to its unfolded state. In that respect, they may act like other enzymes by stabilizing a transient or intermediate state in the reaction pathway, and accordingly, their function in assembling the substrate does not involve or require binding to the final product (the folded state of BamA) as indicated by the assembly of the $BamA_{P12}$ substrate described above.

However, this direct interaction between the unfolded substrate and the lipoproteins is not sufficient to produce folding on its own. When soluble BamB or BamD was added to empty liposomes, no folding of BamA was observed (Figure S5 of the Supporting Information), implying that the membrane localization or orientation of the lipoproteins matters. Furthermore, BamD bound unfolded OmpA, but in this case, binding was not sufficient to produce folding of the substrate OMP (Figures 3A and 4). OmpA does not partition into the membrane even if it is bound near it. BamB and BamD are not capable of completing OmpA's assembly alone, but simply binding unfolded BamA near the membrane is sufficient to facilitate this substrate's assembly. BamA may be unusual or unique in its ability to assemble in a manner independent of other components. We were able to exploit this property of BamA to observe the individual functions of BamB and BamD *in vitro*. This is an advantage of our *in vitro* system in that it allows dissection of an essential machine; we do not have to contend with the pleiotropic effects of mutations and deletions *in vivo* and can isolate the effects of individual components.

DISCUSSION

Here we have shown that BamB and BamD can bind unfolded substrates and that this function facilitates the assembly of BamA. These lipoproteins likely interact with the unfolded substrate in different ways, but both are capable of facilitating BamA assembly by localizing the unfolded substrate to the membrane. Although BamD can also bind unfolded OmpA, it is not sufficient to catalyze the assembly of this substrate *in vitro*. We attribute this difference in assembly requirements to the function of BamA. BamA may be able to assemble aided only by the lipoproteins because it performs some of the OMP assembly mechanism; the fact that it is conserved in all organisms may reflect its role in the later folding and insertion steps of β-barrel assembly. BamD alone cannot assemble OmpA because this substrate relies on a preassembled BamA to complete the later steps of its assembly.

Many OMPs have been shown to assemble spontaneously into lipid bilayers *in vitro*, but their ability to do so depends strongly on the lipid content of the artificial membranes.[26,34,35] *In vivo*, however, all OMPs must assemble into the same membrane. The inability of OmpA to assemble under the same conditions as BamA (i.e., into a membrane containing only BamB or BamD) suggests that the BamA substrate may possess some additional or unusual features. We propose that the structure of BamA in some way facilitates its own assembly such that it is less reliant on a preassembled Bam complex than other OMPs. This spontaneous assembly process is clearly much less efficient than the Bam complex-catalyzed process, but it provides an intriguing solution to the "chicken and egg problem". Perhaps in a primitive organism, an ancestral BamA protein assembled itself, and the other complex components later evolved to adapt BamA to assemble more and different types of other OMPs. In turn, BamA became more reliant on the other Bam components for its assembly, and the spontaneous process became comparatively less efficient and important. By creating an efficient catalyst for β-barrel assembly, it also became possible to segregate β-barrels to a single membrane by making the rate of their assembly into the membrane containing the Bam complex dramatically faster than that into an empty membrane.

By dissecting the Bam complex *in vitro*, we have identified a substrate binding interaction that appears to be important in BamA's assembly. Our next step is to establish whether inhibition of this binding event is sufficient to inhibit OMP assembly.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and five additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Bam, β-barrel assembly machine; OM, outer membrane; IM, inner membrane; OMP, outer membrane protein; DDM, *n*-dodecyl β-D-maltopyranoside; TCEP, tris(2-carboxyethyl)-phosphine; SDS, sodium dodecyl sulfate; LDAO, lauryldimethylamine-*N*-oxide; POTRA, polypeptide transport-associated.

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