Cryo-EM structure of the bacterial Ton motor subcomplex ExbB–ExbD provides information on structure and stoichiometry

Herve Celia¹, Istvan Botos¹, Xiaodan Ni², Tara Fox³,⁴, Natalia De Val³,⁴, Roland Lloubes⁵, Jiansen Jiang²* & Susan K. Buchanan¹*

The TonB–ExbB–ExbD molecular motor harnesses the proton motive force across the bacterial inner membrane to couple energy to transporters at the outer membrane, facilitating uptake of essential nutrients such as iron and cobalamine. TonB physically interacts with the nutrient-loaded transporter to exert a force that opens an import pathway across the outer membrane. Until recently, no high-resolution structural information was available for this unique molecular motor. We published the first crystal structure of ExbB–ExbD in 2016 and showed that five copies of ExbB are arranged as a pentamer around a single copy of ExbD. However, our spectroscopic experiments clearly indicated that two copies of ExbD are present in the complex. To resolve this ambiguity, we used single-particle cryo-electron microscopy to show that the ExbB pentamer encloses a dimer of ExbD in its transmembrane pore, and not a monomer as previously reported. The revised stoichiometry has implications for motor function.
on is a membrane protein complex found in the inner membrane of Gram-negative bacteria. Ton can be described as a molecular motor in which the ExbB and ExbD subunits assemble to form a proton channel and use the proton gradient across the cytoplasmic membrane. The energy derived from proton translocation is propagated through the elongated TonB subunit that spans the periplasmic space and physically interacts with numerous TonB dependent transporters (TBDTs) at the outer membrane, allowing the active transport of rare nutrients into the periplasm.

To gain further information on structure and stoichiometry, we performed a single particle cryo-electron microscopy (cryo-EM) analysis on the full-length ExbB–ExbD complex reconstituted in lipid nanodiscs. The new structure shows that five copies of ExbB are arranged as a pentamer around two copies of ExbD, which sit in the pore. Compared to the crystal structure, the two copies of ExbD sit about 3 Å higher in the pore indicating that vertical, piston-like movement is a possible mode of action. The five copies of ExbB are shifted outward to accommodate both copies of ExbD, with no proton channel available in this conformation. Disulfide crosslinking experiments at various locations on ExbD provide supporting evidence for the observed stoichiometry and arrangement.

Results

Cryo-EM structure of ExbB–ExbD in lipid nanodiscs. The final reconstruction used 85,936 particles and yielded a 3.3 Å overall resolution structure calculated without imposing symmetry (Table 1; Supplementary Figs. 1–3). The structure reveals numerous alpha helices, with clear side chain densities and secondary structure elements, that allowed an atomic model to be built (Fig. 1). As found in the crystal structure, ExbB assembles as a pentamer, but instead of one ExbD TM domain, two TM helices of two adjacent ExbB subunits. The 7 TM helices of two adjacent ExbB subunits are arranged as a pentamer around two copies of ExbD, which sit in the pore. Compared to the crystal structure, the two copies of ExbD sit about 3 Å higher in the pore indicating that vertical, piston-like movement is a possible mode of action. The five copies of ExbB are shifted outward to accommodate both copies of ExbD, with no proton channel available in this conformation. Disulfide crosslinking experiments at various locations on ExbD provide supporting evidence for the observed stoichiometry and arrangement.

Cysteine crosslinking shows two copies of ExbD in the pore. To verify whether this new structure is representative of the whole population of ExbB–ExbD complexes, we performed cysteine crosslinking experiments and introduced single cysteines at three different positions on ExbD, either in the cytoplasm (D10C), the TM region (L40C) or the linker region between the TM and C-terminal domain (P50C). Co-expression and purification with ExbB depleted of cysteine show efficient disulfide crosslink formation for all three constructs (Fig. 2d). SEC experiments confirm that the disulfide bonds occur within the same ExbB–ExbD complex (Fig. 2e, f). While efficient crosslinking with P50C is expected for both configurations, i.e. one or two ExbD TM helices in the pore, crosslinking with D10C or L40C can only occur if both the TM and N-terminal domains of ExbD are located in the periplasmic pore and cytoplasmic cavity of ExbB (Fig. 2e). As assessed by SDS-PAGE, the crosslinking of L40C is not as efficient as that for D10C and P50C. Examination of the cryo-EM structure shows that the two Leu40 side chains are pointing in opposite directions in the pore. The fact that L40C is able to crosslink at all suggests that the ExbD TM helices are mobile and can rotate.

Table 1 Data collection, processing, and refinement statistics

| Deposition ID | EMD-20583; PDB 6TYI |
| Magnification | 130,000 (nominal) |
| Voltage (kV) | 300 |
| Electron exposure (e−/Å²) | 71 |
| Defocus range (µm) | −0.7 to −2.5 |
| Pixel size (Å) | 1.06 |
| Symmetry imposed | C1 |
| Initial particle images (no.) | 3,206,108 |
| Final particle images (no.) | 85,936 |
| Map resolution (Å) | 3.3 |
| FSC threshold | 0.143 |
| Map resolution range (Å) | 3.0–19.0 |
| R.m.s. deviations | |
| Bond length (Å) (# > 4σ) | 0.012 (10) |
| Bond angles (°) (# > 4σ) | 0.901 (1) |
| Validation | |
| Refined model CC | 0.82 |
| MolProbity score | 1.83 |
| Clashscore | 8.55 |
| Poor rotamers (%) | 0.55 |
| Ramachandran plot | |
| Favored (%) | 94.71 |
| Allowed (%) | 5.29 |
| Disallowed (%) | 0 |

likely because the 19-residue linker that connects the TM to the folded C-terminal domain is highly flexible. Four phospholipids were built into grooves defined by the hydrophobic portions of the α6 and α7 TM helices of two adjacent ExbB subunits. The polar head groups of the phospholipids point toward the lateral fenestrations of the complex that are lined up with basic residues (Supplementary Fig. 5).
Discussion

The cryo-EM structure shows that the pentamer of ExbB encloses a dimer of ExbD TM helices in its central pore. The exact stoichiometry of the Ton complex has always been a matter of debate, with different stoichiometries reported ranging from four ExbB and one or two ExbD, five ExbB and two ExbD, or six ExbB and three ExbD5-7. However, recent mass spectrometry experiments performed on native membranes of E. coli cells detected only the pentameric form of ExbB, suggesting that the tetramer and hexamer forms are non-physiological or of very low abundance8.

Because of the reconstitution in lipid nanodiscs, the ExbB–ExbD subcomplex is observed in a natural lipid environment. The cryo-EM map reveals densities that correspond to phospholipids closely associated with the ExbB pentamer. Four phospholipids were built, three PE and one PG, and are part of the cytoplasmic leaflet of the membrane. The crystal structure did not reveal any associated lipids, likely because they were displaced by the detergent.

The ExbD periplasmic domain dimerizes in the ExbB–ExbD complex, and the structure of the soluble periplasmic domains of the closely related MotB and TolR homologues are dimeric5,9,10. It was therefore surprising to find only one TM domain of ExbD in the ExbB pentameric crystal structure, as disulfide cysteine scanning experiments with TolR or MotB suggested that these proteins also dimerize in their membrane embedded region11,12. It is not fully understood why only one TM domain of the ExbD_{deltaperi} construct was found in the ExbB pentameric crystallographic structure, but it is likely related to the fact that this truncated construct lacks the C-terminal periplasmic domain of ExbD, which has been shown to dimerize in vivo13.

The observed densities in the cryo-EM structure are better defined for the ExbB subunits than for ExbD subunits, suggesting that the ExbD subunits are somewhat mobile (Supplementary Fig. 3). The cryo-EM experiment was performed at neutral pH (7.5), while the crystal structure that showed the single ExbD TM domain was obtained with crystals grown at acidic pH (4.5). It is likely that the cryo-EM structure represents the resting state of the Ton complex, as there is no visible channel that would connect the Asp25 residues either to the periplasm or cytoplasm. In the crystal structure obtained at acidic pH, the ExbD TM helix is shifted by about 3 Å compared to the cryo-EM structure, bringing Asp25 closer to the cytoplasmic cavity (Supplementary Fig. 4). As Asp25 is likely to be protonated in the crystal structure, it might represent an intermediate in which the Asp is primed to deliver a proton to the cytoplasm. If correct, this would be indicative of a translational movement along the pore axis of one or both of the ExbD TM helices during the catalytic cycle.

It is hypothesized that the proton translocation through the Ton complex results in movement propagation. The two ExbD TM helices might move up and down the pore axis, and/or rotate during the catalytic process. Inspection of the cryo-EM structure shows that the ExbD TM domains fit tightly in the pore and do...
not have enough space to move in either direction, unless the ExbB subunits move as well. Inspection of the cryo-EM and crystal structures shows that the ExbB pentamer can adopt different conformations (Supplementary Fig. 4, Supplementary Movies 1 and 2). When overexpressed, ExbB is also able to assemble as a hexamer and can form a stable oligomer in the absence of ExbD. This structural plasticity might be important to accommodate the different structural states of the ExbD TM domains.

Taken together, we have demonstrated that the Ton subcomplex consists of a pentamer of ExbB enclosing a dimer of ExbD TM domains. The essential Asp25 residues on ExbD are in close proximity to the conserved Thr 148 and 181 on ExbB. Because there is no apparent channel in this structure through which a proton could be translocated, conformational changes must occur to open such a channel, possibly involving interaction with the TonB subunit. We showed previously that addition of TonB to the ExbB–ExbD subcomplex does not change the stoichiometries for ExbB and ExbD.

Methods

Cloning and purification. Native exbD was cloned into pET26b, exbD harboring a 3’ end TEV protease site followed by 10 His codons was inserted into pCDF-Ib<sup>5</sup>. The D10C, L40C, and P50C cysteine substitutions of ExbD and C25S mutation of ExbB were obtained by quick change PCR using specific primers (primer sequences available upon request)<sup>3,4</sup>. The sequences of all plasmid constructs were verified by sequencing analysis.

The plasmid encoding the MPS1E3D1 apo-lipoprotein was obtained from Addgene and expressed and purified as described<sup>3,15</sup>. The N-terminal histag was cleaved using TEV protease. The resulting MSP1E3D1 was concentrated to 230 µM, flash frozen in liquid nitrogen and stored at −80°C.

The ExbB–ExbD complexes (ExbB–ExbD, ExbR<sub>C25S</sub>–ExbD<sub>D10C</sub>, ExbR<sub>C25S</sub>–ExbD<sub>50C</sub>) were expressed, solubilized with n-Dodecyl β-D-maltoside (DDM) and purified by affinity chromatography as previously described<sup>5</sup>. For the crosslinking analysis, SEC experiments were performed on a Superose6 increase 10/30 column (GE Healthcare) in PBS imidazole 50 mM DDM 0.1% w/v.

**Figure 2**  Arrangement of ExbD α-helices in the ExbB-ExbD subcomplex. a  Ribbon representation of the TM domains of ExbB, viewed from the cytoplasm, colored in orange and yellow. The atoms of the essential Asp25 residues are represented in ball and sticks. The ring of conserved threonines 148 on ExbB TM α6 and 181 on TM α7 are shown as ball and sticks. b  Same as A but viewed perpendicular to the membrane plane. For clarity only two ExbB subunits are shown. c  Section through a surface representation of the ExbB pentamer (cyan) and the two ExbD TM domains (orange and yellow) are shown as ball and sticks. d  SDS-PAGE of DDM solubilized and purified ExbB-ExbD complexes. The first lane is the control with no cysteine on ExbD, the second, third and fourth lane are ExbR<sub>C25S</sub>–ExbD<sub>D10C</sub>, ExbR<sub>C25S</sub>–ExbD<sub>L40C</sub> and ExbR<sub>C25S</sub>–ExbD<sub>P50C</sub> respectively. e  Ribbon representation of the solution structure of the ExbD soluble periplasmic domain (in green, pdb code 2PFU)<sup>4</sup> and ExbB-ExbD. The positions of the D10, L40, and P50 residues of ExbD are indicated with arrows. f  SEC elution profiles of the DDM solubilized and purified ExbB-ExbD complexes. The X axis is expressed as column volume (Superose6 increase 10/30), the Y axis is in milli-absorbance units at 280 nm.
The second data collection was performed with a Titan Krios G3 cryo-electron microscope (Thermo-Fisher) operated at 300 kV at the MCEP (NIH). Micrographs were exposed as dose-fractionated movies with Ctf recombination with a Gatan K2 Summit direct electron detector operated in counting mode at a nominal magnification of \(130,000\) (calibrated pixel size of 1.06 Å on the sample level). A Gatan Imaging Filter (GIF) Quantum 1S was included with the K2 Summit camera and the slit width was set to 20 e−/pixel/s. The dose rate on the camera was set to \(8\) e−/pixel/s. The total exposure time for each movie was 10 s fractionated into 50 frames with 0.2 s exposure time for each frame. A total of 5244 movies were acquired with automation using Leginon17.

**Image processing.** The movie frames from the first data collection were gain corrected and aligned using MotionCor218, and defocus determination was performed on averaged images with CTFFIND419. A total of 1,867,538 particles were picked using the convolution neural network option for particle picking in EMAN220,21. Particles were extracted as 128 × 128 pixels boxes and processed using RELION322 with few modifications in Supplementary Fig. 1. A total of 3,206,108 particles in the dose weighted average images were picked with Gautomatch23 using the result from the previous cryo-EM 3D reconstruction as the starting model. The particles were separated into five subsets by 3D classiﬁcation with C1 symmetry. The good particles from each 2D classiﬁcation with C1 symmetry for 70 iterations. The beginning 30 iterations and following 20 iterations were with a dynamic mask using both RELION3 and cryoSPARC25. The 3D autoreﬁnement with C1 symmetry and the soft mask using RELION3 generated a reconstruction at 3.5 Å. The non-uniform reﬁnement with C1 symmetry and a dynamic mask using cryoSPARC2 obtained a similar structure at 3.3 Å resolution. The resolution calculation was based on the “gold-standard” FSC at 0.143 criterion with automatic soft mask. The cryoEM maps were sharpened with B-factor and low-pass filtered using the relion_postprocess program in RELION3 or the Local Filtering function in cryoSPARC2. The local resolution was calculated by ResMap24 or by cryoSPARC2 with an algorithm similar to the locres program25 using two cryoEM maps independently reﬁned from halves of data. CryoEM data collection, reﬁnement, and validation statistics are given in Table S1.

**Model building and reﬁnement.** Model building into the ExbB–ExbD 3.5 Å map was started by ﬁtting the pentameric ExbB crystal structure (PDB:5SV0) into the map with UCSF Chimera26 and real-space reﬁning it in Phenix27. The resulting structure was rebuilt in Coot28 using the more detailed, Phenix auto-sharpened 3.3 Å map. Two ExbD molecules were built into the map in Coot, based on the TM helix of the ExbD crystal structure (PDB:5SV1). Pores were individually built in Coot then the whole structure was real-space reﬁned in Phenix.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** Atomic coordinates and structure factors for ExbBD have been deposited in the EMDB and wwPDB under accession codes EMD-20583 and PDB 6TYI. Source data for all ﬁgures and files are available from the authors upon reasonable request.

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Author contributions
H.C. expressed and purified the proteins, prepared nanodisc samples, prepared EM grids, collected and analyzed data, and wrote the manuscript. I.B. collected and analyzed data, constructed the model, and wrote the manuscript. T.F. and N.D.V. prepared EM grids and collected data. X.N. and J.J. collected and processed the EM data, and wrote the manuscript. R.L. designed the cysteine substitutions and commented on the manuscript. S.K.B. directed the work and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to J.J. or S.K.B.

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