INTRODUCTION

β-barrel outer membrane proteins (β-OMPs), representing one of the two major categories of integral membrane proteins in all domains of life, play critical roles in nutrition acquisition, protein import/export, and other fundamental biological processes.1-3 Distinct from α-helical membrane proteins, β-OMPs exclusively reside in the OMs of Gram-negative bacteria,
mitochondria, and chloroplast of eukaryotes, where their recognition, folding, and insertion are mediated by the BAM complex, SAM complex, and TOB complex, respectively.\textsuperscript{1,4} Phylogenetic analysis indicates that these β-OMP assembly complexes contain a conserved Omp85 family protein,\textsuperscript{2,5-8} suggesting that β-OMP biogenesis may share a similar mechanism in both Gram-negative bacteria and eukaryotes.

The Gram-negative bacteria \textit{Escherichia coli} serves as a model organism for study of the mechanism underlying β-OMP biogenesis. In \textit{E. coli}, β-OMP polypeptides are synthesized in the cytoplasm, and are subsequently translocated across the cytoplasmic membrane via the SecYEG translocon.\textsuperscript{9} Chaperones such as SurA in the periplasm escort the β-OMP polypeptides to the periplasmic side of the OM, where they are recognized and further inserted into the OM by an OM-localized five-protein complex, the BAM complex.\textsuperscript{3} The BAM complex comprises an integral β-barrel OMP BamA and four lipoproteins BamB, BamC, BamD, and BamE,\textsuperscript{3,6-8,10} of which both BamA and BamD are essential for cell survival.\textsuperscript{3,11} BamA, the only conserved component in both Gram-negative bacteria and eukaryotes, comprises two characteristic regions: a set of five polypeptide transport-associated (POTRA) domains and a C-terminal β-barrel transmembrane domain.\textsuperscript{12} In the past decade, significant progress has been made on structural determination of the individual components of the BAM complex, including BamA,\textsuperscript{13-16} BamB,\textsuperscript{17-20} BamC,\textsuperscript{19,21} BamD,\textsuperscript{19,21-23} BamE,\textsuperscript{21,24} and various isolated POTRA domains of BamA,\textsuperscript{16,25-30} the BAM subcomplexes (BamCD and BamACDE),\textsuperscript{31,32} and the complete BAM complex (BamABCDE).\textsuperscript{31,33,34} These studies show that the conformation of the BamA β-barrel, the arrangement of the BamA POTRA domains, the orientation of the BamA β-barrel relative to the periplasmic components of the BAM complex, and the implied membrane thickness surrounding the gate of the BamA β-barrel vary pronouncedly, suggesting that the BAM complex has a highly dynamic nature and these conformations might represent different functional states in the β-OMP assembly cycle.\textsuperscript{31-34} Multiple lines of evidence further indicate that the first β-strand of BamA β-barrel (β\textsubscript{1}BamA) may bind to the last β-strand of an incoming β-OMP substrate,\textsuperscript{35-41} leading to a proposed β-signal hypothesis during β-OMP biogenesis.\textsuperscript{36,42} Recently, a breakthrough has been made by cryo-EM structure determination of a folding intermediate trapped on the BAM complex, snapshotting an important step in β-OMP biogenesis.\textsuperscript{43} Here, we report two structures of the BAM complex in detergents and in nanodisks, and two crystal structures of the BAM complex bound with different substrates. Structural analysis indicates that membrane composition surrounding the BAM complex could modulate the overall conformation of the BAM complex, further highlighting low energy barriers among different conformational states. The structures of the BAM complex with bound substrate and the related functional analysis provide evidences to show that the last β-strand of the incoming β-OMP substrate binds with the β\textsubscript{1}BamA strand in the BAM complex upon its release from the chaperone-bound state.

## 2 | MATERIALS AND METHODS

### 2.1 | Cloning, expression, and purification of the BAM complex and the BAM-substrate complexes

The cloning, expression, and purification of the BAM complex followed a protocol described previously.\textsuperscript{33} The only difference is that the eluted protein sample was applied to size exclusion chromatography column (Superose 6 10/300, GE Healthcare) that was pre-equilibrated with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.74% cymal-4, and 1.0% OG (Anatrace) prior to crystallization. To obtain different BAM-substrate complexes, the coding sequences of the first β-strand of OmpLA (β\textsubscript{1}OmpLA, including residues P\textsubscript{54}YDTNYLIYTNTSDLN\textsubscript{68}), the last β-strand of OmpLA (β\textsubscript{12}OmpLA, including residues F\textsubscript{274}NQTRVGVGMNLDL\textsubscript{289}), the first β-strand of OmpA (β\textsubscript{1}OmpA, including residues D\textsubscript{25}NTWYGAKLGSWQ\textsubscript{39}), or the last β-strand of OmpA (β\textsubscript{8}OmpA, including residues N\textsubscript{1190}GMLSGVSRYFG\textsubscript{1192}) were fused to the C-terminus of BamA via a flexible linker coding sequence for GS\textsubscript{GS}GS\textsubscript{GS}. The DNA coding sequences of BamA-β\textsubscript{1}OmpLA, BamA-β\textsubscript{12}OmpLA, BamA-β\textsubscript{1}OmpA, or BamA-β\textsubscript{8}OmpA, together with vectors that allows the co-expression of five proteins with one plasmid in bacteria.\textsuperscript{44} The expression and purification of different BAM-substrate complexes are similar to that for the BAM complex described previously.\textsuperscript{33} All the protein samples were concentrated to approximately 15 mg mL\textsuperscript{-1} using a 100 kDa cutoff spin concentrator for crystallization trails.

### 2.2 | Crystallization, data collection, and structural determination of the BAM complex and the BAM-substrate complexes

Crystallization of the BAM complex and the BAM-substrate complexes was conducted at 16°C using the hanging drop vapor diffusion method by mixing the protein and precipitants at a ratio of 1:1. The BAM complex, the BamA-β\textsubscript{1}OmpLA BCDE complex, the BamA-β\textsubscript{12}OmpLA complex, the BamA-β\textsubscript{1}OmpA BCDE complex, and the BamA-β\textsubscript{8}OmpLA complex were all crystallized in a buffer containing 100 mM Tris-HCl (pH 7.4), 27%-30% PEG400, and 100 mM NaCl. The BAM complex (in mixing detergents of 0.74% cymal-4 and 1.0% OG) crystals appeared in 3 days and grew to their final size in approximately...
1 week, whereas crystals of the BAM-substrate complexes (in mixing detergents of 1.0% OG, 0.05% LDAO, and 0.8% C₄E₄) appeared in about 10 days, and grew to their final size normally within a month. X-ray diffraction data were collected at Shanghai Synchrotron Radiation Facility beamline BL-19U and BL-17U (SSRF, Shanghai, China). Diffraction data were obtained from a single crystal diffracted at a wavelength of either 0.97915 Å or 0.97919 Å at 100 K. Data were processed using HKL2000 and the structure was solved by molecular replacement using Phaser coordinates of the BAM complex (PDB code: 5D00) as search model. Iterative rounds of refinement were accomplished using Phenix. Manually building of models was done using Coot. All figures depicting the structure were generated with Pymol. Data collection and crystallographic statistics for the BAM complex, the BamA-β12OmpLA complex, and the BamA-β8 OmpA complex are listed in Table S1; Data collection and crystallographic statistics for the BamA-β1 OmpLA complex and the BamA-β1 OmpA complex are listed in Table S3.

### 2.3 Cryo-EM specimen preparation, data acquisition, and processing

The purified BAM complex sample in a buffer containing 20 mM Tris pH 8.0, 150 mM NaCl, and 0.025% DDM was reconstituted into lipid nanodiscs following the published protocol. In brief, saposin, the BAM complex, and E coli total lipids (Anatrace) were mixed at a molar ratio of 12:1:40, respectively, and incubated on ice for 1 hour. Detergents were removed by adding Bio-Beads SM2 (Bio-Rad) to a concentration of 100 mg mL⁻¹ followed by gentle agitation. The Bio-Beads were replaced with fresh ones every 4 hours, twice in total. After detergent removal, the sample was loaded onto a Superose 6 10/300 GL column pre-equilibrated with buffer (20 mM Tris pH 8.0 and 150 mM NaCl) and the elution peak corresponding to the reconstituted BAM complex was collected for electron cryo-microscopy analysis.

The reconstituted BAM complex was concentrated to ~0.8 mg mL⁻¹ for cryo-EM specimen preparation. Approximately 3.5 μL aliquots of protein sample were applied to glow-discharged Quantifoil Cu R2.0/2.0, 400-mesh holey carbon grid (Quantifoil, Micro Tools GmbH, Germany). Grids were flash plunged into liquid ethane at ~−184°C after being blotted for 4.0 seconds by automatic plunging device EMGP (Leica). The dataset of the BAM complex in nanodisks was collected in FEI Talos Arctica equipped with direct detector K2 summit (Gatan) after GIF quantum energy filter (energy width of 20 eV) at magnifications of 22,500 yielding binned pixel sizes of 1.0 Å. The defocus was set from −1.5 to −2.0 μm. Each micrograph was dose-fractionated to 30 frames under a dose rate of 8 e/\text{pixel/s}, with a total exposure time of 6.25 seconds, resulting in a total dose of about 50 e/Å². The beam-induced motion was corrected by MotionCor2. A total of 2,791 motion-corrected, dose-weighted micrographs were kept after inspection by eyes. A total of 1,669,059 particles were picked from selected micrographs using RELION for 2D classification. After two rounds reference-free 2D classification, 638,574 particles were kept from well featured classes for further data processing. The initial model for 3D classification was generated from the BAM complex (PDB code: 5LJO). The refinement of all selected particles from 3D classification yielded a 4.2-Å EM map in RELION-3.0.

For building the model of the BAM complex, the crystal structure of the BamA β-barrel from the previous crystal structure of the BAM complex (PDB code: 5AYW) and the modeled structure of the periplasmic components from the previous cryo-EM structure (PDB code: 5LJO) were fitted into the cryo-EM map by Chimera and the whole model was manually adjusted in Coot according to the density. The model was further refined in real space by PHENIX. Cryo-EM data collection and refinement are presented in the Table S2.

### 2.4 Complementation assays

The bamA-depleted JCM320 E coli cells were transformed with pQLink-BamABCDE plasmid encoding a mutant BamA or the wild-type BamA under kanamycin selection, plated on LB-agar plates with kanamycin (50 μg mL⁻¹) in the presence of l-arabinose (0.05%) and grown for 12 hours at 37°C. Single colony was picked up and inoculated into 5 mL Luria broth (LB) with 0.05% L-arabinose and 50 μg mL⁻¹ kanamycin. When cells grew to a density of OD₆₀₀nm = 1.0, cells were spun down and washed with 5 mL LB medium twice. The washed cells were resuspended in different volumes of LB medium to ensure that each sample has the same starting cell density of OD₆₀₀nm = 0.5, and were subsequently serially diluted (1:10, 1:100, 1:1000, 1:10,000, and 1:100,000), and 2 μL spotted on LB plates containing 50 μg mL⁻¹ kanamycin and 10 μM IPTG, respectively. As control, serially diluted cells were spotted on LB plates that contains 0.05% L-arabinose instead of 10 μM IPTG. All the plates were incubated at 37°C overnight.

### 2.5 Western blot analysis of the expression levels of BamA proteins in E coli strain JCM320 cells

To compare the relative protein expression levels of BamA variants, plasmids pQLink-BamABCDE expressing either
wild-type or BamA mutants (all contain an N-terminal 6-histidine tag after signal peptide sequence of BamA) were transformed into *E. coli* strain JCM320 cells, and spread on LB agar plates that contained 50 µg mL⁻¹ kanamycin, 0.05% l-arabinose, and 10 µM IPTG. After incubation at 37°C for 12 hours, colonies were scraped off of the plates, transferred to Eppendorf tubes, and the cell density was adjusted to OD₅₆₀ = 1.0 by addition of 1x PBS buffer. Cells (15 µL) from each sample were allocated into Eppendorf tube, and boiled for 5 minutes prior to 12% SDS-PAGE electrophoresis. The proteins were then transferred to a PVDF membrane and blocked using TBST buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 8% skim milk for 2 hours. The PVDF membrane was then incubated with anti-His antibody (1:5000) (TIANGEN) at room temperature for 2 hours. Followed by washing the PVDF membrane twice with TBST buffer, the PVDF membrane was incubated with horseradish-peroxidase-conjugated secondary antibody (1:5000) (Trans) at room temperature for 2 hours. The PVDF membranes were exposed using enhanced chemiluminescence reagents (EasySee Western Blot Kit, Trans).

### 2.6 Sample preparation for detection of inter-strand disulfide bond in the BamA(B426C)-β12OmpLA(V283C)BCDE complex by mass spectrometry

The detection of inter-strand disulfide bond in the BamA(B426C)-β12OmpLA(V283C)BCDE complex was performed on MALDI-TOF/TOF (Bruker, Germany). Because there is no trypsin-cutting site within the sequence of the three β-strands (including residues N422-T461) of the BamA β-barrel, a E435R point mutation was introduced into the BamA to facilitate mass spectrometric detection of the peptide fragment, and the resulted BamA(E435R)-β12OmpLABCDE complex will produce a peptide fragment that includes residues NTGSFNFGIGYGT after trypsin proteolysis. Next, two cysteine mutations (BamA(B426C) and OmpLA(V283C)) were also introduced into the BamA(E435R)-β12OmpLABCDE complex.

The protein bands were excised from 12% SDS-PAGE gels (containing no reducing reagents in the loading dye) and divided into two aliquots. After dehydration, the gel plugs were incubated in 25 mM NH₄HCO₃ with and without 5 mM DTT, respectively, for 45 minutes. Then, both samples were alkylated with 40 mM iodoacetamide in 25 mM NH₄HCO₃ for 45 minutes at room temperature in the dark and then, digested overnight with trypsin (40 ng for each band) at 37°C. The reactions were terminated by adding trifluoroacetic acid to a final concentration of 1%, and desalted using C18 Zip-Tip microcolumns (Millipore). The samples were then loaded into the instrument in a crystalline matrix of α-cyano-4-hydroxycinnamic acid (5 mg mL⁻¹). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric detection was achieved using an AXIMA-CFR Plus mass spectrometer from KRATOS Analytical (Shimadzu). The MS spectra were manually inspected and compared.

### 3 RESULTS

#### 3.1 Structures of the BAM complex in detergents and in nanodisks

Previous structural studies show that the cryo-EM structure of the BAM complex in *n*-dodecyl-β-D-maltopyranoside (DDM) is strikingly different from the two crystal structures of the BAM complex in mixing detergents of octyl β-D-glucopyranoside (β-OG), n-octyltetraoxyethylene (C₈E₄), and n-lauryl-N,N-dimethyamine-N-Oxide (LDAO).³¹,³³,³⁴ In the cryo-EM structure, the BamA β-barrel adopts a laterally open conformation and has a different orientation relative to the periplasmic components of the BAM complex,³⁴ in sharp contrast to those observed in the crystal structures.³¹,³³ These differences prompt us to investigate the structures of the BAM complex in different detergent combinations and in lipids. To this end, we crystallized the BAM complex in a condition that contains mixing detergents of cymal-4 and β-OG, and determined its structure (denoted as the BAM⁰m⁰l+OG complex) at a 3.0-Å resolution with molecular replacement using the whole BAM complex (PDB code: 5D00) as search model (Table S1). Molecular replacement shows that the BAM⁰m⁰l+OG complex not only contains all the components observed in the previous crystal structures, but also reveals extra densities that correspond to the BamC domains that are invisible in previous BAM crystal structures³¹,³³ and cryo-EM structure.³⁴

BamC, a multidomain lipoprotein, is composed of BamC-UN (an unstructured region), BamC-N, α-helical linker, and BamC-C (Figure 1A). In the BamACDE complex, the N-terminal domains of BamC, including BamC-UN, BamC-N, α-helical linker, and bind BamD, whereas the most C-terminal domain BamC-C is in close contact with POTRA2 of BamA.³¹,³² In contrast, in the BAM⁰m⁰l+OG complex, the BamC-N domain, which is invisible in previous crystal structures and cryo-EM structure of the BAM complex, relocates to a place and interacts with POTRA1 domain of BamA (Figure 1B), distinct from what is observed in crystal structure of the BamACDE complex (Figure 1C). However, the BamC-N domain in the BAM⁰m⁰l+OG complex is incomplete, with its first β-strand that consists of the BamC-N domain invisible (Figure 1D). Crystal packing analysis indicates that the other end of the BamC-N domain is also in close contact with the POTRA4 domain of BamA of a neighboring BAM complex, which may stabilize...
FIGURE 1  Structures of the BAM complex in detergents and in nanodisks. A, Schematic structure (upper) and crystal structure of BamC (bottom). Based on the crystal structure of the BamACDE complex, BamC consists of four domains/regions: BamC-UN, BamC-N, α-helical linker, and BamC-C. Different domains/regions of BamC are highlighted by different colors. B, Cartoon representation of the BAMcymal+OG complex structure showing the locations of BamC-UN and BamC-N (magenta). The invisible residues between the two domains are indicated with a dotted line. Components of the BAM complex are shown in different colors. C, An overlay of BamD in the BAMcymal+OG complex with that in the BamACDE complex showing that BamC-UN binds with BamD similarly in the two structures, but BamC-N locates differently. D, An alignment of the BamC-N domains in the structure of the BAMcymal+OG complex and in the BamACDE complex highlighting that BamC-N in the BAMcymal+OG complex misses the N-terminal loop and the first β-strand of the domain (blue). E, Fitting of two cryo-EM maps of the BAM complex showing the striking difference in densities corresponding to the BamA β-barrel region in the two structures (marked with a dotted circle). The cryo-EM maps of the BAM complex in DDM and in nanodisks are colored in green and cyan, respectively. F, Comparison of the BamA β-barrel structures (marked with dotted circles) of the BAM complex in two cryo-EM structures. The BamA β-barrel domain of the BAM complex in nanodisks (right) adopts a lateral closed conformation, in contrast to that in the previous cryo-EM structure obtained in DDM (left).
the conformation of the BamC-N domain in the structure (Figure S1). Considering that the cryo-EM structure of the BAM complex in DDM differs significantly in the structure of the BamA β-barrel and its orientation relative to the periplasmic components to those in three available crystal structures, we reason that detergents have differential effects on the overall conformation and integrity of the BAM complex as well as the conformation of individual components such as BamC and the BamA β-barrel in the BAM complex. This hypothesis prompts us to further study the structure of the BAM complex in a more native-like environment such as in nanodisks.
FIGURE 2  Crystal structure of the BamA-β12OmpLA-BCDE complex. A, The designed BamA-β1OmpLA and BamA-β12OmpLA fusion proteins. The crystal structure of OmpLA (PDB code: 1QD6) is shown on the left. β1OmpLA and β12OmpLA in the OmpLA structure are highlighted in blue and red, respectively. β1OmpLA and β12OmpLA not only contain residues that consists of strands β1 and β12, but also include different numbers of extra residues from their respective N-terminal loops and C-terminal loops. Detailed amino acid sequences of the two fusion proteins BamA-β1OmpLA and BamA-β12OmpLA are shown on the right. B, Cartoon representation of the BamA-β12OmpLA complex structure showing the secondary structure and location of β12OmpLA (red) in the structure. Components of the BAM complex are shown in different colors. β12OmpLA forms an antiparallel β-strand to the β1BamA strand. C, 2Fo − Fc difference Fourier electron density (blue mesh, contoured at 2.0 σ) of β12OmpLA. Residues of β12OmpLA (magenta) are shown in stick model. Numbering of the β12OmpLA residues in the structure according to the full-length OmpLA sequence with its N-terminal DDLM34 containing density for a single copy each of BamA, BamB, BamC, BamD, and BamE (Figure 1E). As expected, the map also contains a doughnut-shaped density that surrounds the BamA β-barrel, consistent with a nanodisk in both size and appearance.57 A preliminary examination of the density by fitting of the crystal structure of BamA β-barrel (PDB code: 5AYW)33 and the modeled structure of the periplasmic components from a previous cryo-EM structure (PDB code: 5LJO),34 confirmed that its periplasmic architecture of the complex is similar to that in the previous cryo-EM structure. However, the densities corresponding to the BamA β-barrel of the BAM complex are strikingly different to those in the previous cryo-EM map (Figure 1E). A pseudoatomic model was built into the 4.2-Å resolution density map (Table S1) and it clearly reveals that the BamA β-barrel domain adopts a laterally closed conformation, in sharp contrast to that in the cryo-EM structure of the BAM complex in DDM (Figure 1F). To explore the conformational heterogeneity of the cryo-EM density data sets, we performed extensive three-dimensional classification,38 but no subclasses representing different conformations was identified. The striking conformational difference of the BamA β-barrel in the two cryo-EM structures further support the hypothesis that lipids and detergents may have differential effects on the overall conformations of the BAM complex, highlighting a highly dynamic nature of the BAM complex.

3.2 Structures of the BAM complex with bound substrates

While numerous biochemical and biophysical studies show that the β1BamA interacts the last but not the first β-strand of a β-OMP substrate,35-41 it is unclear how the BAM complex achieves this specificity. To address this issue, it is important to obtain the structures of the substrate-bound BAM complex. As assembly of a complete β-barrel OMP substrate by the BAM complex is a highly efficient process in bacteria and the incomplete β-barrel OMP substrate is readily degraded by proteases in the periplasm,59 it is difficult to obtain sufficient amount of the substrate-bound BAM complex with correct stoichiometry for structural characterization via co-expression of the BAM complex with a β-OMP substrate. Moreover, obtaining the substrate-bound BAM complex by in vitro reconstitution faces the insolubility problem of the unfolded β-OMP substrates. To circumvent these difficulties, we developed a fusion-protein strategy to obtain sufficient amount of the substrate-bound BAM complex and ensure a correct stoichiometry between the BAM complex and the substrate for structural characterization.

Previous studies indicate that a specific amino acid sequence in the last β-strand of a β-barrel OMP is important for its recognition by BamA.35-42 We selected the outer membrane phospholipase (OmpLA), a 12-stranded β-barrel OMP, as substrate, and fused its protein sequences that correspond to either the first β-strand (β1OmpLA) or the last β-strand (β12OmpLA) of OmpLA to the C-terminus of BamA (β16BamA) via a flexible 10-residue linker, GSGSGSGSGS (Figure 2A). The DNA coding sequences of BamA-β1OmpLA (or BamA-β12OmpLA), BamB, BamC, BamD and BamE from E coli strain K12 MG1655 were cloned into pQlink vector that allows co-expression of five proteins with one plasmid in bacteria.44 We purified and crystallized the BamA-β1OmpLA-BCDE complex and the BamA-β12OmpLA-BCDE complex, and further determined their structures at resolutions of 3.3 and 3.2 Å, respectively (Tables S1 and S3). Molecular replacement using the BAM complex as search model revealed that extra densities appear along the β1BamA strand in the map of the BamA-β1OmpLA-BCDE complex, but no extra densities were observed in the map of the BamA-β12OmpLA-BCDE complex (Figure S4A). Model building and refinement clearly reveal that residues in the β12OmpLA strand form an antiparallel β-strand with the β1BamA strand, but there are no densities for the flexible linker (−GSGSGSGGS−) that connects the β12OmpLA strand with the C-terminus of BamA (Figure 2B,C).

To further confirm the sequence registry of the β12OmpLA strand built in the model, two cysteine mutations
Mass spectrometric analysis of the purified double-cysteine mutation-containing BamA-β1 OmpLA BCDE complex clearly exhibits a peak with molecular weight (MW) of 2611.3 Da under nonreducing condition (Figure 2D, upper panels). This MW exactly matches the sum MW of the two peptide
fragments (BamA-N_422TGSCNFGIGYGT_434 and OmpLA-V_279GVGCMNLDLF_289) that are cross-linked by a disulfide bond. In contrast, the peak corresponding to the MW of 2611.3 Da disappeared under reducing condition, but a peak with a molecular mass of 1503.7 Da, which matches the peptide BamA-N_422TGSCNFGIGYGT_434, emerged (Figure 2D, bottom panels). The results of mass spectrometric analysis confirmed that the β12OmpLA interact with the β1BamA strand in the newly purified proteins, and the registry of the β12OmpLA sequence was correctly built.

To provide more evidences to further support that BamA may recognize the last but not the first β-strand of an β-barrel OMP substrate, we selected OmpA, an 8-stranded β-OMP, as substrate. The first β-strand (β1OmpA) and last β-strand (β8OmpA) of OmpA have no sequence identities to the β1OmpLA and β12OmpLA, respectively (Tables S1 and S3). In line with structural observations in BamA-β1OmpLABCDE and in BamA-β12OmpLABCDE, the β8OmpA sequence forms an antiparallel strand with the β1BamA strand in BamA-β8OmpABCDE (Figure 3B,C), but the β1OmpLA is completely invisible in BamA-β1OmpABCDE (Figure 3A). Using a similar fusion-protein strategy, we determined the crystal structures of the BamA-β1OmpABCDEx5 complex and the BamA-β8OmpABCDE complex at resolutions of 3.2 and 3.3 Å, respectively (Figures 3D and S5). Taken together, the two substrates are able to rescue the growth of the bamA-depleted E coli strain JCM3203 when transformed.

Since the incoming β-OMP substrates are recognized by β1BamA in the BAM complex via antiparallel β-strand interactions, we made a series of single-point mutations to probe the effects on the growth of the E coli strain JCM320 in absence of L-arabinose (Figure S6A, left panel) in the complementation assay. These residues are located at the beginning of the β1BamA strand, which form regular hydrogen bonds with substrates in both BamA-β12OmpLABCDE and BamA-β8OmpABCDE. In contrast, single-point mutants I430P, G431P, and Y432P, which is located at the end of β1BamA, have no noticeable effects on the growth of the E coli strain JCM320 in the complementation assay (Figure 4A). Nonetheless, a set of double mutants (I430P + G431P, I430P + Y432P, G431P + Y432P, F426P + I430P, F426P + G431P, and F426P + Y432P) also caused cell death, suggesting that these positions might also be involved in certain stages during β-OMP biogenesis (Figures 4A and S6A). We also tested a series of mutants at different positions in the strand β16 of BamA. These mutants (F802P, Q803P, F804P, N805P, and I806P) are all lethal to bamA-depleted JCM320 E coli cells in the absence of L-arabinose (Figure S6A).
In line with this, residues F802-I806 of BamA (substrate) form an anti-parallel strand with β1 BamA (of BAM complex) as revealed by the recent cryo-EM structure of a substrate trapped on the BAM complex that snapshots a later intermediate stage of β-OMP biogenesis. To rule out the possibility that the impaired growth of the \emph{E. coli} strain JCM320 resulted from their decreased protein expression levels caused by mutation, we carried out western blotting to compare the expression levels of these mutants with that of the wild-type BamA. As show in Figures 4A and S6A (low panel), mutation itself did not cause a significant variation of the protein expression levels of BamA mutants. Taken together, the results of complementation assays are consistent with the structural observations that the N-terminal exposed-edges of the β1 BamA strand may be important for associating with the incoming β-OMP substrates upon their release from periplasmic chaperone.

4 | DISCUSSION

β-OMP assembly by the BAM complex is a multistep process that involves binding chaperone-substrates, recognition of substrates, folding, and inserting them into the OM. The strikingly different conformations of the BAM complex may represent its distinct functional states during each β-OMP assembly cycle in vivo. The currently observed strikingly different conformations of the BAM complex in detergents and in nanodisks further support the notion that the BAM complex is highly dynamic, and implicate that the membrane compositions surrounding the BAM complex might play roles in modulating the function of the BAM complex. The dynamic nature of the BAM complex also implies low energy barriers between different functional states of the BAM complex, which could allow ready interconversion between these functional states in a cellular energy-free environment.

In order to understand the mechanism of β-OMP assembly, it is necessary to break the process into steps and determine how the BAM complex facilitates each step. Therefore, it would be useful to observe substrates binding to the BAM complex at intermediate stages of folding and inserting into the membrane. In this regard, the structures of the BAM complex with bound substrate are important for understanding how the BAM complex recognizes its substrates upon their release from the chaperone-bound state. The structures of BamA-β12 OmpLABCDE and BamA-β8 OmpABCDE consistently pinpoint that the N-terminal exposed-edge of the β1 BamA strand may serve as a consensus site for initial association with the last but not the first β-strand of an β-OMP substrate, in line with previous biophysical and biochemical studies and the proposed β-signal hypothesis during β-OMP biogenesis. While a number of previous studies indicated that the most C-terminal Phe residue in the last β-strand of a β-OMP substrate could be important for associating with the BAM complex, we did not observe any interactions between F289 in β12 OmpLA and BamA in BamA-β12 OmpLABCDE. Consistent with our structure observations, F289A mutation in BamA-β12 OmpLABCDE also did not affect the interaction of β1 BamA and β12 OmpLA based on mass spectrometric analysis (Figure S7). In particular, our structures are also consistent with the recent cryo-EM structure of a folding intermediate trapped on the BAM complex in which the β1 BamA strand of the BAM complex anti-parallelly interacts with the last β-strand of the substrate. Although the structures of BamA-β12 OmpLABCDE and BamA-β8 OmpABCDE were obtained by fusing the substrates to the C-terminus of BamA, the 10-residue long flexible linker sequence (GSGSGSGSGS), along with the 2-4 N-terminal loop residues of the fused substrates,
should allow the fused substrates have sufficient freedom to access any motifs in the BAM complex within an approximately 40-Å distance to the C-terminus of BamA. As such, the two structures of the BAM-substrate complexes might represent an early intermediate state of the substrate that is recognized by the BAM complex upon its release from the chaperone-bound state.

In both BamA-β12OmpLABCDE and BamA-β8OmpABCDE, we did not observe conformational changes in the region of BamA β-barrel as compared to that in the apo-BAM complex, and none of the substrates adopted a complete β-strand conformation as what are observed in the crystal structures of OmpLA 60 and OmpA. 61 We reason that the initial substrate recognition by the β1BamA strand and the induced partially folding of the last β-strand of the substrates might be unable to provide sufficient energy for inducing conformational changes of the BAM complex; the last β-strand of a β-OMP substrate may only be important for initial recognition by the BAM complex, but also may play important roles in closing to form a complete β-barrel. In agreement with this hypothesis, the cryo-EM structure of a folding intermediate trapped on the BAM complex in which the substrate is a nearly completed β-OMP reveals significant conformational changes in the region of BamA β-barrel of the BAM complex. 43 Taken together, although events including subsequent substrate folding, release, and insertion into the OM during β-OMP assembly await further biochemical and structural characterization, our current structures of the substrate-bound BAM complex highlight that the N-terminal exposed-edges of β1BamA in the BAM complex might be important for associating with β-OMP substrates, and potentially functioning as a template that triggers the initial folding of the last β-strand of an incoming β-OMP substrate upon its release from the periplasmic chaperone.

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CONFLICT OF INTEREST
The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS
Y. Huang supervised the project; L. Xiao, L. Han, H. Zhou, and Q. Luo performed protein purification, crystallization and diffraction data collection; L. Xiao, B. Li, and X. Zhang contributed the cryo-EM data collection and map calculation; L. Xiao, L. Han, and M. Zhang built the model and refined all the structures; Y. Huang wrote the manuscript. All the authors contributed to manuscript preparation and data analysis. The coordinates, diffraction data of the BAM complex, the BamA-β12OmpLABCDE complex, and the BamA-β8OmpABCDE complex have been deposited in the Protein Data Bank with the accession codes 6LYS, 6LYQ, and 6LYR, respectively; Density map of the BAM complex is available through the EMDB with entry code EMD-30018 and its coordinates are deposited in the Protein Data Bank (PDB) with the accession codes 6LYU.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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