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Reconstituted *Escherichia coli* Bam complex catalyzes multiple rounds of β-barrel assembly

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Abstract

β-barrel proteins are folded and inserted into the outer membranes of *E. coli* by the multi-protein Bam complex. The Bam complex has been purified and functionally reconstituted in vitro. Here we report assay conditions for this reconstitution that increase the folding yield ten-fold and allow us to monitor the time course of folding directly. We use these conditions to analyze the effect of a mutation in the Bam complex and to demonstrate the ability of the reconstituted complex to catalyze more than one round of substrate assembly without any additional cellular components.

Integral membrane proteins in the outer membranes of Gram-negative bacteria have β-barrel structures and perform a variety of functions including creating pores in an otherwise impermeable membrane. These outer membrane proteins (OMPs) are synthesized in the cytoplasm, translocated across the inner membrane, and transported across the aqueous inter-membrane periplasmic space in an unfolded state (1, 2). They are folded and inserted into the outer membrane by the Bam complex, which, in *E. coli*, consists of five proteins, BamA–E (3–6). BamA is an integral β-barrel protein that also contains an N-terminal periplasmic region composed of five polypeptide transport associated (POTRA) domains. The other four Bam proteins are lipoproteins and associate with the POTRA domains of BamA (7). Genetic deletions of BamB, C, and E can be generated, but BamA and BamD are essential for cell survival. Orthologs of BamA are found in the outer membranes of all Gram-negative bacteria and in the mitochondria and chloroplasts of eukaryotes (8–11). These orthologs are responsible for assembling β-barrels in their respective membranes, and the mechanism by which they function is believed to be conserved.

Crystal and NMR structures have indicated that the POTRA domains of BamA can bind peptides with β-structure and may thereby template folding of substrates (7, 12–14). Recent structures of the lipoprotein components of the Bam complex have led to several hypotheses about how these proteins interact with one another and with substrate OMPs (15–22). It has been suggested that BamB may also template β-strand formation, affect the conformation of the POTRA domains to bind substrates or chaperones optimally, or channel substrates towards BamA (15–17). A pocket in BamD may bind the C-terminal sequence of substrate OMPs, which has been suggested to act as a targeting sequence for the Bam complex (18, 21, 23, 24). It may be attractive to propose a role for BamD in selectively recognizing OMPs

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Supporting Information. Experimental procedures, SDS-PAGE analysis of Bam complex proteoliposomes, experiments illustrating the effects of additional SurA and urea and demonstrating turnover of the Bam complex and the time course of folding at high substrate concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

in order to explain its essentiality; however, in order to develop a better understanding of the mechanism of OMP assembly, these hypotheses need to be evaluated in a functional system.

We have reported methods of over-expressing and purifying the Bam proteins to yield a homogeneous complex and demonstrated that proteoliposomes containing this complex can fold and insert an OMP (25). We used OmpT as the substrate OMP because it has protease activity when it is folded. We monitored cleavage of a fluorogenic peptide as a reporter of the folded state of OmpT. The enzymatic, proteolytic reaction amplified the signal of folded OmpT in real-time, and the folding kinetics were evaluated indirectly by using the derivative of the fluorescent signal. The activity of the Bam complex was also demonstrated in a gel-based assay using radiolabeled substrate protein, but the low (5–7%) yields of folded product precluded direct observation of the kinetics of folding. The substrate concentrations used were also sub-saturating, which made it impossible to study turnover of the Bam complex. Therefore, we have investigated how the conditions of the assay affect folding in order to increase the yield.

The protease activity of OmpT is dependent upon lipopolysaccharide (LPS) (26), and we included LPS in the previously described assay in order to produce a significant fluorescent signal. However, LPS is not required for the folding of OmpT. In fact, when it is omitted from the assay buffer, we observe a large increase in the folding yield as determined by comparing the densities of the folded and unfolded radiolabeled OmpT bands on semi-native SDS-PAGE. By this method, we observe that approximately 60% of the OmpT substrate is folded in the presence of the wild-type, five-protein Bam complex after 30 minutes (Figure 1). No folding is observed in the absence of Bam complex, and as we previously reported, a sub-complex lacking BamB is much less efficient. We can now directly monitor the accumulation of folded OmpT over time. The kinetics of OmpT folding agree with those determined using the real-time fluorescence assay; folding occurs rapidly during the first two minutes of the experiment and is virtually complete after ten minutes. Although the gel-based assay is discontinuous, it directly reports on the folding reaction and does not require that the substrate possess enzymatic activity.

The improved assay conditions also enable study of Bam sub-complexes that have lower activities. We have evaluated one mutation (denoted *bamA6*) that is a duplication of Q217 and K218 in a long loop in the third POTRA domain of BamA (27). This two amino acid insertion results in the assembly of approximately ten percent less of the abundant OMPs, OmpA and LamB, in the outer membrane; a *bamB* null mutation, by comparison, more severely decreases the levels of OmpA and LamB (27). BamA6BCDE complex in proteoliposomes assembles significantly less OmpT than the wild-type complex but more than the BamACDE sub-complex (Figures 1 and S1). The proteoliposomes are identical in their composition and preparation except for two inserted amino acids in BamA, but they demonstrate different activities consistent with the in vivo behavior of their respective complexes. In vivo, the lower OMP levels cannot be directly attributed to a difference in Bam complex activity because the mutations induce the envelope stress response, which reduces the synthesis of OMPs. In vitro, however, the same amount of OmpT should be folding-competent in each of the reactions. Therefore, the BamA6BCDE and BamACDE complexes must be less functional than the wild-type complex. We have demonstrated that the *bamA6* and *bamB* null mutations directly affect the activity of the Bam complex; BamB and the long loop in the third POTRA domain of BamA play a role in binding and/or folding the substrate.

The bulk mass of OMP substrates are delivered to the Bam complex by SurA, a periplasmic chaperone, and we previously reported that preincubating urea-denatured OmpT with SurA increases the yield of folded product upon dilution into the proteoliposome solutions (25, 28,

29). Although the concentration of this chaperone is important in generating a folding-competent substrate, a relatively high concentration of urea also improves the folding yield. When denatured OmpT was preincubated with an excess of SurA in the presence of increasing concentrations of urea from 0.5 to 2 M, the yield of folded OmpT increased with urea concentration (Figures 2A & S2). Accordingly, when the SurA-OmpT substrate is diluted (upon its addition to the proteoliposome solutions), it begins to lose folding competence. We observe that if the SurA-OmpT substrate is diluted prior to addition of the BamABCDE proteoliposomes, the folding yield decreases (Figures 2B and S3). If the interaction with SurA is dynamic, higher concentrations of urea may be necessary to maintain the substrate in a foldable state while SurA dissociates and reassociates.

Urea is required for generating a folding competent substrate in vitro, but it has detrimental effects on the activity of the Bam complex. The Bam complex remains active in the presence of relatively low concentrations of urea; when a second aliquot of substrate is added 15 minutes into the folding reaction, it is folded as efficiently as the first aliquot (Figure 2C). However, at final urea concentrations above 1 M, we observe a decrease in the rate of OmpT folding and in the final yield of folded product (Figures 2A and S4). Urea may cause some unfolding or dissociation of components of the Bam complex or interfere with the interaction of the substrate with the Bam complex. If the substrate is bound by formation of hydrogen bonds (perhaps by β-strand augmentation), urea would certainly alter the affinity of that interaction (7). The requirement for an intermediate urea concentration is an artifact of the in vitro system, but it must be addressed in order to study turnover of the Bam complex.

We attempted to determine whether the reconstituted Bam complex can turnover by adding higher concentrations of SurA-OmpT to the Bam complex while keeping the final urea concentrations under 1 M (Figures 3, S5, and S6). The yield of folded protein increased linearly with substrate under these conditions, and at the highest concentrations, the amount of folded OmpT produced was greater than the amount of BamABCDE in the reaction. Therefore, the Bam complex is capable of folding multiple OmpT molecules in vitro. If all of the reconstituted Bam complexes are active, these results represent ~1.6 turnovers of the Bam machine. However, it is possible that not all of the complexes are active and that a smaller percentage of them are responsible for the observed folding and thus are performing a larger number of turnovers. For example, in initial reconstitutions of the preprotein translocase, only ~15% of the reconstituted machines were active, but they were capable of catalyzing ~22 rounds of translocation (30). Regardless of exactly how many turnovers the reconstituted Bam complex is performing, it is clear that no additional cellular components, including ATP, are required to complete the assembly cycle.

The efficiency and essentiality of OMP assembly make it challenging to study this process in vivo. Mutations in or deletions of the proteins in the assembly pathway often produce pleiotropic phenotypes due to gross changes in the composition of the OM; thus the roles of the individual components of the pathway have been difficult to deconvolute. An in vitro assay allows analysis of the effects of individual proteins in isolation (i.e. without altering other properties of the membrane). The correlation between the effects of mutations or deletions in the Bam complex in vitro and in vivo indicates that the reconstituted system reasonably reproduces several aspects of the in vivo process.

At present, we have only reconstituted the folding of one OMP substrate. We have learned that the way in which the OMP substrate is prepared significantly affects the efficiency of folding in vitro; therefore, it may be necessary to alter the preincubation conditions in order to study the folding and insertion of other OMP substrates, which may have different requirements for folding competence. However, the reconstitution conditions recapitulate

the in vivo environment of the Bam complex sufficiently well to distinguish the effects of mutations in its component proteins and to begin to study its mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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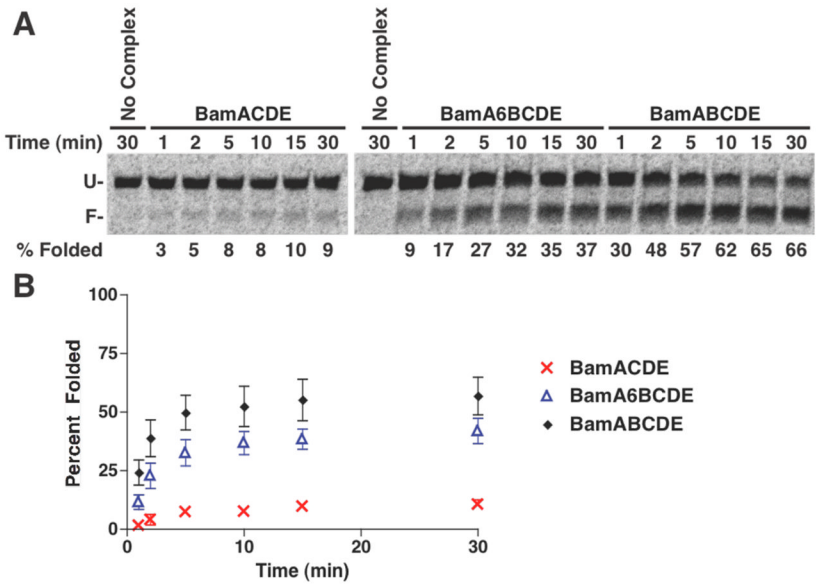

Figure 1.

Folding of [^{35}S]-labeled OmpT by wild-type and deficient Bam complexes can be monitored directly. A. Autoradiogram of time course of OmpT folding reactions in proteoliposomes containing BamACDE, BamA6BCDE, or BamABCDE. Folding reactions were stopped with 1.5% SDS buffer at the indicated time points and run on SDS-PAGE without prior boiling. The folded OmpT product migrates as the band of lower apparent molecular weight. (U: unfolded OmpT, F: folded OmpT). The percent yield in each lane was determined by comparing the densities of the folded and unfolded bands. B. The average yield of folded OmpT produced over time in three separate experiments is depicted. The error bars indicate the standard deviation among these experiments.

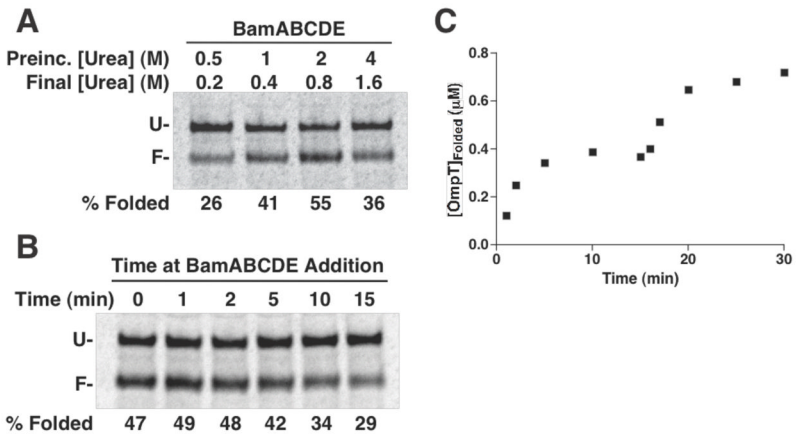

Figure 2.

The SurA-OmpT substrate becomes less folding competent upon urea dilution. In all three experiments, urea-denatured OmpT was preincubated with a 25-fold excess of SurA and then diluted into solutions containing BamABCDE proteoliposomes. Reactions in A and B were stopped at t = 30 minutes. A. Autoradiogram of OmpT folding following preincubation with SurA and increasing amounts of urea. The folding yield increases with urea concentration until the final concentration in the reaction exceeds ~1 M. B. The SurA-OmpT substrate loses folding competence over time following dilution. The preincubated SurA-OmpT substrate was diluted into buffer, and proteoliposomes containing the Bam complex were then added at the indicated time points. Less OmpT folds the longer it is allowed to incubate at dilute conditions. C. Folding stops after ~10 minutes, but the Bam complex is still active. One aliquot of SurA-OmpT was added at t = 0 min and a second aliquot was added at t = 15 min. The folding yield was determined at the indicated time points by SDS-PAGE and plotted against time.

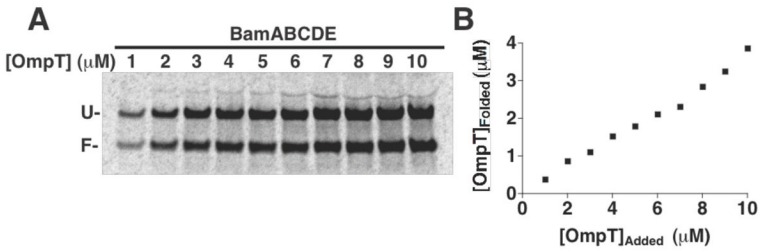

Figure 3.

Reconstituted BamABCDE can fold multiple OmpT molecules. A. Autoradiogram of folding reactions containing increasing amounts of SurA-OmpT substrate. OmpT was preincubated with a 10-fold excess of SurA and then diluted into solutions containing ~2.5 μM BamABCDE in proteoliposomes. Reactions were stopped after 30 minutes and run on SDS-PAGE without prior boiling. B. The yield of folded OmpT produced in the experiment described in A increases linearly with the substrate concentration. At the highest substrate concentrations, the amount of folded OmpT produced exceeds the amount of BamABCDE in the reaction.