

Reconstitution of Outer Membrane Protein Assembly from Purified Components

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β -barrel membrane proteins in Gram-negative bacteria, mitochondria, and chloroplasts are assembled by highly conserved multi-protein complexes. The mechanism by which these molecular machines fold and insert their substrates is poorly understood. It has not been possible to dissect the folding and insertion pathway because the process has not been reproduced in a biochemical system. We purified the components that fold and insert *Escherichia coli* outer membrane proteins and reconstituted β -barrel protein assembly in proteoliposomes using the enzymatic activity of a protein substrate to report on its folding state. The assembly of this protein occurred without an energy source but required a soluble chaperone in addition to the multi-protein assembly complex.

The outer membranes of Gram-negative bacteria and the mitochondria and chloroplasts of higher eukaryotes contain proteins with β -barrel structure, which are assembled in their respective membranes by multi-protein machines (1–6). The folding and insertion of these β -barrels must be coordinated because they would have many unsatisfied hydrogen bonds in the membrane if they were inserted in an unfolded state, but conversely, they would be “inside out” if they folded first in the aqueous environment and were subsequently inserted. In order to understand how β -barrel proteins assemble into membranes, we purified the proteins comprising the *Escherichia coli* outer membrane protein (OMP)–folding machinery and established a reconstituted system to monitor the activity of this machinery.

The β -barrel assembly machine (Bam) in *E. coli* consists of an integral β -barrel protein, BamA (formerly YaeT), and four lipoproteins, BamB, -C, -D, and -E (formerly YfgL, NlpB, YfiO, and SmpA, respectively). Only BamA and BamD are essential for cell survival, but deleting or depleting any member of the complex causes defects in OMP assembly (6–9). BamA has homologs in prokaryotes and eukaryotes and contains five periplasmic polypeptide transport–associated (POTRA) domains, which scaffold the Bam lipoproteins (2–5, 10). Unfolded OMPs are delivered to this complex after their synthesis in the cytoplasm and translocation across the inner membrane by the secretion machinery (SecYEG) (Fig. 1A) (11). The chaperones, SurA, Skp, and DegP, prevent unfolded OMPs that are released from the Sec machine from aggregating and misfolding as they transit the periplasm. These chaperones are thought to transport OMPs in two parallel but separate pathways: one that relies on SurA and one that involves both Skp and DegP (12, 13). Most OMPs can be handled by either pathway, but SurA delivers the bulk of OMPs to the OM (14).

The process of OMP assembly can be monitored in isolated mitochondria (15) and in vivo in *E. coli* (16). We sought to develop an in vitro system to study the function of the Bam proteins. Expressing all five *bam* genes in a single strain produced a mixture of complexes and subcomplexes that could not be easily separated. Previous lipoprotein and POTRA domain–deletion experiments indicated that BamA binds BamB independently from BamCDE (8, 10); thus,

we expressed the subcomplexes BamAB and BamCDE separately and reconstructed the full complex in vitro (17). The reconstructed complex was identical to the native complex, as analyzed with blue native polyacrylamide gel electrophoresis (BN-PAGE) (Fig. 1B). The purified Bam complex had an apparent molecular weight that is too small to accommodate more than one copy of BamA, and the ratio of BamA:B:C:D was 1:1:1:1 (fig. S1). Either one or two copies of BamE may have been present; its small size prevents a definitive conclusion. The Bam complex may exist as a higher-order oligomer in the membrane in vivo, but the relative stoichiometry is expected to be as we have determined.

Purified Bam complex was incorporated into liposomes, as others have done to reconstitute complexes that handle membrane proteins (18–22). To follow OMP assembly in the proteoliposomes, we used a substrate OMP that possesses enzymatic activity, OmpT. OmpT is a β -barrel outer membrane protease that cleaves peptides between two consecutive basic residues, and its activity can be monitored by using a fluorogenic peptide (23). Urea-denatured OmpT was incubated with the periplasmic chaperone

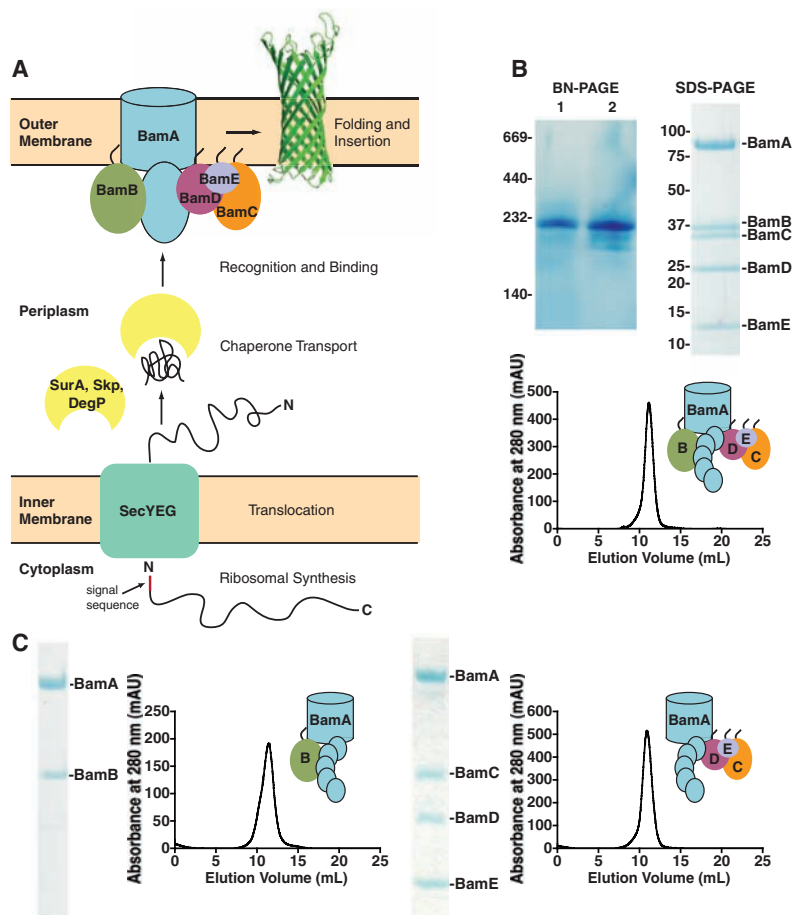


Fig. 1. The OMP assembly pathway and the purified Bam complex and subcomplexes. **(A)** The *E. coli* OMP biogenesis pathway. **(B)** Blue native gel analysis, SDS-PAGE, and gel filtration chromatogram of the purified Bam complex. The complex isolated from cells expressing the complex at basal levels (lane 1) and the overproduced and reconstructed complex (lane 2). **(C)** SDS-PAGE and gel filtration chromatograms of the purified two- and four-protein Bam subcomplexes.

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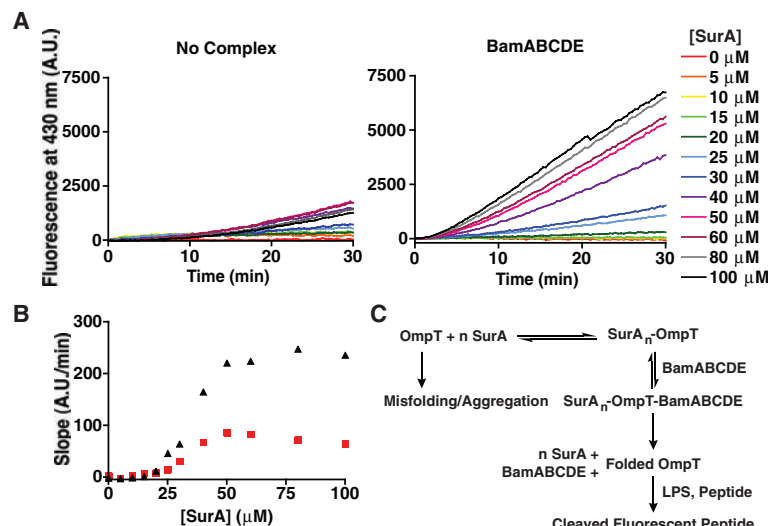


Fig. 2. SurA and the Bam complex facilitate OmpT assembly in proteoliposomes. **(A)** Preincubated solutions of urea-denatured OmpT with SurA were diluted [at time (t) = 0 min] into (left) liposomes or (right) proteoliposomes that contain the Bam complex. OmpT was present in each reaction at a final concentration of 10 μ M, and the concentration of SurA was varied from 0 to 100 μ M. **(B)** The amount of active OmpT (reflected in the slope from 15 to 30 min) in the reactions in (A) as a function of SurA concentration in the presence (black triangles) or absence (red squares) of the Bam complex. **(C)** Schematic of the reconstitution reaction pathway.

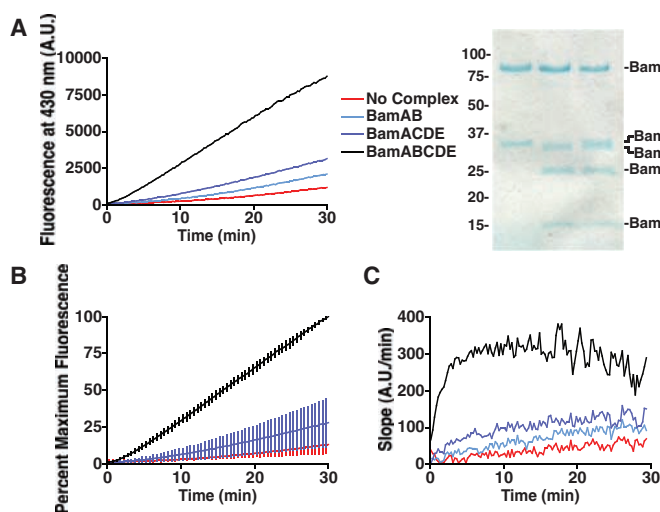
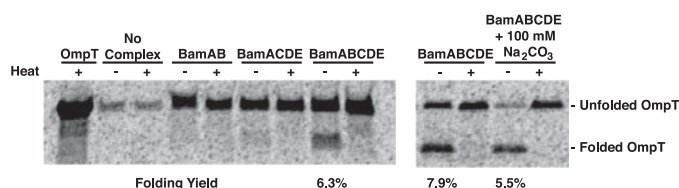


Fig. 3. OmpT assembly requires specific components of the Bam complex. **(A)** Fluorescence produced in the presence of the Bam complex and subcomplexes (left). OmpT and SurA are diluted to final concentrations of 10 μ M and 100 μ M, respectively. SDS-PAGE of these proteoliposomes indicates that they contain equal amounts of the complexes (right). **(B)** Eight different experiments were normalized to their maximum fluorescence values and then averaged. The error bars represent the SD among these experiments. **(C)** The gradient of the fluorescence data in (A).

Fig. 4. OmpT assembled by the Bam complex is folded on SDS-PAGE and resistant to membrane extraction. Shown are pellets of the reactions of SurA and 35 S-labeled OmpT with proteoliposomes containing the Bam complexes. 35 S-labeled OmpT and SurA were diluted to final concentrations of 0.4 μ M and 100 μ M, respectively. Extraction in 100 mM sodium carbonate results in a threefold enrichment of the folded material relative to the unfolded material.



SurA, and this chaperone-OmpT complex was diluted into solutions containing the proteoliposomes, the fluorogenic peptide, and lipopolysaccharide (LPS), which is required for OmpT activity (17). OmpT assembly was observed as an increase in the rate of fluorescence production.

OmpT activity increased with the concentration of SurA and in the presence of the Bam complex (Fig. 2A and figs. S2 and S4). OmpT activity saturated at the same SurA concentration in the presence or absence of the complex, which suggests that SurA plays a general role in delivering the unfolded protein in a state that can be folded and also that the Bam complex handles OmpT bound to SurA (Fig. 2, B and C). The concentrations of SurA at which we saw a substantial improvement in OmpT assembly (above 20 μ M) are comparable with the reported binding constants for SurA and model peptides (1 to 14 μ M) (24, 25). OmpT contains several putative SurA-binding sites (26, 27), and the sigmoidal relationship may indicate that multiple SurA molecules are involved in generating a SurA_n-OmpT complex that can be folded efficiently.

We then wanted to determine whether the assembly observed in the presence of the Bam complex reflected the function of the complex and not a nonspecific property of the proteoliposomes (28, 29). We compared the activity of the five-protein Bam complex with subcomplexes containing two (BamAB) and four proteins (BamACDE), which had comparable stability as judged by their behavior on gel filtration chromatography (Fig. 1C). The components of these subcomplexes were incorporated into proteoliposomes in equal proportion to those of the five-protein complex (Fig. 3A). Cleavage of the fluorogenic substrate was greatly reduced in proteoliposomes containing the subcomplexes as compared with those containing the five-protein complex (Fig. 3A and fig. S3). The activity of the four-protein complex was always less than that of the five-protein complex (Fig. 3B). In the first five minutes of the experiment, OmpT assembly was orders of magnitude faster in the presence of the five-protein complex (Fig. 3C). These large differences in activity at early time points are relevant given that in vivo pulse-chase experiments indicate that OMP assembly occurs in the cell on a similar time scale of 30 s to several minutes (30, 31). The four-protein complex appeared to have some activity on this time scale, which BamB dramatically improved. Although there was a background rate of OmpT folding in the absence of the Bam complex, the Bam proteins significantly improved the kinetics of OmpT assembly.

In order to verify that increased fluorescence correlated with increased amounts of folded OmpT, we ran the products of our folding reactions on SDS-PAGE gels without and with prior heat denaturation. β -barrel proteins remain folded on SDS-PAGE if they are not boiled and consequently run faster than their unfolded forms. Purified 35 S-labeled OmpT was incubated with SurA and then diluted into proteoliposomes, as in the fluorescence experiments. After 30 min, the

reaction solutions were centrifuged and the pellets were run on SDS-PAGE (Fig. 4 and fig. S5). Much more folded OmpT was observed in the reaction of SurA_Δ-OmpT with the full Bam complex than in the reactions with the subcomplexes. The yield of folded protein represented by the faster migrating band was about 7%, as determined by five separate experiments. The folded OmpT was resistant to extraction from the pellet by incubation in 100 mM sodium carbonate for 30 min, suggesting that the folded protein had been integrated into the membrane.

The nonessential lipoprotein BamB is important in the assembly of OMP substrates delivered by SurA; the five-protein complex had significantly higher activity than the four-protein complex, BamACDE. Thus, some or all of the BamCDE proteins are required for OmpT assembly, but BamB appears to have a specific function that, when coordinated with the other components, increases the activity of the complex. The functional importance of both SurA and BamB is consistent with *in vivo* observations that these proteins play related but not redundant roles in OMP biogenesis. Deleting *surA* or *bamB* produces identical phenotypes with respect to the kinetics of conversion of unfolded mature LamB to folded monomers (31). In addition, simultaneous deletion of the *surA* and *bamB* genes results in a phenotype that is severely defective in the assembly of OMPs and much sicker than either single-deletion strain (32, 33). Our results also suggest that SurA and BamB both affect the efficiency of OMP assembly. Our highly simplified system thus appears to recapitulate elements of the cellular process and clearly demonstrates that a nonessential protein can alter the activity of essential proteins to regulate the efficiency of the process that they catalyze.

We do not know if the Bam complex in proteoliposomes can process multiple substrate molecules, but clearly the Bam complex can assemble a β -barrel protein in the membrane without an input of energy. In contrast, the inner membrane secretion machinery couples energy-driven translocation to insertion (11). Although it remains possible that β -barrel assembly may be coupled to an energy-driven process in the cell, it is remarkable that these six proteins (the Bam complex and a chaperone) are sufficient to perform the folding and insertion process. Together, they perform a chemical transformation that we do not understand, and that they are able to do so without energy suggests that their structure inherently facilitates OMP assembly.

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Additive Genetic Breeding Values Correlate with the Load of Partially Deleterious Mutations

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The mutation-selection–balance model predicts most additive genetic variation to arise from numerous mildly deleterious mutations of small effect. Correspondingly, “good genes” models of sexual selection and recent models for the evolution of sex are built on the assumption that mutational loads and breeding values for fitness-related traits are correlated. In support of this concept, inbreeding depression was negatively genetically correlated with breeding values for traits under natural and sexual selection in the weevil *Callosobruchus maculatus*. The correlations were stronger in males and strongest for condition. These results confirm the role of existing, partially recessive mutations in maintaining additive genetic variation in outbred populations, reveal the nature of good genes under sexual selection, and show how sexual selection can offset the cost of sex.

Partially recessive, deleterious mutations of small effect are thought to be responsible for much of the observed additive genetic variation in traits that are closely associated with fitness (1–4). These mutations are also central to “good genes” models of sexual selection, which posit that the additive genetic breeding values of males must correlate negatively with mutational load (5–8). The contribution of nonadditive allelic effects to additive genetic variation through mutation-selection balance has empirical support from mutation-accumulation experiments (9) and molecular-evolution surveys (10), but not from recent biometric analyses (11–13).

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Here we develop Fox’s proposal (14) for detecting among-family variation in inbreeding depression. Inbreeding depression is the reduction in fitness observed in the offspring of related (compared with unrelated) parents (4, 15) and is believed to be predominantly caused by the increased homozygosity of numerous, at least partially recessive, deleterious mutations of small effect (4, 15). By estimating the correlation between the load of mutations carried by a pair of parents and their breeding value, we were able to test the central assumption of good genes models of sexual selection and contribute to our understanding of the maintenance of genetic variation.

We used a seven-generation pedigree of cow-pea weevils *Callosobruchus maculatus* (Coleoptera: Bruchidae) reared on black-eye beans (*Vigna unguiculata*) to estimate predicted breeding values, heritabilities, and inbreeding depression in a range of life-history (LH) and morphological

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