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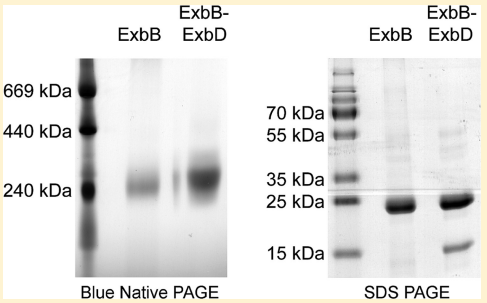
Oligomeric Structure of ExbB and ExbB-ExbD Isolated from *Escherichia coli* As Revealed by LILBID Mass Spectrometry

Avijit Pramanik,*[,†] Waldemar Hauf,[†] Jan Hoffmann,[‡] Mihaela Cernescu,[‡] Bernhard Brutschy,[‡] and Volkmar Braun[†]

[†]Max Planck Institute for Developmental Biology, D-72076 Tübingen, Germany

[‡]Institute of Physical and Theoretical Chemistry, Johann Wolfgang Goethe University, D-60438 Frankfurt am Main, Germany

ABSTRACT: Energy-coupled transporters in the outer membrane of *Escherichia coli* and other Gram-negative bacteria allow the entry of scarce substrates, toxic proteins, and bacterial viruses (phages) into the cells. The required energy is derived from the proton-motive force of the cytoplasmic membrane, which is coupled to the outer membrane via the ExbB-ExbD-TonB protein complex. Knowledge of the structure of this complex is required to elucidate the mechanisms of energy harvesting in the cytoplasmic membrane and energy transfer to the outer membrane transporters. Here we solubilized an ExbB oligomer and an ExbB-ExbD subcomplex from the cytoplasmic membrane with the detergent undecyl maltoside. Using laser-induced liquid bead ion desorption mass spectrometry (LILBID-MS), we determined at moderate desorption laser energies the oligomeric structure of ExbB to be mainly hexameric ($ExbB_6$), with minor amounts of trimeric ($ExbB_3$), dimeric ($ExbB_2$), and monomeric ($ExbB_1$) oligomers. Under the same conditions ExbB-ExbD formed a subcomplex consisting of $ExbB_6ExbD_1$, with a minor amount of $ExbB_5ExbD_1$. At higher desorption laser intensities, $ExbB_1$ and $ExbD_1$ and traces of $ExbB_3ExbD_1$, $ExbB_2ExbD_1$, $ExbB_1ExbD_1$, $ExbB_3$, and $ExbB_2$ were observed. Since the $ExbB_6$ complex and the $ExbB_6ExbD_1$ complex remained stable during solubilization and subsequent chromatographic purification on nickel–nitrilotriacetate agarose, Strep-Tactin, and Superdex 200, and during native blue gel electrophoresis, we concluded that $ExbB_6$ and $ExbB_6ExbD_1$ are subcomplexes on which the final complex including TonB is assembled.



Nutrients pass through the outer membrane of Gram-negative bacteria such as *Escherichia coli* by diffusion, facilitated diffusion, or energy-coupled transport. Diffusion is mediated by permanently open porin proteins, whereas energy-coupled transport is catalyzed by transport proteins consisting of a β-barrel with a pore that is tightly closed by a globular domain, called the plug. These transport proteins import nutrients of low abundance, such as heme, ferric siderophores, vitamin B_{12}, nickel, or carbohydrates, or substrates that are too large to diffuse through the porins, such as the colicin protein toxins (reviewed in refs 1−5).

For energy-coupled import into the periplasm (the compartment between the outer membrane and the cytoplasmic membrane), the substrates must be released from their high-affinity binding sites on the transport proteins close to the cell surface, and the plug must move to open a channel. The energy required by these reactions is not generated in the outer membrane, where the formation of a transmembrane potential is prevented by the pores, through which compounds of ≤700 Da pass freely. Also, no energy-generating systems that form energy-rich compounds, i.e., ATP, are found in the periplasm. Instead, the energy required for transport is derived from the electrochemical potential of the cytoplasmic membrane through the action of the proteins TonB, ExbB, and ExbD. These three proteins presumably form a complex that reacts to the proton-motive force across the cytoplasmic membrane by changing the complex conformation, which in turn alters the structure of the outer membrane transport proteins such that the substrates are released from their binding sites and the pore in the β-barrel is opened for the substrates to enter the periplasm.

Radiolabeling of these proteins has revealed that much more ExbB is formed than ExbD and TonB,[6] and determination of the protein concentrations in cells has indicated a 7:2:1 ratio for ExbB:ExbD:TonB.[7] It is unclear whether these numbers reflect the stoichiometry of the proteins in the complex or include also proteins not yet assembled in the complex. The three proteins interact with each other as shown by stabilization of TonB and ExbD by ExbB[6,8] and the formation of TonB homodimers, TonB-ExbD heterodimers, ExbD homodimers, and ExbB-ExbD heterodimers in cells treated with formaldehyde.[9] TonB dimers have also been observed in vivo in an assay with TonB fused to ToxR, which in the dimeric form activates transcription of the *ctx* cholera toxin gene.[10] Cysteine-cross-linking of ExbB and TonB depends on an unimpaired response to the proton-motive force, which indicates a functionally relevant dimerization.[11] Larger cross-linked complexes have not been observed,

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possibly because the low yields of cross-linked products prevented identification of complexes larger than dimers.

ExbB has three transmembrane segments in the cytoplasmic membrane, with its N-terminus in the periplasm and its C-terminus in the cytoplasm (Figure 1).[12] In contrast, the

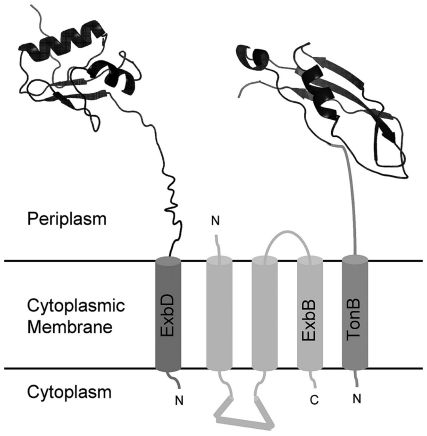

Figure 1. Transmembrane topology of ExbB,[12] ExbD,[13] and TonB[4] in *Escherichia coli*. The structure of the ExbD fragment in the periplasm (residues 44−141) determined by NMR spectroscopy[25] and the crystal structure of a TonB fragment (residues 158−235) bound to FhuA[15] are shown.

periplasmic TonB and ExbD proteins have a single, N-terminal transmembrane domain through which they are anchored to the cytoplasmic membrane (Figure 1).[13,14] The C-terminal domain of TonB interacts with the outer membrane transport proteins, as revealed by genetic suppressor analysis, cross-linking between introduced cysteine residues, cross-linking with formaldehyde,[1−5] and crystal structure determination of transport proteins with bound C-terminal fragments of TonB.[15,16]

Since ExbB stabilizes TonB and ExbD and is synthesized in surplus over these two proteins, we predicted that ExbB is the scaffold for the entire complex. Indeed, analysis of isolated ExbB, solubilized in 0.1% decyl maltoside, by size-exclusion chromatography, native gel electrophoresis, small-angle X-ray scattering, and transmission electron microscopy indicates that isolated ExbB forms a stable homo-oligomer of four to six monomers.[17]

In the present study, we further investigated the oligomeric structure of ExbB and of ExbB bound to ExbD using laser-induced liquid bead ion desorption mass spectrometry (LILBID-MS). This novel method has been successfully utilized to analyze the composition of protein complexes, such as a mitochondrial TOM complex,[18] a cytochrome bc_1 complex,[19] and the c-ring of an F_1F_0-ATP synthase.[20] We solubilized the ExbB-ExbD complex with undecyl maltoside which solubilized ExbB together with ExbD, in contrast to the previously used decyl maltoside, which did not solubilize the ExbB-ExbD complex reproducibly. Our results indicated that $ExbB_6$ and $ExbB_6ExbD_1$ are subcomplexes which form the foundation for the assembly of the complete native complex.

EXPERIMENTAL PROCEDURES

Cloning of the *exbB*, *exbD*, and *tonB* Genes. Construction of the plasmids pAP06 *exbB exbD* and pAP09 *exbB* used for expression of the ExbB and ExbD proteins has been previously described.[17] In short, the *exbB-exbD* operon was amplified from *E. coli* genomic DNA using the polymerase chain reaction (PCR), introducing a StrepII tag sequence at the encoded C-terminus of ExbD. The PCR product was cloned between the *Nco*I and *Eco*RI sites of vector pET-28a, and the resulting plasmid pAP44 was introduced into *E. coli* strain TOP10 (Invitrogen) by transformation. Then, the ribosomal binding site of *exbD* was improved by replacing AGGA, which overlaps with the last two codons of *exbB,* with AGGAGA placed downstream of *exbB,* and a C-terminal His tag (His_6-Thr-Gly)-encoding sequence was added at the 3′ end of *exbB* to generate pAP06. Plasmid pAP09 was generated by deleting *exbD* from pAP06 by PCR.

E. coli tonB was cloned into plasmid pARA; this plasmid has the origin of replication of pACYC177, which is compatible with that of pET28a-based constructs, confers ampicillin resistance, and harbors *araC,* followed by a P_{BAD} promoter and a multiple cloning site from the pBAD (Invitrogen) vector. Plasmid pAP52 *tonB* was constructed by amplifying chromosomal *E. coli tonB* by PCR with the primer pairs TGACT*TCATGA*CCCTTGATTTACCTCG and CGG*AAGCTT*TTGACTTTCTGCTTACTG. The DNA fragment was digested with *Pag*I and *Hin*dIII and cloned between the *Nco*I and *Hin*dIII sites of pARA.

Plasmid pAP06 or pAP09 alone and together with pAP52 were expressed in the *E. coli* C43 mutant, which lacks the chromosomal *exbB* and *tonB* genes. Deletion of chromosomal *exbB* largely prevents expression of *exbD*.[17] Chromosomal *tonB* was deleted as previously described for deletion of *exbB*.[17] The correctness of the constructs was verified by DNA sequencing.

Protein Expression, Solubilization, and Purification. Cells of *E. coli* C43 Δ*exbB* Δ*tonB* harboring plasmid pAP09, pAP06, or pAP52 were grown overnight at 37 °C in 200 mL of LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter). The overnight culture was used to inoculate 5 L of LB medium. The culture was incubated with aeration at 37 °C to an optical density at 600 nm of 0.8. Synthesis of ExbB or ExbB-ExbD was induced by adding 1 mM isopropyl *β*-D-thiogalactopyranoside (IPTG), and incubation was continued for 4 h at 30 °C. Expression of *tonB* on pAP52 was induced by addition of 0.02% arabinose at an optical density at 600 nm of 0.8 and incubation continued for 4 h. Cells were harvested by centrifugation, and the pellet was frozen at −80 °C until used. The cell pellet was suspended in 30 mL of HSG buffer (20 mM Hepes pH 7.3, 200 mM NaCl, 10% glycerol); 0.04% undecyl maltoside; 1 mM phenylmethanesulfonyl fluoride; and one vial of Protease-inhibitor Mix per 100 mL, as recommended by the supplier (Serva, Heidelberg). Cells were disrupted by three passages through a French pressure cell at 15000 psi. Intact cells were removed by centrifugation at 4000*g* for 10 min, and the membrane fraction was pelleted by centrifugation at 186000*g* for 60 min, washed with HSG supplemented with 0.5 M NaCl, and then suspended in 40 mL of HSG with 1% undecyl maltoside by incubating for 3 h at 4 °C. The insoluble membrane fraction was removed by centrifugation at 186000*g* for 60 min. The supernatant was loaded onto a 20 mL nickel−nitrilotriacetate agarose column equilibrated with HSG containing 0.04% undecyl maltoside. The column was washed with 40 mL of HSG/0.04% undecyl maltoside, and the bound protein was eluted with a gradient of 0−200 mM imidazole in HSG/0.04% undecyl maltoside. The fractions were monitored spectrophotometrically at 280 nm. The peak fractions were pooled and concentrated to 7 mL with Amicon 50 kDa filters;

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the concentrate was loaded onto a Superdex 200 (26/60) column equilibrated with HSG/0.04% undecyl maltoside and chromatographed with the same buffer. The collected fractions were concentrated and kept frozen at −80 °C until used. The ExbB-ExbD complex was purified by nickel−nitrilotriacetate agarose chromatography and subsequent chromatography on a Strep-Tactin Superflow cartridge H-PR 5 mL (IBA GmbH, Göttingen) via the StrepII tag at the C-terminus of ExbD. The protein sample was dissolved in HSG buffer containing 0.04% undecyl maltoside, adsorbed to the column, and washed. The bound proteins were eluted with HSG buffer containing 2 mM desthiobiotin (IBA GmbH).

Protein Electrophoresis. Proteins samples were examined under denaturing, reducing conditions by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE[21]). Native protein complexes were separated on 4−16% bis-Tris blue-native precast polyacrylamide gels (Invitrogen) by electrophoresis for 6 h at 200 V.[22,23]

Analytical Size-Exclusion Chromatography. Proteins were separated by analytical size-exclusion chromatography on GE Healthcare 10/300 GL columns prepacked with Superose 6 matrix. Proteins samples (200 μL) were loaded onto the column pre-equilibrated with HSG buffer containing 0.04% undecyl maltoside and were separated by eluting with one column volume (24 mL) of the same buffer.

Determination of Undecyl Maltoside Bound to ExbB. The amount of undecyl maltoside bound to ExbB was determined using the anthrone colorimetric method by which the maltoside headgroup is quantified, as described previously for protein bound decyl maltoside.[17]

Mass Spectrometry. LILBID-MS was carried out as described previously.[18,24] Briefly, microdroplets (diameter 50 μm) of protein solutions were produced at 10 Hz by a piezo-driven droplet generator and introduced into vacuum via differential pumping stages. Therein the droplets were irradiated one by one by synchronized high-power mid-infrared laser pulses of 5 ns pulse length which were generated in a home-built optical parametric oscillator (OPO) using a $LiNbO_3$ crystal and a Nd:YAG pump laser. The wavelength was adjusted to the absorption maximum of water at around 3 μm corresponding to the excitation of its stretching vibrations. At threshold intensity (some 100 MW/cm^2) the droplets are disrupted, resulting in the emission of ions from liquid into gas phase where they are mass analyzed in a time-of-flight (TOF) reflectron mass spectrometer. To detect large biomolecules a Daly-type ion detector was used working up to an m/z range in the low 10^6 region. At low laser intensities LILBID gently desorbs ions out of the liquid (soft mode) and is able to detect specific noncovalently assembled protein complexes. In this mode stoichiometries as in condensed phase have been observed. At higher laser intensities (moderate) the complexes are thermolyzed into subcomplexes and further into the covalent subunits (harsh mode). The analogue signals from the detector were recorded by a fast transient recorder. For data acquisition and analysis a user-written Labview program was used. The signal-to-noise ratio was improved by subtracting an unstructured background caused by metastable loss of water and buffer molecules from the original ion spectra. The differences in spectra were smoothened by averaging the signal over a preset number of channels of the transient recorder with the smoothing interval always lying within the time resolution of the TOF mass spectrometer. The spectra were calibrated with bovine serum albumin. ExbB and ExbB-ExbD (3 mg per mL) were analyzed in 20 mM NH_4HCO_3, 0.04% undecyl maltoside, pH 8. This buffer with 0.05% dodecyl maltoside was previously used for LILBID-MS of the mitochondrial TOM complex.[18]

Examination of the ExbB Amino Acid Sequence. The amino acid sequence of purified ExbB protein on SDS−polyacrylamide gels was examined by digesting the protein in situ with trypsin and analyzing the products using a Q-ToF instrument (maXis, Bruker Daltonics) coupled to a nanoLC-Ultra System von Eksigent (Guido Sauer, Max Planck Institute for Developmental Biology) and an LTQ-Orbitrap XL mass spectrometer (Mirita Franz-Wachtel, Proteome Center, University of Tübingen).

Western Blotting. Proteins from the membrane fraction or solubilized proteins were separated on SDS PAGE, followed by electroblotting on a nitrocellulose membrane (Whatman). After blocking with 1% bovine serum albumin blots were probed with anti-TonB serum raised in rabbits against the *E. coli* TonB periplasmic fragment. Following thorough wash, the immunoblots were treated with goat Anti-Rabbit-IgG coupled to alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc.). Immunoblots were then developed with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and visually inspected for TonB.

RESULTS

Isolation of ExbB and ExbB-ExbD. The *E. coli* C43 Δ*exbB* deletion mutant was transformed with the cloned genes *exbB* (pAP09) and *exbB exbD* (pAP06) to test whether ExbBD-dependent activities were restored. Transformants were tested for sensitivity to albomycin and colicin M which require the ExbBD functions for killing cells. ExbB and ExbB with a C-terminally fused His_6 tag did not increase the residual activity of the mutant (clear growth inhibition zone at a colicin M dilution titer of 2^3, no inhibition zone with undiluted albomycin as the untransformed mutant), whereas the *exbB exbD* transformants were fully active as wild type (colicin M and albomycin at dilutions of 2^8). The requirement for coexpression of ExbD was caused by a low level of residual ExbD activity due to a strong polar effect of the *exbB* deletion on the downstream *exbD* expression.

For the isolation of ExbB alone and ExbB and ExbD together *E. coli* C43 Δ*exbB* Δ*tonB* was transformed with plasmid pAP09 *exbB* or pAP06 *exbB exbD,* respectively. Transcription of the plasmid-encoded *exbB and exbB exbD* genes occurred by the T7 RNA polymerase under lactose control and was induced by IPTG.

ExbB was solubilized in undecyl maltoside and not as previously described in decyl maltoside[17] since after coexpression with ExbD constant stoichiometric amounts of ExbD were bound to ExbB which was not the case when decyl maltoside was used (data not shown). ExbB was purified by affinity chromatography on a nickel−nitrilotriacetate agarose column and subsequently by size-exclusion chromatography on Superdex 200. The same procedure was used for the isolation of the ExbB-ExbD complex, except that an additional Strep-Tactin affinity chromatography was performed after the nickel−nitrilotriacetate agarose chromatography, taking advantage of the C-terminal StrepII tag on ExbD. Regardless of whether ExbB-ExbD was first chromatographed on nickel−nitrilotriacetate agarose and then on Strep-Tactin or vice versa, the ExbB-ExbD complex was similar, as revealed by LILBID (see later).

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In buffer containing undecyl maltoside, both ExbB and the ExbB-ExbD complex were stable.

ExbB and the ExbB-ExbD complex each eluted as a single peak after analytical size-exclusion chromatography on a Superose 6 matrix (Figure 2). The ExbB-ExbD peak eluted

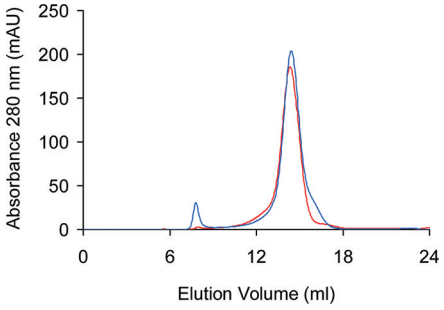

Figure 2. Size-exclusion chromatography of ExbB (blue line) and the ExbB-ExbD complex (red line) on a Superose 6 10/300 GL prepacked column (GE Healthcare). The protein complex was solubilized in 20 mM Hepes, 200 mM NaCl, 10% glycerol, 0.04% undecyl maltoside, pH 7.3. The elution was monitored spectrophotometrically at 280 nm.

slightly ahead of the ExbB peak. The small peak from the ExbB sample at the size-exclusion limit also formed when the main peak was rechromatographed after centrifugation at 18000*g* for 20 min. This peak was at the exclusion limit of Superose 6 (M_r 4×10^7) and thus represented aggregates of ExbB. The ExbB-ExbD preparation did not show spontaneous aggregate formation during chromatography. Both complexes have somewhat different solubility properties, as revealed also by their different behavior in decyl maltoside and undecyl maltoside. Comparison of the elution position of the ExbB-ExbD complex with the elution positions of standard proteins with known M_r value indicated a M_r of 253 000; the M_r value of ExbB was similarly determined to be 240 000 (Figure 3). These

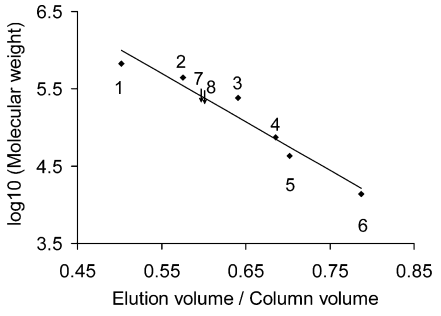

Figure 3. Estimation of the M_r of the ExbB complex and the ExbB-ExbD complex by size-exclusion chromatography. The proteins were from the peak fraction shown in Figure 2. Size standards: thyroglobulin (1, M_r 669 000), ferritin (2, M_r 440 000), catalase (3, M_r 240 000), conalbumin (4, M_r 75 000), egg albumin (5, M_r 43 000), and RNase A (6, M_r 14 000). Log molar mass was plotted against V_e/V_t (elution volume/total column volume). The arrows indicate the elution positions of the ExbB complex (arrow, 8) and the ExbB-ExbD complex (arrow, 7).

values correspond to 6 and 5.7 ExbB monomers in the complex when one takes into account the 35% (w/w) undecyl maltoside bound to ExbB and ExbB-ExbD and the M_r of ExbB with the His tag (27.3 kDa) and ExbD with the StrepII tag (16.4 kDa).

The peak fractions of ExbB and ExbB-ExbD (Figure 2) each yielded a single band after blue native gel electrophoresis

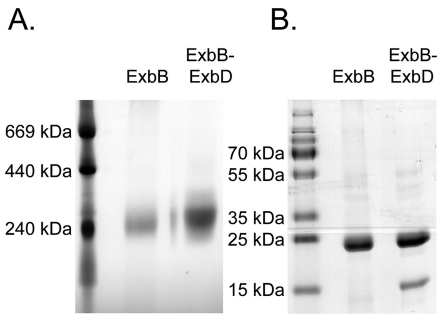

Figure 4. (A) Blue native gel electrophoresis of ExbB and the ExbB-ExbD complex solubilized in undecyl maltoside. (B) SDS-PAGE of purified ExbB and ExbB-ExbD after size-exclusion chromatography. The left lanes contain standard proteins. The proteins were stained with Coomassie blue.

(Figure 4A). After SDS-PAGE the ExbB-ExbD peak fraction yielded two Coomassie stained protein bands at the expected positions for ExbB and ExbD (Figure 4B). The ExbB band intensity was much stronger than the ExbD band intensity.

We attempted to isolate a TonB-ExbB-ExbD complex. The three proteins were synthesized in *E. coli* C43 Δ*exbB* Δ*tonB* transformed with plasmids pAP06 *exbB exbD* and pAP52 *tonB*. Transcription of the *exbB exbD* and *tonB* genes was induced by addition of IPTG and arabinose to the culture. A cocktail of protease inhibitors was added to prevent protein degradation. Under these conditions ExbB formed a strong band after electrophoresis of whole cells on SDS−polyacrylamide gels, but a TonB band could not be identified by staining (not shown) but was observed after immunoblotting with anti-TonB antiserum (not shown). Only a fraction of TonB was solubilized by undecyl maltoside, which solubilized the ExbB-ExbD complex. Therefore, we did not further attempt to isolate a TonB-ExbB-ExbD complex, and a strain with a chromosomal *exbB tonB* double deletion was used for purification of ExbB and the ExbB-ExbD complex to avoid any nonstoichiometric amounts of TonB in the samples.

Oligomeric Structure of ExbB and ExbB-ExbD. The oligomeric structure of ExbB and ExbB-ExbD was analyzed using LILBID-MS. The laser intensity was adjusted such that detergent molecules stripped off from the noncovalently bound protein complexes, which remained associated at low to moderate intensity and gradually dissociated at higher intensities. The spectra showed a set of peaks over the mass range of 0−171 000 m/z. These peaks could clearly be assigned to the ExbB and ExbD proteins and their complexes of various defined sizes. At moderate laser intensity, ExbB displayed a mass of 154.8 kDa, which nearly corresponds to a hexamer (B_6). The hexamers appeared in a distribution of negative charge states from −1 to −4 (Figure 5A). At higher laser intensities, the hexamers dissociated into $ExbB_3$, $ExbB_2$, and $ExbB_1$ (Figure 5B).

LILBID-MS of ExbB-ExbD at moderate laser intensities revealed a major $ExbB_6ExbD_1$ complex and a minor $ExbB_5ExbD_1$ complex (Figure 6A). At higher laser intensities, $ExbB_1$ and $ExbD_1$ were the major products, and traces of $ExbB_2$, $ExbB_3$, $ExbB_2ExbD_1$, and $ExbB_3ExbD_1$ were observed in the spectrum (Figure 6B). ExbD was firmly attached to ExbB and remained associated with ExbB even after dissociation of $ExbB_6$ into smaller fragments. These results indicated that the major products were $ExbB_6$ and $ExbB_6ExbD_1$, and at higher laser intensities, $ExbB_6$ and $ExbB_6ExbD_1$ dissociated into smaller

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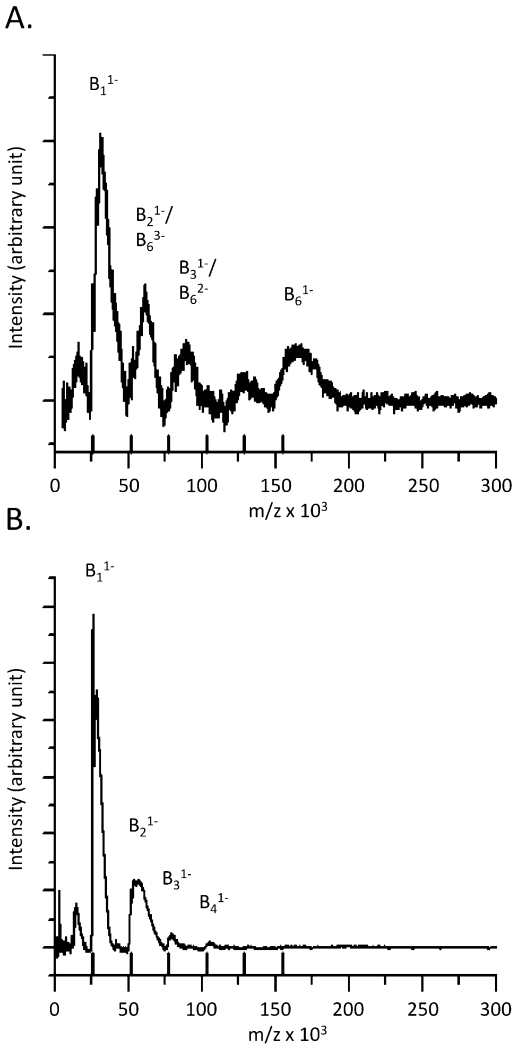

Figure 5. (A) LILBID-mass anion spectrum of purified ExbB at moderate laser intensity. The negatively charged molecules from −1 to −4 correspond to a size of 154.8 kDa. (B) LILBID-mass anion spectrum of purified ExbB using higher laser intensity. The black bars below the experimental trace indicate the theoretical mass for multiples of the ExbB mass from 1 to 6. Peak signals attributed to ExbB ions are denoted as B with oligomerization numbers as subscript and charges as superscript.

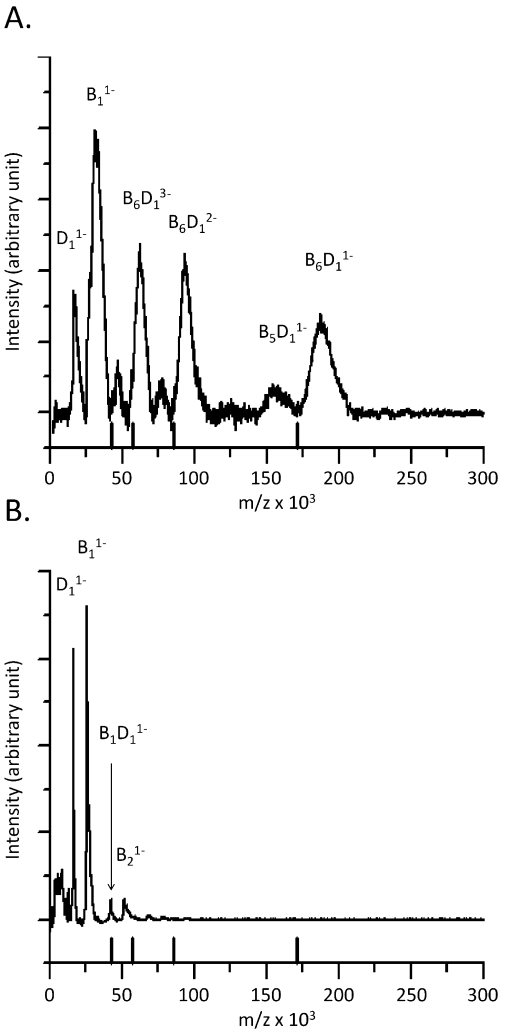

Figure 6. (A) LILBID-mass anion spectrum of purified ExbB-ExbD at moderate laser intensity. The negatively charged molecules from to −4 correspond to a size of 171.2 kDa ($ExbB_6ExbD_1$) and 145.4 kDa ($ExbB_5ExbD_1$). (B) LILBID-mass anion spectrum of purified ExbB-ExbD at higher laser intensity. The black bars below the experimental trace indicate the theoretical mass/charge ratio for $ExbB_6ExbD_1$ ions with charge −1 to −4. Peak signals attributed to ExbB and ExbD ions are denoted as B and D, respectively, with oligomerization numbers as subscript and charges as superscript.

fragments. Since chemical cross-linking identified ExbD dimers, we examined carefully our LILBID data for the presence of ExbD dimers. We observed no dimers even at laser intensities lower than those shown in Figure 6 at which more loosely bound ExbD should remain associated with ExbD or ExbB.

The size of the ExbD monomer determined by LILBID-MS corresponded to a calculated mass of 16.4 kDa. The ExbB monomer determined by LILBID-MS had a mass of 25.8 kDa, which was smaller than the expected 27.3 kDa deduced from the *exbB* nucleotide sequence and considering the C-terminal $(His)_6$-Thr-Gly tag. Accordingly, $ExbB_6$ was smaller (154.8 kDa) than the expected mass (163.8 kDa).

To determine the reasons for the size discrepancy of ExbB, we digested the ExbB protein with trypsin after SDS-PAGE and examined the resulting peptides by mass spectrometry. The observed peptides covered 35% of the *E. coli* ExbB sequence. Several mass spectrometric analyses with different ExbB samples revealed full-length ExbB only in low yields and higher yields of N-terminally truncated ExbB proteins starting at positions 7, 14, and 16. Apparently, ExbB was proteolytically degraded at the N-terminus despite the cocktail of protease inhibitors present throughout the entire purification procedure.

DISCUSSION

In this paper determination of the ExbB and ExbB ExbD oligomer structures rests on two conditions: (1) use of undecyl maltoside to cosolubilize ExbB with ExbD and (2) use of LILBID-MS which is not affected by detergents. We previously solubilized ExbB in decyl maltoside, isolated the protein, and determined its oligomeric structure.[17] The methods used in the former study (size-exclusion chromatography, small-angle X-ray scattering, electron microscopy, and blue native gel electrophoresis) measured the protein−detergent complex. To calculate the size of the ExbB oligomer, we determined the amount of detergent bound to ExbB by quantifying the maltoside group of the detergent in the equilibration buffer and

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in the protein sample after extensive dialysis. Per ExbB molecule, 38−44 decyl maltoside molecules were bound. Taking this and the limited accuracy of the methods into account, we calculated an oligomeric ExbB consisting of 4−6 subunits. Here we provide direct evidence for association between ExbB and ExbD.

In the present study, we tested both decyl maltoside and undecyl maltoside in their ability to solubilize the ExbB-ExbD complex. When decyl maltoside was used, the ExbB-ExbD complex was not stable. In contrast, with undecyl maltoside, the complex was stable during solubilization from the cytoplasmic membrane and subsequent purification by three column chromatographies.

We analyzed the oligomeric structure of the intact ExbB-ExbD complex and also that of ExbB using a more precise method, LILBID-MS, than used in our previous study. With this method, laser desorption of membrane proteins solubilized in detergent and solvated in microdroplets of adequate buffer strips off the detergent from the proteins in variable amounts depending on the applied laser intensity. At very low intensity residual detergent molecules stay attached, visible by an increased width of the mass peaks. At high laser intensity all detergent was stripped off and the protein complex disassembled into the monomeric proteins. Hence, the mass of the protein without detergent molecules is determined by the low mass onset of a mass peak which results in the true molar mass that does not need to be corrected for the amount of bound detergent. Furthermore, the laser energy for ablation can be adjusted such that dissociated subcomplexes are formed.

At moderate laser energy, ExbB formed a hexamer, consistent with the upper estimate of the oligomeric structure determined previously. At higher laser energies, the hexamer dissociated into trimers, dimers, and monomers. ExbB-ExbD at moderate laser energy formed an $ExbB_6ExbD_1$ oligomer and a minor amount of $ExbB_5ExbD_1$ oligomer. At higher laser energies, the $ExbB_1$ and $ExbD_1$ monomers were the dominant forms, and minor amounts of $ExbB_1ExbD_1$, $ExbB_2$, $ExbB_2ExbD_1$, $ExbB_3$, and $ExbB_3ExbD_1$ subcomplexes were observed. Upon dissociation of $ExbB_6ExbD_1$ into these smaller fragments, some ExbD remained associated with ExbB even down to the $ExbB_1ExbD_1$ complex. We conclude that the ExbB hexamer is the basic form to which one ExbD molecule is tightly bound. For the formation of the complete complex TonB and possibly an additional ExbD is added. Dimerization of ExbD has been demonstrated by chemical cross-linking of ExbD dimers in vivo[9] and by using NMR diffusion of dimers of an isolated periplasmic fragment (residues 44−141) of ExbD at pH 7 and low protein concentration (0.2 mM).[25] The NMR solution structure of ExbD monomers could only be determined at pH 3 because otherwise the isolated ExbD protein fragments aggregated strongly.[25] In the isolated ExbBD complex only one ExbD was found which does not exclude that a second ExbD exists which is more loosely bound and dissociates from the complex during isolation. It is feasible that ExbD bound to ExbB remains bound to ExbB and ExbD bound to ExbD is released. This would mean that the complex is asymmetric in that only one ExbD binds to ExbB, or the affinities of the two ExbD molecules to ExbB differ.

Our attempts to solubilize the intact ExbB-ExbD-TonB complex from the cytoplasmic membrane with the detergent undecyl maltoside failed possibly because TonB was not as firmly integrated into the complex as ExbB and ExbD. The amount of TonB associated with the complex may be estimated from the ratio of 7:2:1 for radiolabeled ExbB:ExbD:TonB in cell lysates.[7] To account for the observation of chemically cross-linked ExbD dimers and TonB dimers[9] and TonB dimers coupled to the cholera toxin activator,[10] the final functional complex in the cytoplasmic membrane may even be composed of $ExbB_{12}$ $ExbD_2$ $TonB_2$.

This study has been performed with the complete ExbB and ExbD proteins. Previous structural studies on ExbD and TonB used the soluble periplasmic parts of the proteins of which the N-terminal membrane anchor had been removed. However, the ExbB protein isolated in our current study was slightly truncated at the N-terminus, as compared to the deduced amino acid sequence of the *exbB* gene. Proteolysis was not prevented by addition of protease inhibitors to all steps of the purification procedure. In our previous study of the transmembrane topology of ExbB in the cytoplasmic membrane,[12] we showed that the N-terminal residues 1−15 of ExbB extend into the periplasm and are susceptible to proteolysis; aminopeptidase K truncated the 26.6 kDa ExbB in spheroplasts to 26 kDa. For the assembly of the isolated complex the N-terminus does not seem to play an important role.

E. coli contains another transport system that uses energy provided by the proton-motive force and functions similarly as ExbB-ExbD-TonB.[26] This Tol system is essential for the uptake of group A colicins and infection by single-stranded filamentous DNA bacteriophages and plays a role in the stability of the outer membrane and cell division. The Tol system consists of the proteins TolA, TolQ, TolR, TolB, and Pal.[26] TolQ and TolR exhibit sequence similarities to ExbB and ExbD, respectively,[27] have the same transmembrane topology as ExbB-ExbD,[28] and can partially replace the functions of ExbB and ExbD.[29] In contrast, TolA and TonB differ in sequence but an N-terminal membrane-anchored portion of TolA fused to an N-terminally truncated TonB yields a functional TonB.[30] Cross-linking and suppressor studies demonstrated interaction of the TolQRA proteins (summarized in ref 26). These studies led to a tentative TolQ-TolR-TolA stoichiometry of 4:2:1 to 6:2:1.[31,32] Recently, the solution structure of a TolR fragment (residues 59−130) of *Haemophilus influenzae* was determined by NMR. The hydrophobic N-terminus was deleted to improve solubility. The fragment formed a dimer.[33] Very recently, a putative $TolQ_6$ $TolR_2$ model was used to take into account identification of TolR dimers, chemically cross-linked TolQ multimers, and TolQ cysteine scanning data.[34] However, the authors point out that the data are not sufficient to draw firm conclusions on the stoichiometry of the complex. Because of the structural and functional similarities of the Ton and Tol dependent transport systems, we assume that the oligomeric structures of ExbB-ExbD-TonB and TolQ-TolR-TolA are similar.

AUTHOR INFORMATION

Corresponding Author

*Tel: +49-7071601342. Fax: +49-7071601349. E-mail: avijit.pramanik@tuebingen.mpg.de.

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■ ABBREVIATIONS

LILBID-MS, laser-induced liquid bead ion desorption mass spectrometry; ATP, adenosine triphosphate; PCR, polymerase chain reaction; His_6, hexahistidine; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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