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From Homodimer to Heterodimer and Back: Elucidating the TonB Energy Transduction Cycle

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ABSTRACT

The TonB system actively transports large, scarce, and important nutrients through outer membrane (OM) transporters of Gram-negative bacteria using the proton gradient of the cytoplasmic membrane (CM). In *Escherichia coli*, the CM proteins ExbB and ExbD harness and transfer proton motive force energy to the CM protein TonB, which spans the periplasmic space and cyclically binds OM transporters. TonB has two activity domains: the amino-terminal transmembrane domain with residue H20 and the periplasmic carboxy terminus, through which it binds to OM transporters. TonB is inactivated by all substitutions at residue H20 except H20N. Here, we show that while TonB trapped as a homodimer through its amino-terminal domain retained full activity, trapping TonB through its carboxy terminus inactivated it by preventing conformational changes needed for interaction with OM transporters. Surprisingly, inactive TonB H20A had little effect on homodimerization through the amino terminus and instead decreased TonB carboxy-terminal homodimer formation prior to reinitiation of an energy transduction cycle. That result suggested that the TonB carboxy terminus ultimately interacts with OM transporters as a monomer. Our findings also suggested the existence of a separate equimolar pool of ExbD homodimers that are not in contact with TonB. A model is proposed where interaction of TonB homodimers with ExbD homodimers initiates the energy transduction cycle, and, ultimately, the ExbD carboxy terminus modulates interactions of a monomeric TonB carboxy terminus with OM transporters. After TonB exchanges its interaction with ExbD for interaction with a transporter, ExbD homodimers undergo a separate cycle needed to re-energize them.

IMPORTANCE

Canonical mechanisms of active transport across cytoplasmic membranes employ ion gradients or hydrolysis of ATP for energy. Gram-negative bacterial outer membranes lack these resources. The TonB system embodies a novel means of active transport across the outer membrane for nutrients that are too large, too scarce, or too important for diffusion-limited transport. A proton gradient across the cytoplasmic membrane is converted by a multiprotein complex into mechanical energy that drives high-affinity active transport across the outer membrane. This system is also of interest since one of its uses in pathogenic bacteria is for competition with the host for the essential element iron. Understanding the mechanism of the TonB system will allow design of antibiotics targeting iron acquisition.

The ability to acquire iron is the basis of a tug-of-war between host and bacterial pathogen (1, 2). Successful pathogens can acquire iron from their hosts, and in the case of Gram-negative bacteria, that is usually mediated by the TonB system, thus making the understanding of its mechanism an imperative goal (3, 4) and an attractive target for antibiotic development (5).

In broad terms, the TonB system harnesses the proton motive force (PMF) of the cytoplasmic membrane (CM) to energize active transport across a largely unenergized outer membrane (OM). It is thus a means by which Gram-negative bacteria overcome the diffusion limitations imposed by the luxury of having a protective OM where most nutrients diffuse through porins (for a review, see reference 6). It is widespread among Gram-negative bacteria (7). A TonB system has one or more characteristic OM active transporters, each specific for one or a few transport substrates. *Escherichia coli* K-12, the model organism in these studies, has seven such TonB-gated transporters, with the current record-holder being *Bacteroides thetaiotaomicron*, which has over 120 predicted transporters (8). The transporters consist of a 22-stranded β-barrel whose lumen is occluded by an internal globular domain (see FepA in Fig. 1). The mechanism by which transport occurs and the specific role that PMF plays have remained mysterious.

The novel aspects of this nonclassical transport system are the requirement for integral membrane proteins in both the cytoplasmic and OM and the spatial displacement of the energy source from its point of utilization. The PMF of the CM is transduced to TonB-gated OM transporters by three integral CM proteins: TonB, ExbB, and ExbD (Fig. 1). *E. coli* TonB has two functional domains, an amino-terminal transmembrane domain (TMD; residues 12 to 32) required for its energization and a periplasmic carboxy-terminal domain from residue 150 to 239 that is essential

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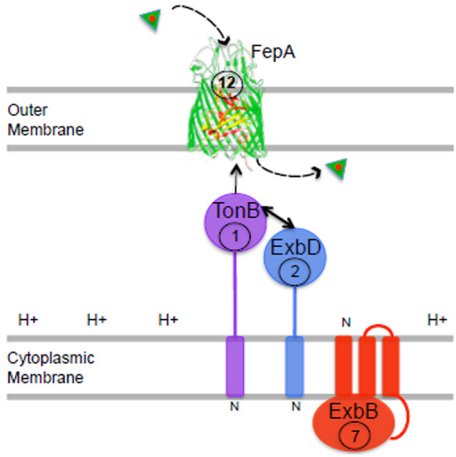


FIG 1 Known proteins in the TonB system. FepA is the OM transporter for the siderophore enterochelin (also called enterobactin) (triangles). Enterochelin is synthesized and excreted by *E. coli* to capture iron. TonB, ExbD, and ExbB are integral CM proteins that harness the PMF of the CM for the active transport of ferric enterochelin across the OM through FepA. Numbers in circles on each protein represent the per-cell ratios of the proteins under iron limiting conditions (12).

for energization of TonB-gated transporters by direct contact *in vivo* (9–11). TonB is the limiting protein relative to the transporters (12) (Fig. 1), and there is competition between transporters for energization by TonB (13). Thus, the interaction of TonB with TonB-gated transporters must be transient and cyclic *in vivo*, with TonB undergoing a "search" for ligand-loaded OM transporters (14, 15). Consistent with that idea, the TonB carboxy terminus goes through multiple conformational changes in the course of energizing an OM transport event (14). At least one segment of the energy transduction cycle involves formation of carboxy-terminal homodimers (16–18). Nonetheless, the role of homodimerization of the TonB carboxy terminus and where it fits into the energy transduction cycle are not understood. The TonB transmembrane domain also plays an important role in homodimerization *in vivo* (18).

Residue H20 in the TonB TMD is of particular importance because it is the sole residue in all of TonB whose deletion or replacement with anything except asparagine results in global loss of TonB activity, regardless of the assay used to detect it (17, 19, 20).

ExbD likewise has two functional domains (Fig. 1), an amino terminal TMD that contains an essential (B. Jana, M. Xie, and K. Postle, unpublished observations) and conserved D25 residue and a periplasmic carboxy-terminal domain that exists in both homodimeric and heterodimeric (with TonB) forms. ExbD employs energy derived from CM PMF to modulate the conformation of the TonB carboxy-terminal domain (21, 22) such that it productively contacts ligand-loaded TonB-gated transporters and enables active transport of nutrients into the periplasmic space.

ExbB serves as the scaffold upon which TonB and ExbD assemble (Fig. 1). It has three TMDs, with the majority of the protein located in the cytoplasm and is the only independently proteolytically stable protein of the three. Although PMF energizes TonB-dependent transport across the OM, ExbB TMDs play no direct role in proton translocation (23). Cytoplasmic ExbB sequences are important for signal transduction between the cytoplasm and the periplasmic interaction between TonB and ExbD (24, 25).

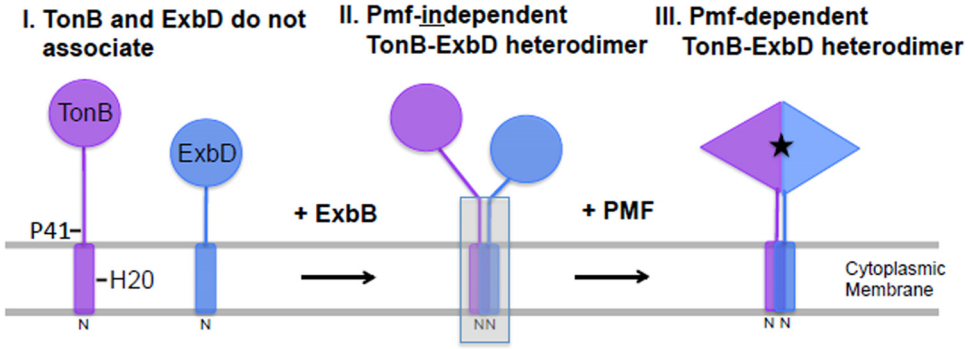
Three stages in the initial energization of TonB have been identified *in vivo* based on detection of a proteinase K-resistant form of TonB and its ability to formaldehyde cross-link to ExbD through their periplasmic domains. Figure 2 summarizes our knowledge prior to this study. In stage I, TonB and ExbD do not detectably interact (26). An H20A substitution in the TonB TMD inactivates TonB and leaves it stalled at or prior to stage I, defined as being sensitive to proteinase K in spheroplasts and unable to formaldehyde cross-link with ExbD. In stage II, through the mediation of ExbB, TonB and ExbD homodimers form a heteromultimeric complex where ExbD protects the amino-terminal two-thirds of TonB from digestion by exogenously added proteinase K. This stage is detectable when PMF has been collapsed by addition of protonophores or when the D25N mutation is present in the ExbD TMD. Consistent with that, PMF is essential for the transition from stage II to stage III, which is a rearrangement of the TonB-ExbD periplasmic domain interactions such that, for the first time in the energy transduction cycle, they can be cross-linked *in vivo* with formaldehyde. In stage III, the TonB conformation has once again become fully sensitive to proteinase K. In stage IV (not shown), the TonB carboxy terminus exchanges its interaction with ExbD for contact with a TonB-gated transporter. It is clear that while contact of TonB carboxy termini with OM transporters is necessary, it is not sufficient. Binding of purified TonB carboxy termini to purified transporters does not support active transport.

In this study, we investigated the dynamics of TonB oligomerization during an energy transduction cycle. We show that TonB moved through an energy transduction cycle homodimerized through its amino-terminal region and with a dynamic carboxy terminus that transited from obligatory homodimer to heterodimers with ExbD or OM transporters and back to an obligatory homodimer. The effect of the H20A mutation was to decrease obligatory homodimerization and hence prevent TonB activity. Combining previous results with the current study leads us to a model wherein the homodimeric TonB carboxy terminus and the homodimeric ExbD carboxy terminus come together to initiate an energy transduction cycle by forming the TonB-ExbD heterodimer required to configure the TonB carboxy terminus for productive interaction with a TonB-gated transporter. The model suggests that the TonB carboxy terminus transduces energy as a monomer, since H20 is required to re-form the carboxy-terminal homodimer prior to or at stage I. Application of this new cyclic model to the previously determined per-cell TonB-ExbD-ExbB ratios of 1:2:7 suggests that ExbD homodimers undergo corresponding cycles of depletion and re-energization as TonB goes through its energy transduction cycles.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this study are described in Table S1 in the supplemental material. DH5α was used as the host strain for constructed plasmids. The plasmids used in this study are listed in Table S1, with the details of their construction provided in the table footnote.

Media and culture conditions. Plasmid-bearing strains were maintained on Luria-Bertani (LB) broth or agar plates supplemented with 34 μg/ml chloramphenicol (27). T-broth was made as described previously, and M9 minimal salts were supplemented with 0.5% glycerol, 0.2% Casamino Acids (wt/vol), 40 μg/ml tryptophan, 0.4 μg/ml thiamine, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 1.85 μM $FeCl_3$ (28). L-Arabinose was added to induce TonB expression to approximate chromosomal levels. All cultures were grown aerobically at 37°C with 275 rpm shaking. Cells were



	Stage I	Stage II	Stage III
Proteinase K- resistant TonB fragment	-	+	-
TonB-ExbD formaldehyde crosslink ★	-	-	+

FIG 2 Model for early events in TonB energization, prior to the studies in this paper (adapted from reference 26 with permission of the publisher). In all stages, the oligomeric state of TonB is unknown. In stage I, ExbD forms homodimers through its periplasmic domain; however, dimerization through the TMD has not been characterized. There does not appear to be any contact between TonB and ExbD. In stage II, ExbB facilitates PMF-independent TonB-ExbD heterodimer formation through their periplasmic domains. This interaction renders TonB residues ~1 to 155 (covered by gray box) resistant to exogenously added proteinase K in spheroplasts (23, 26, 33). In the presence of PMF, the stage II TonB-ExbD heterodimer undergoes a conformational rearrangement such that the two periplasmic domains can now be formaldehyde cross-linked into the TonB-ExbD complex (32) that characterizes stage III. In this configuration, TonB is once again fully sensitive to proteinase K. Stage IV (not shown) is the interaction of the TonB carboxy terminus in an unknown oligomeric state with TonB-gated outer membrane transporters.

grown to mid-exponential phase to an A_{550} of 0.4 to 0.5 as measured on a Spectronic 20 spectrophotometer using a 1.5-cm path length.

Disulfide cross-linking of TonB Cys substitutions. All TonB Cys substitutions were coexpressed with a C18G substitution to prevent intramolecular cross-linking between the native TonB C18 and the engineered carboxy-terminal Cys residue (16). TonB Cys substitutions with a wild-type or H20A TMD were analyzed for disulfide cross-linking in KP1344 (W3110 Δ*tonB*). Stationary-phase cultures grown in T-broth supplemented with 34 μg/ml chloramphenicol were subcultured 1:100 into fresh T-broth supplemented with chloramphenicol and various concentrations of L-arabinose to induce TonB expression to near chromosomal levels. Cells at 0.2 A_{550}-ml were harvested (e.g., if A_{550} = 0.40, 0.2/0.4 = 500 μl cells) when the A_{550} of the culture reached 0.40 to 0.50, and proteins were precipitated with an equivalent volume of 20% trichloroacetic acid (TCA). An equivalent sample was harvested and TCA precipitated as a control for total protein levels. Although TCA was not previously used to harvest TonB triplet dimers (16), it was used here to facilitate equal recovery of bacterial samples. Addition of TCA did not artificially enhance triplet dimer formation compared to untreated samples (16) (data not shown). Triplet dimers are a set of disulfide-linked complexes that migrate on gels with three different apparent masses and are proposed to represent three different trapped transitional conformations of TonB that can form homodimers *in vivo*. Pellets were suspended in Laemmli sample buffer (LSB) (29) supplemented with 50 mM iodoacetamide (to analyze cross-linked species) or LSB with 2-mercaptoethanol (to analyze total protein levels) and incubated for 10 min at 95°C. Cross-links were detected as the presence of higher-molecular-weight complexes on immunoblots of 11% nonreducing SDS-polyacrylamide gels.

^{55}Fe-enterochelin transport. Initial rates of ^{55}Fe-enterochelin transport were determined in triplicate in the same manner as described previously for ferrichrome transport (28). Two samples of each culture were TCA precipitated immediately prior to assay to determine extent of disulfide-linked dimer formation (nonreducing samples) and total TonB expression levels (reducing samples). Cross-linked and un-cross-linked TonB was visualized on immunoblots of SDS-polyacrylamide gels with the anti-TonB monoclonal antibody 4H4 (30).

Proteinase K accessibility. Whole cells expressing chromosomally encoded or plasmid-encoded TonB derivatives were converted to intact spheroplasts and treated with or without 60 μM carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) for 5 min on ice and with or without proteinase K for 3 or 15 min as described previously (26). TCA-precipitated proteins were suspended in Laemmli sample buffer containing 50 mM iodoacetamide and incubated for 10 min at 95°C. Proteinase K-resistant forms associated with TonB F125C and P41C were visualized on immunoblots of nonreducing SDS-polyacrylamide gels with the anti-TonB monoclonal antibody 4H4.

***In vivo* formaldehyde cross-linking.** Cross-linking assays were performed as described previously (28). Cells were subcultured 1:100 in M9 minimal medium and grown to an A_{550} of 0.50. Cells were then resuspended in sodium phosphate buffer (pH 6.8), and paraformaldehyde was added to a final concentration of 1%. After incubation for 15 min at room temperature, cells were collected by centrifugation, immersed in Laemmli sample buffer containing 50 mM iodoacetamide (nonreducing samples) or 1% β-mercaptoethanol (reducing samples), and incubated for 5 min at 60°C. TonB-ExbD complexes were visualized on immunoblots of nonreducing SDS-polyacrylamide gels with anti-ExbD polyclonal antibodies. Steady-state levels of ExbD and TonB were analyzed on immunoblots of reducing samples with anti-ExbD or anti-TonB antibodies.

Sucrose density gradient fractionation. Strains expressing plasmid-encoded TonB derivatives were subcultured 1:100 into supplemented M9 with 34 μg/ml chloramphenicol and L-arabinose to achieve expression near chromosomal levels. Cells were grown to an A_{550} of 0.50, pelleted by centrifugation, and suspended in 10 mM HEPES buffer, pH 7.8. Concentrated cells were then passed through a French press at 20,000 lb/in^2 three times (or until a clear lysate was obtained). Samples were then loaded onto a sucrose gradient consisting of 25%, 30%, 35%, 40%, 45%, 50%, and 56% sucrose supplemented with 50 mM iodoacetamide and centrifuged at 35,000 rpm for 15 h. Fractions were collected, and a portion of each

fraction was TCA precipitated. Proteins were visualized on immunoblots of nonreducing SDS-polyacrylamide gels with anti-TonB antibodies. Immunoblots were also probed with anti-ExbB and/or anti-CorA antibodies as markers for CM fractions; stained membranes served to identify OM fractions (31).

RESULTS

TonB homodimerized through its amino terminus retains activity during the energy transduction cycle. We wanted to determine whether Cys substitutions at residues near the TonB amino terminus could form homodimers. To answer that question, we engineered a P41C substitution in the amino terminus near the periplasmic side of the TMD (Fig. 2). Because the majority of TonB P41C (~70 to 80%) was present in the homodimer form on nonreducing gels (Fig. 3A), it was possible to determine if TonB was active as a homodimer tethered through the amino terminus. If the level of iron transport activity observed could be achieved only by the combined steady-state levels of monomer plus homodimer, then the homodimeric form must be active. We know that select Cys substitutions within TonB carboxy-terminal residues 186 to 230 form homodimers, but since the levels of homodimer formation are relatively low, we do not know if the trapped homodimers are active or inactive (17).

We expressed TonB P41C to approximately the same chromosomal level seen for W3110 (Fig. 3A, bottom), where for purposes of comparison, the monomeric and homodimeric forms were collapsed into a single band on the reducing gel. The level of iron transport activity observed with wild-type TonB was proportional to its level of expression (Fig. 3, lanes WT A, WT B, and WT C). TonB P41C could support a level of iron transport that was the same as or slightly higher than that of W3110 or wild-type plasmid-encoded TonB (Fig. 3B), even though most of TonB P41C was present *in vivo* as a trapped homodimer, visible on a nonreducing gel (Fig. 3A, top; compare monomer levels for strain Chrom [W3110 expressing chromosomally encoded TonB] to those of WT B and the P41C mutant). If the TonB P41C monomer was the sole source of iron transport activity, the transport rate supported by TonB P41C monomer should be ~20 to 30% of the wild-type rate. Instead, TonB P41C supported ~100% of the wild-type rate (Fig. 3B; compare P41C to Chrom and WT B), indicating that the TonB P41C homodimers must have contributed to the iron transport activity as well as the monomers and suggesting that the homodimers supported stages I to IV of TonB activity.

As a further test of that idea, TonB P41C homodimers should be capable of progressing to stage II (Fig. 2), characterized by the production of a proteinase K-resistant TonB fragment when spheroplasts are exposed to proteinase K after the PMF has been collapsed by addition of protonophores (14, 26). TonB P41C homodimers formed a proteinase K-resistant fragment in the presence of CCCP of ~35 kDa (see Fig. S1 in the supplemental material). Thus, like wild-type TonB, the P41C homodimers were capable of interacting with ExbD in a PMF-independent fashion.

The TonB P41C homodimers were also able to proceed to stage III, which is characterized by formaldehyde cross-linking between the periplasmic domains of TonB and ExbD *in vivo* (32). A formaldehyde cross-link between TonB P41C homodimers and ExbD was faintly visible (see Fig. S2 in the supplemental material). Taken together, the results of the enterochelin transport, proteinase K, and formaldehyde cross-linking assays indicated that the periplasmic region near the TMD of TonB remained in a homodimerized state throughout a complete energy transduction cycle, which would include binding and release of TonB-gated transporters. It should be noted that a lack of complexes representing higher oligomers does not mean they do not exist at undetectable levels.

By the same reasoning, the presence of TonB F125C homodimers did not decrease the level of ^{55}Fe-enterochelin transport (Fig. 3B). Although the proportion of homodimerized TonB F125C was less than that of TonB P41C, it constituted a sufficient

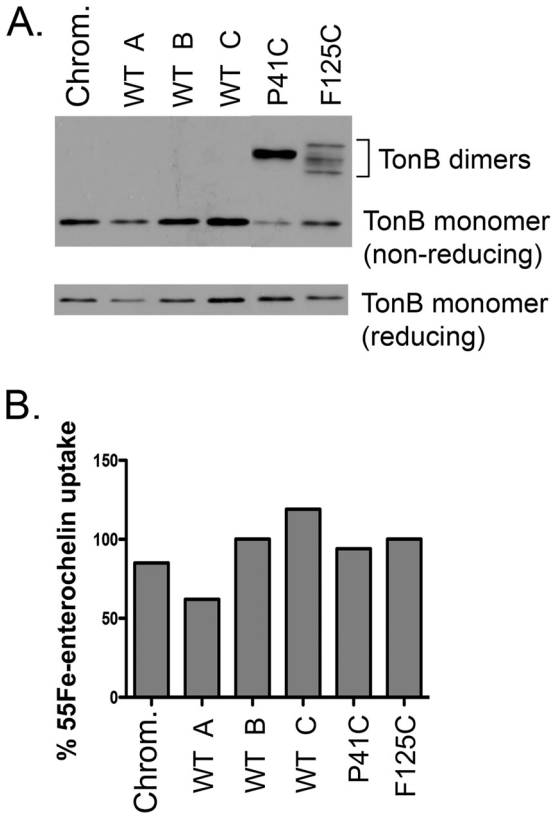


FIG 3 TonB homodimers formed through P41C and F125C are active. ^{55}Fe-enterochelin transport assays were performed as described in Materials and Methods with the following strains: Chrom (W3110 expressing chromosomally encoded TonB), WT (KP1344/pKP568, plasmid-encoded wild-type TonB), the P41C strain (KP1344/pKP1785), and the F125C strain (KP1344/pKP1070). All strains were assayed in triplicate within a given experiment. All plasmid-encoded TonB derivatives, including WT, contain a C18G substitution, which does not affect TonB activity. L-Arabinose was added to induce expression of plasmid-encoded TonB as follows: 0.0004% for WT A, 0.0006% for WT B, 0.0008% for WT C, 0.0006% for the P41C strain, and 0.0008% for the F125C strain. (A) Steady-state levels of TonB in the assayed cultures were determined on immunoblots of 11% nonreducing SDS-polyacrylamide gels with the anti-TonB monoclonal antibody 4H4. Positions of monomeric and dimeric forms of TonB are indicated on the right (top). Steady-state levels of TonB proteins from the same samples were also evaluated on immunoblots of 11% reducing SDS-polyacrylamide gels with the anti-TonB monoclonal antibody 4H4 (bottom). (B) Initial enterochelin uptake rates are reported as the percent activity relative to plasmid-encoded wild-type TonB (WT B), because its expression most nearly matched that of chromosomal TonB, and initial rates shown for P41C are the averages from two independent experiments ranging from 165 ± 16 to 202 ± 24 cpm/0.05 A_{550}-ml cells/minute. The initial rates of transport (in the same units) for the other strains are 166 ± 11 for W3110, 120 ± 22 for WT A, 195 ± 10 for WT B, 233 ± 9 for WT C, and 193 ± 11 for F125C.

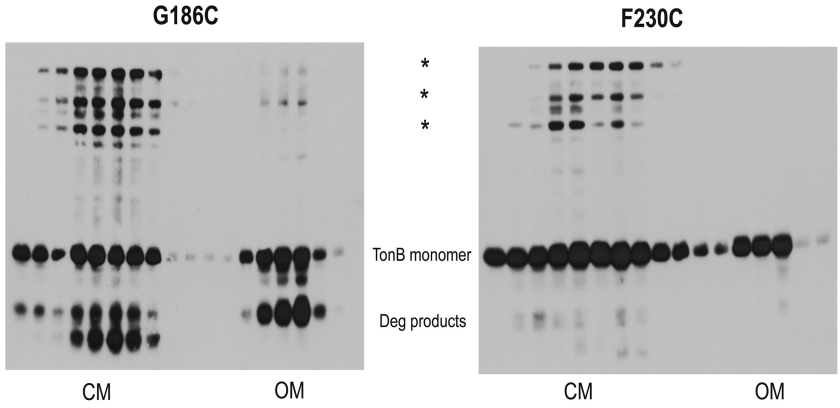


FIG 4 Disulfide-linked triplet dimers formed through G186C and F230C exhibit little or no association with the outer membrane. KP1344 cells expressing TonB C18G F230C (pKP570) and TonB C18G G186C (pKP612) were fractionated as described in Materials and Methods. Disulfide-linked triplet dimers (*) were visualized on immunoblots of nonreducing SDS-polyacrylamide gels with the TonB monoclonal antibody 4F1. SP, soluble proteins; CM, cytoplasmic membrane; OM, outer membrane.

proportion to suggest that F125C homodimers might also be active (Fig. 3A, compare monomer levels in lanes WT B and F125C).

TonB F125C was also capable of forming the proteinase K-resistant fragment (see Fig. S3 in the supplemental material). Unlike P41C, which forms a high proportion of homodimer relative to monomer (Fig. 3A), TonB F125C homodimers were not sufficiently abundant to allow detection of a formaldehyde cross-link between the F125C homodimers and ExbD (see Fig. S2 in the supplemental material). However, it is likely that the trapped TonB F125C homodimers were functional throughout the energy transduction cycle, which was supported by their ability to fractionate with the OM in sucrose density gradient fractionations as described below.

The finding that TonB retains the ability to move through its energy transduction cycle as an amino-terminal homodimer completely changed our thinking about the energy transduction cycle. All the data on ExbD or its paralogues TolR and MotB have shown them to form homodimers *in vivo* (33–35). The cellular ratio in the TonB system is 1:2:7 for TonB-ExbD-ExbB (12). As long as TonB functioned as a monomer in some stages, these ratios made sense. The finding that TonB P41C retained full activity as an amino-terminal homodimer throughout the energy transduction cycle meant that there were 2 ExbD homodimers for every 1 TonB homodimer. Thus, a separate pool of ExbD homodimers existed that was not associated with TonB homodimers. A model that addresses a potential role of that pool of ExbD homodimers is presented in Discussion.

TonB homodimerized through its carboxy terminus is inactive because required conformational change is prevented. TonB remains associated with the CM throughout its energy transduction cycle in whole cells (36). However, when wild-type cells are lysed by French press and fractionated on sucrose density gradients, approximately 1/3 of the full-length TonB is found in OM fractions. The OM-associated TonB represents a species that is so tightly bound (most likely to TonB-gated transporters in stage IV) that it is pulled away from its CM association with ExbB/D at the moment when the cells are lysed.

The TonB carboxy-terminal residues that interact with TonB-gated transporters span the region from ~150 to 239. Of 90 Cys substitutions in that region, substitutions at 5 residues—G186C, F202C, W213C, Y215C, and F230C—form disulfide-linked homodimers. Outside that region, TonB F125C also forms disulfide-linked homodimers (17). Homodimerization through these 6 residues is evident as a set of disulfide-linked complexes that migrate on gels with three different apparent masses, referred to here as triplet dimers. The three homodimeric complexes are proposed to represent three different trapped transitional conformations of TonB that can form homodimers but do not ultimately represent the form of TonB that binds to transporters (16, 17).

In a previous study, triplet homodimers of TonB F202C, TonB W213C, and TonB Y215C were unable to associate with the OM, suggesting that they were inactive (16). That inactivity could result from two possible causes. In one case, residues F202, W213, and Y215 are located within the boundaries of a putative amphipathic helix (residues 199 to 216) that may be required for binding to TonB-gated transporters (37–39). If so, homodimer formation through Cys residues in the amphipathic helix could distort it sufficiently that it no longer recognizes OM transporters in stage IV. Alternatively, trapping the TonB carboxy terminus in homodimeric form in stage I could prevent more global conformational changes subsequently required for productive binding to OM transporters.

To discriminate between those two possibilities, strains expressing TonB G186C and TonB F230C, located outside the amphipathic helix, were fractionated on sucrose density gradients, and localization of homodimers was evaluated on immunoblots of nonreducing SDS-polyacrylamide gels (Fig. 4). Like the previously observed triplet dimers formed by TonB F202C, TonB W213C, and TonB Y215C, those formed by TonB F230C were exclusively confined to CM fractions. When the disulfide bonds were formed through TonB G186C, TonB triplet dimers were similarly almost exclusively confined to the CM. The monomeric forms in each Cys substitution fractionated like wild-type TonB between cytoplasmic and outer membranes. This ruled out the possibility that the Cys substitutions themselves blocked OM association. These data suggested that when the TonB homodimers were trapped through carboxy-terminal disulfide bond substitutions, TonB was inactivated and ultimately unable to associate with the OM in stage IV.

In similar fractionation experiments, a portion of the TonB

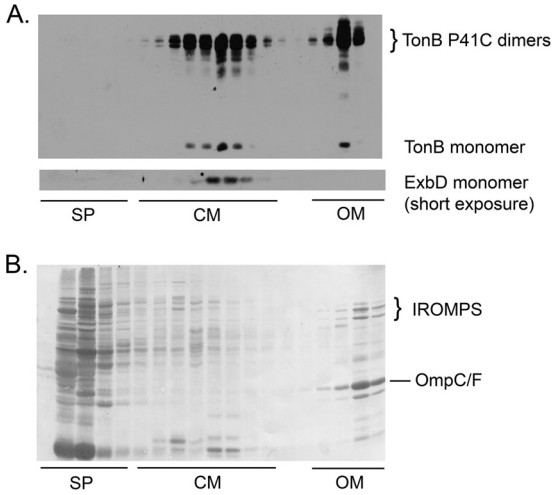


FIG 5 Disulfide-linked dimers formed through P41C associate with the outer membrane. (A) Strain KP1344 expressing TonB P41C was fractionated as described in Materials and Methods. Disulfide-linked dimers were visualized on immunoblots of nonreducing SDS-polyacrylamide gels with the monoclonal antibody 4H4. ExbD was a marker for cytoplasmic membrane fractions on immunoblots with anti-ExbD antibodies. (B) A Coomassie-stained polyvinylidene difluoride membrane is provided to show that the iron-regulated outer membrane proteins (IROMPS) and OmpC and -F were present in the outer membrane fractions.

P41C triplet homodimers (Fig. 5) and TonB F125C triplet homodimers (see Fig. S4 in the supplemental material) still associated with the OM, consistent with the other data showing that they were active. The results also indicated that homodimerization *per se* did not prevent fractionation with the OM. When combined, the results demonstrated that TonB activity was unaffected when amino termini were trapped as homodimers but that TonB activity was prevented when carboxy termini were similarly trapped. Thus, TonB can be divided into two domains: the amino terminus, which can remain associated as a homodimer throughout an energy transduction cycle, and the carboxy terminus, which homodimerizes and also undergoes conformational changes during energy transduction.

TMD residue H20 is not important for homodimerization through the amino terminus. To better understand the role of the TonB TMD, we focused our attention on residue H20. The H20A mutation stalls TonB prior to or at stage I (Fig. 2) (26). Because TonB trapped near its TMD by P41C disulfide cross-links was active, we hypothesized that TMD residue H20 would be important for homodimerization through that residue. If that was true, then preventing homodimerization of the TMD could be the key to the inactivity of TonB H20A. The possibility that residue H20 plays a direct role in proton translocation had been ruled out previously (20).

The H20A substitution was combined with P41C, and the resultant TonB double substitution was expressed to near chromosomal levels and assayed for the ability to form homodimers on immunoblots of nonreducing SDS-polyacrylamide gels compared to TonB P41C in the presence of wild-type H20. TonB P41C was resolved into dimers with three slightly different apparent masses that were reminiscent of triplet homodimers at the carboxy terminus (Fig. 6). In the presence of the H20A mutation, the overall level of TonB P41C homodimerization was similar to that of the wild type, but with the lower-mass complex decreasing and a higher-mass complex increasing (Fig. 6). Contrary to our expectations, these results suggested that, even though the H20A mutation inactivated TonB P41C (data not shown), it did not prevent homodimerization at the amino terminus, and we should examine homodimer formation through the carboxy terminus.

TMD residue H20 is important for TonB triplet dimer formation through the carboxy terminus. To test whether H20A affected formation of triplet homodimers through the TonB carboxy terminus, it was combined with TonB F125C and the set of 90 previously characterized carboxy-terminal Cys substitutions from residues 150 to 239 (17). All 91 Cys substitutions combined with H20A were expressed to near chromosomal levels and assayed for the ability to form homodimers on immunoblots of nonreducing SDS-polyacrylamide gels compared to the Cys substitutions when wild-type H20 was present. In contrast to results with P41C, the H20A substitution significantly diminished each member of the triplet homodimers arising from the six Cys substitutions (F125C, G186C, F202C, W213C, Y215C, and F230C) that previously formed triplet dimers most efficiently (17) (Fig. 6). Data for TonB G186C typify the triplet homodimers that were seen for F125C, F202C, W213C, Y215C, and F230C on longer exposures, with the top complex being most abundant and the bottom complex being least abundant. In Fig. 6, TonB Y163C and TonB F180C show typical low levels of triplet dimer formation observed for about half of the remaining 85 Cys substitutions, for which homodimers could be detected upon lengthy development of the immunoblot (see Fig. S5 in the supplemental material). Importantly, this low level of homodimerization was also decreased by the presence of the H20A mutation (see Fig. S5 in the supplemental material). The rest of the Cys substitutions exhibited no detectable homodimerization.

These results suggested that the H20A mutation did not simply cause conformational changes that prevented a few residues from interacting but rather uniformly decreased homodimerization of the entire TonB carboxy terminus. Because the H20A mutation prevents TonB from progressing to stage II (26), it suggested that the TonB carboxy-terminal homodimerization occurred prior to or in stage I and was essential for energy transduction. The effect of H20A was clearly an indirect one, suggesting that H20 in the TMD was somehow important for signals to be transmitted that allowed homodimerization of the TonB carboxy terminus. TonB H20A can still formaldehyde cross-link to ExbB and, like wild-type TonB, is unstable in a ΔExbB strain (20, 32). The H20A mutation also inhibited the ability of ToxR-TonB to dimerize and induce expression from a *ctx*::*lacZ* fusion (see Fig. S6 in the supplemental material), suggesting that the ability of ToxR-TonB to form homodimers was largely mediated through the TonB carboxy terminus. A potential reason for the residual low-level dimerization of ToxR-TonB H20A is that the TonB carboxy terminus can dimerize by itself (18).

Two out of three TonB carboxy-terminal triplet homodimers depend on ExbD for formation. The triplet dimers formed by TonB could represent different conformations that are formed through the TonB carboxy terminus (17). If this is the case, based on the data for initial stages in TonB energization (Fig. 2), the absence of ExbD should affect the triplet homodimers differentially. The absence of ExbD should not interfere with TonB homodimerization prior to or in stage I, whereas it may be involved in stage II. ExbD homodimerizes

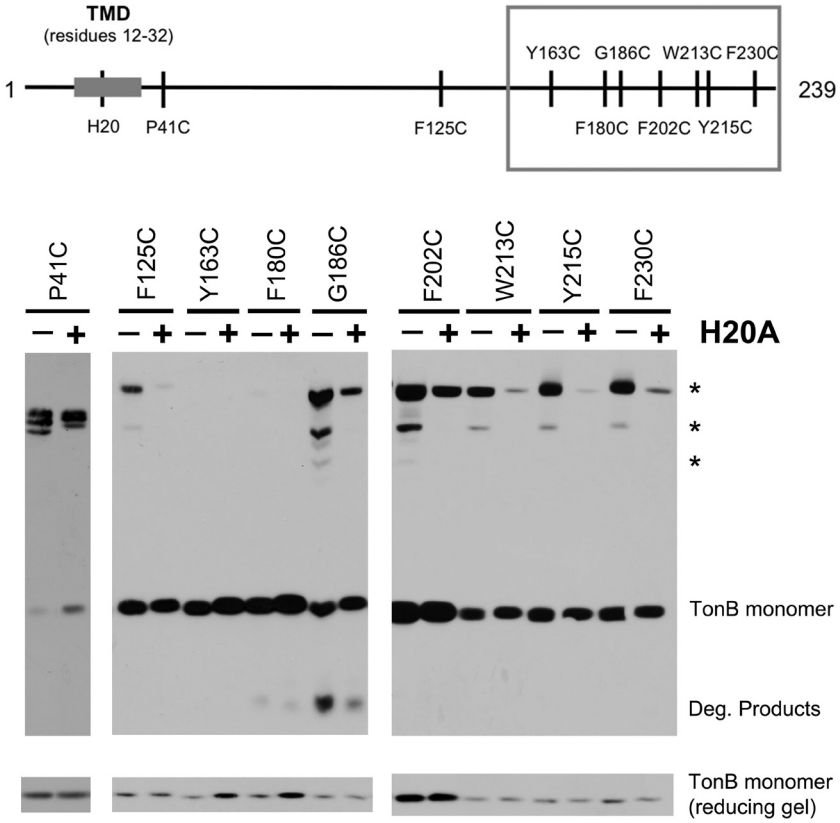


FIG 6 TonB H20A reduces cross-linking of TonB Cys substitutions in the carboxy terminus but not the amino terminus. A schematic of the TonB primary amino acid sequence is shown at the top. Positions of the transmembrane domain (TMD), residue H20, the carboxy terminus (boxed residues 150 to 239), and locations of the diagnostic Cys substitutions are labeled. The majority of the TonB protein, residues 33 to 239, is located in the periplasm. Disulfide cross-linking was performed as described in Materials and Methods. cTonB, chromosomal TonB (W3110/pKP477). Plasmid-encoded Cys substitutions were combined with a wild-type (−) TMD (17) or H20A (+) TMD (this study) and expressed in KP1344 (W3110 Δ*tonB*) with L-arabinose to induce expression of TonB derivatives as follows: P41C (0.0004%), H20A P41C (0.00035%), F125C (0.00045%), H20A F125C (0.0005%), Y163C (0.0004%), H20A Y163C (0.00056%), F180C (0.0005%), H20A F180C (0.00055%), G186C (0.00075%), H20A G186C (0.0008%), F202C (0.00045%), H20A F202C (0.00055%), W213C (0.00036%), H20A W213C (0.00055%), Y215C (0.00045%), H20A Y215C (0.0005%), F230C (0.0005%), and H20A F230C (0.0006%). Samples were divided and suspended in either nonreducing or reducing Laemmli sample buffer (LSB), loaded onto separate 11% SDS-polyacrylamide gels, and probed with the anti-TonB monoclonal antibody 4H4 (30). The figure is a composite of samples from three separate experiments (divided by spaces), and each sample was expressed to near chromosomal levels. However, in some cases, it was necessary to dilute overexpressed samples prior to loading so that the total amounts of TonB monomer in the reducing gels were approximately equal for all samples. In all cases, the same proportion of the H20 and the corresponding H20A variant samples were loaded on the gels. Disulfide-cross-linked triplet dimers are labeled with asterisks. Deg. Products, endogenous proteolysis degradation products.

($ExbD_2$) through carboxy-terminal residues and forms heterodimers with TonB through a nearly identical set of residues, suggesting that one ExbD carboxy terminus swaps its homodimeric partner to bind a TonB at a site(s) within the region of residues 150 to 239 (33).

To test whether $ExbD_2$ was required for the formation of any TonB triplet homodimers, the ability of TonB Cys substitutions to homodimerize was assessed in the Δ*tonB* Δ*exbD* Δ*tolQRA* strain KP1509. The *tolQR* genes were deleted because TolQR can contribute up to 10% of TonB activity in the absence of ExbD. We then compared the degree of homodimer formation under conditions where steady-state levels of TonB on reducing gels were similar (Fig. 7).

For the carboxy-terminal substitutions F125C, G186C, F202C, W213C, Y215C, and F230C, the abundance of the homodimers with the highest apparent mass remained largely unaffected by the absence of ExbD, suggesting that they formed prior to or in stage I, where there is no interaction with ExbD (Fig. 2). As expected, the absence of ExbD did not influence formation of the P41C homodimers (Fig. 7). They probably formed as soon as they were exported to the CM and assembled onto the ExbB scaffold (40). In contrast, the abundance of the two homodimers of lower apparent mass was diminished for each of the carboxy-terminal Cys substitutions (Fig. 7) (this was also evident on longer exposures [data not shown]), suggesting that ExbD played a role in their formation and that they thus occurred in stage II. These results support our previous suggestion that the triplet homodimers represent three different conformations of the carboxy-terminal TonB homodimer (17). The data further suggest that they reflect interactions in at least two different stages in the energy transduction cycle. The difference in abundance of the triplet homodimers was also consistent with the direction of the energy transduction cycle. If the most abundant of the homodimers forms prior to or in stage I, it leaves a smaller proportion that can form the homodimers with lower apparent masses in stage II.

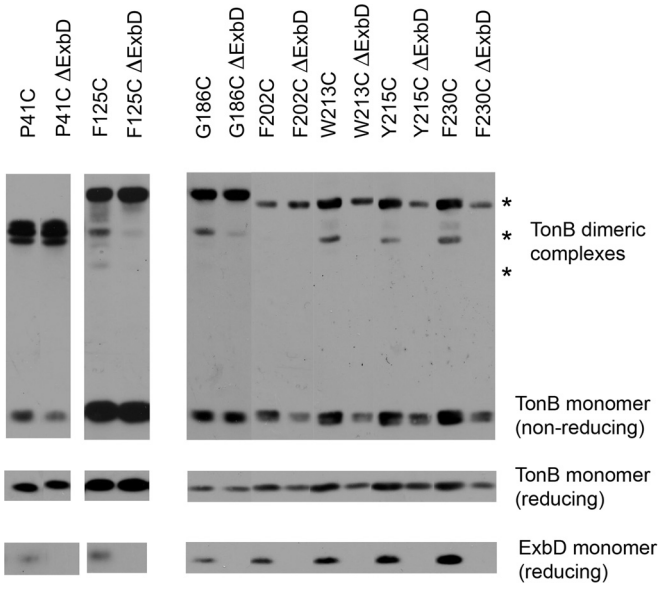


FIG 7 Deletion of ExbD does not prevent dimer formation through TonB P41C or F125C. Disulfide cross-linking was performed in T-broth as described in Materials and Methods. Cys substitutions were expressed in strains with (KP1344) or without (KP1509) ExbD. L-Arabinose was added to induce expression of substitutions as follows: KP1344 P41C (0.0004%), KP1509 P41C (0.0005%), KP1344 F125C (0.00045), KP1509 F125C (0.0006), KP1344 G186C (0.0007%), KP1509 G186C (0.0007%), KP1344 F202C (0.00055%), KP1509 F202C (0.00045%), KP1344 W213C (0.0005%), KP1509 W213C (0.0004%), KP1344 Y215C (0.00055%), KP1509 Y215C (0.00045%), KP1344 F230C (0.00055%), and KP1509 F230C (0.00045%). All samples were expressed to near chromosomal levels. Triplet homodimers (*) were visualized as higher-molecular-mass complexes on anti-TonB immunoblots of 11% nonreducing SDS-polyacrylamide gels with the monoclonal antibody 4H4. An equivalent sample from each strain was suspended in gel sample buffer containing the reductant 2-mercaptoethanol for determination of steady-state levels of TonB (middle) and ExbD (bottom; polyclonal anti-ExbD antibodies). The figure is a composite of three separate immunoblots, but each pair of wild-type ExbD-ΔExbD comparisons was from the same immunoblot.

DISCUSSION

The results in this paper allow us to propose a complete TonB-dependent energy transduction cycle. The model rests upon our earlier identification of three stages in the initial energization of TonB (Fig. 2). Our findings in this study allow us to incorporate for the first time the long-standing and puzzling cellular TonB-ExbD-ExbB ratios of 1:2:7, which have largely been ignored, and to understand the roles of residue H20 in the TonB TMD and of TonB carboxy-terminal homodimerization, and they support the idea that ExbD homodimers move in and out of association with TonB based on a need to be energetically replenished.

Discrepancies between cellular ratios and *in vitro* complex compositions provide important insights. ExbB and ExbD exist with two sets of paralogues, TolQ and TolR and MotA and MotB in *E. coli* K-12. TolQ and TolR are required for OM integrity (41) and cell division (42). MotA and MotB are stators in the flagellar motor (43, 44). Specific residues in the last two TMDs of ExbB/TolQ/MotA and the essential Asp in the single TMD of ExbD/TolR/MotB proteins are highly conserved (23, 45, 46). All three protein complexes use PMF as an energy source. In addition, ExbB-ExbD and TolQ-TolR functionally substitute for each other to some extent in energizing TonB and TolA (a TonB paralogue), respectively (47–49). Based on these similarities, they likely have similar mechanisms and can be considered a group. MotA has an additional TMD at its amino terminus that may compensate for the absence of corresponding TonB and TolA proteins in the flagellar stator (50). The *in vivo* cellular ratios of both ExbB-ExbD-TonB and TolQ-TolR-TolA are 7:2:1 (12, 51), and the ratio of MotA to MotB is nearly the same, at 4:1 (43), suggesting that these ratios have mechanistic implications.

However, the cellular ExbB-ExbD-TonB ratios seen *in vivo* are at odds with an abundance of *in vitro* data, suggesting that the basic composition of complexes of ExbB and ExbD or its paralogues consist of 4 ExbB and 2 ExbD, giving a ratio of 2:1, not 7:2 (35, 52, 53). Consistent with the 4:2 composition of an *in vitro* ExbB-ExbD complex, ExbB formaldehyde cross-links *in vivo* as a dimer of homodimers to yield $ExbB_4$ (24), and the carboxy terminus of ExbD cross-links as a homodimer, $ExbD_2$ (32). Based on these observations, we propose that the tetramer of ExbB is the basic and unchanging scaffold on which TonB or ExbD assemble (24, 52, 54, 55). A composition of 6 ExbB to 1 ExbD *in vitro* has been proposed by another group, although it was not clear if that complex ultimately represents a functional scaffold (56).

In this study, we showed that TonB can remain homodimerized throughout the energy transduction cycle, suggesting that the quantitative relationship that must be considered is a TonB-ExbD-ExbB ratio of 2:4:14. Based on that relationship, the likely existence of ExbB as a tetramer, and our previous observation that ExbD also forms carboxy-terminal homodimers, a single putative complex of $TonB_2$-$ExbD_2$-$ExbB_4$ would leave 2 ExbD and 10 ExbB unaccounted for. These apparent and unbalanced excesses of ExbD and ExbB relative to TonB, combined with our current and previous results, are accounted for in the following model.

Model for the TonB energy transduction cycle. (i) Separate $TonB_2$-$ExbB_4$ and $ExbD_2$-$ExbB_4$, complexes. Stage I, where wild-type TonB is homodimerized through both its amino and carboxy termini, is defined by a lack of interaction between TonB and ExbD, since none has been detected, and the inactive ExbD mutant ExbD D25N has no effect on TonB in stage I, whereas the D25N mutation stalls TonB in stage II, where TonB-ExbD interactions are first detected (26). We propose that, in stage I, $TonB_2$ and $ExbD_2$ are therefore each found in independent complexes with $ExbB_4$ (Fig. 8). Consistent with that, absence of TonB does not prevent ExbD carboxy-terminal homodimerization, and the absence of ExbD does not prevent TonB homodimerization through its amino terminus (this work and references 26, 32, and 33). ExbB is required for the proteolytic stability of both TonB and ExbD, suggesting that each is always in complex with $ExbB_4$ (57). In addition, overexpression of ExbB prevents formation of the stage III formaldehyde-cross-linked TonB-ExbD heterodimer, potentially by favoring formation of the interacting partners $TonB_2$-$ExbB_4$ and $ExbD_2$-$ExbB_4$ (A. Ollis and K. Postle, unpublished data). The existence of separate $TonB_2$-$ExbB_4$ and $ExbD_2$-$ExbB_4$ complexes in stage I accounts for 8 ExbB total and thus leaves 2 ExbD and now 6 ExbB unaccounted for.

(ii) TonB homodimers are required to form a dimer of TonB-ExbD heterodimers. Previously, we knew that the TonB carboxy terminus could form disulfide-linked homodimers *in vivo* (16). Also previously, we showed that $ExbD_2$ appears to exchange one of its homodimeric carboxy termini with a single TonB carboxy terminus to form a TonB-ExbD carboxy-terminal

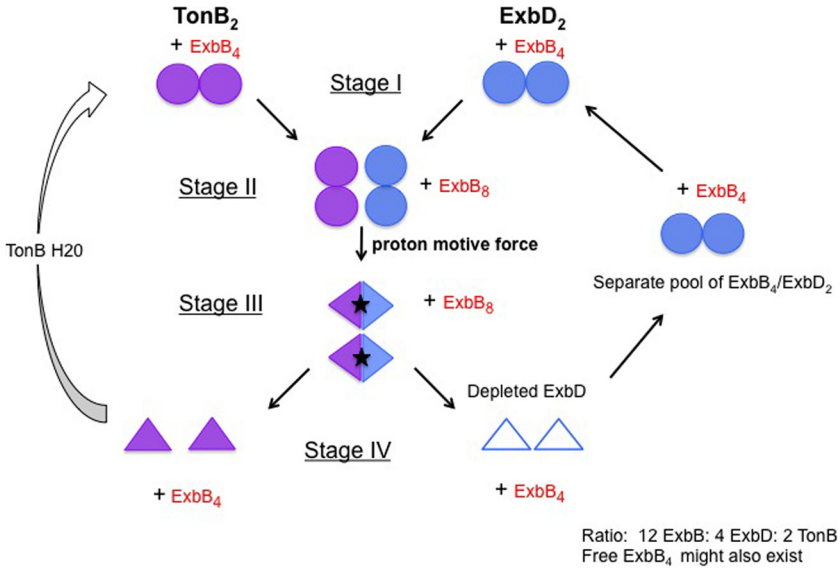


FIG 8 Model for dynamics of TonB and ExbD carboxy-terminal interactions during the energy transduction cycle. TonB amino-terminal domains remain homodimerized throughout the cycle. Arrangements of the periplasmic carboxy-terminal domains of $TonB_2$ and $ExbD_2$ homodimers are shown. Purple circles and triangles are TonB carboxy termini. Blue circles and triangles are ExbD carboxy termini. In stage I (Fig. 2), the TonB carboxy termini form homodimers, mediated by H20 in the TonB TMD. ExbB tetramers ($ExbB_4$) independently stabilize both $TonB_2$ and $ExbD_2$ homodimers. Residue H20 is required for homodimerization of the TonB carboxy terminus, which in turn is required for formation of TonB-ExbD heterodimers in stages II and III. Stages II and III consist of a dimer of heterodimers. Stage IV is binding of a monomeric carboxy terminus of TonB to a TonB-gated transporter, such that transport across the outer membrane occurs. After a transport event, H20 is required for re-formation of TonB dimers in stage I. $ExbD_2$ is deenergized after this event (empty triangles) and needs to be recycled. We speculate that $ExbD_2$ moves in and out of the complex, escorted by the ExbB tetramer. A separate pool of recycled $ExbB_4$-$ExbD_2$ exists to replenish stage I $ExbB_4$-$ExbD_2$. See Discussion for details.

heterodimer (33), but we did not understand where in the energy transduction cycle the disulfide-linked, carboxy-terminal TonB homodimers formed, what their relationship to ExbD carboxy-terminal homodimers was, or whether they were active.

The results of this study showed that the TonB carboxy terminus was homodimeric in stage I, since the H20A mutation that stalled TonB at or before stage I also decreased TonB carboxy-terminal homodimerization. This study also demonstrated that TonB carboxy-terminal homodimers formed prior to or in stage I cannot remain trapped and still associate with the OM in fractionation experiments. Taken together, the data reinforced our previous idea that $ExbD_2$ and TonB carboxy termini switch partners to form the previously characterized TonB-ExbD heterodimers in stage III (33). They now suggested that the TonB carboxy terminus must also be homodimeric to accomplish that domain swap. Such stage III TonB-ExbD heterodimers have been identified both through formaldehyde cross-linking (32) and through disulfide cross-linking of engineered carboxy-terminal Cys residues. For example, TonB F202C and ExbD K108C are each involved in formation of both their respective TonB or ExbD homodimers and also ExbD-TonB heterodimers (17, 21, 33). A mechanism whereby homodimers are prerequisites for conversion into heterodimers would be similar to the interaction between homodimeric yeast copper chaperone yCCS and homodimeric superoxide dismutase SOD1, which results in the activation of the heterodimeric yCCS-SOD1 complex (58, 59).

(iii) A separate pool of $ExbD_2$-$ExbB_4$ exists that does not interact with $TonB_2$. Because TonB appeared to be always homodimerized through its amino terminus, and ExbD (and its paralogues) form homodimers, cellular TonB-ExbD-ExbB ratios of 2:4:14 indicate that there must be an equimolar pool of $ExbD_2$ that is not in contact with $TonB_2$. It is known that ExbB and ExbD do not have separable phenotypes (60). We speculate that $ExbD_2$-$ExbB_4$ which has participated with $TonB_2$-$ExbB_4$ in stages II and III is subsequently released at the end of stage III feeds into the pool of "spent" $ExbD_2$-$ExbB_4$ and is somehow restored to activity (Fig. 8). Consistent with this idea, the ExbD paralogue MotB forms membrane pools from which it associates and dissociates with the flagellar motor for unknown reasons (61). Why might the $ExbD_2$-$ExbB_4$ complex need to be recycled? Perhaps this is the means by which the TonB carboxy terminus is exchanged from its interaction with ExbD to bind a TonB-gated transporter in stage IV.

Although the TonB system clearly requires PMF for transport across the OM, neither TonB nor ExbB TMDs have residues that participate in proton translocation (20, 23). The ExbD TMD has the sole candidate to be on a proton translocation pathway, Asp25, which is conserved among other ExbDs and the paralogues TolR and MotB. Consistent with that, inactive ExbD D25N stalls TonB-ExbD at stage II, unable to proceed to stage III, the stage that requires PMF (Fig. 2) (26). This separate pool of $ExbD_2$-$ExbB_4$ helps to explain the need for separate $TonB_2$-$ExbB_4$ and $ExbD_2$-$ExbB_4$ complexes in stage I. At the end of an energy transduction cycle, the $TonB_2$ carboxy terminus is being rehomodimerized and the $ExbD_2$ is being re-energized so that they can re-engage in stage II and subsequently form the stage III TonB-ExbD heterodimers that lead to stage IV.

The existence of a separate equimolar pool of $ExbD_2$-$ExbB_4$ without any TonB brings the total tally to 2 TonB, 4 ExbD, and 12 ExbB, leaving only 2 ExbB unaccounted for. An ExbB homodimer is a stable, detectable subunit of the homotetramer (24). Free pools of $ExbB_2$ as precursors to ExbB tetramers could make up the

deficit. Alternatively, there may be a small pool of $ExbB_4$ homotetramers that feeds into the system.

(iv) *In vivo*, the TonB carboxy terminus likely interacts as a monomer with a TonB-gated transporter. The oligomeric state of purified TonB carboxy-terminal domains alone and during interaction with purified TonB-gated transporters has been studied *in vitro* without the emergence of a consistent picture. In solution, purified TonB carboxy termini of various lengths bind to transporters as both homodimers (62–65) and monomers (66) or both (67). The carboxy-terminal ~150 residues crystallize as a homodimer (68, 69) but in a conformation which does not exist *in vivo* (17). This region of TonB also crystallizes as a monomer in cocrystals with transporters (38, 39), which may not entirely represent the *in vivo* situation, since Arg residues identified as important in TonB-transporter cocrystal structures play no role *in vivo* (70). Furthermore, the purified TonB carboxy terminus has not been sufficient to reconstitute active transport through a TonB-gated transporter *in vitro*. In retrospect, these results are not surprising, given the dependence of TonB on its TMD, ExbB, ExbD, and the PMF. Thus, the question of whether the TonB carboxy terminus functions as a monomer or a dimer to energize transport across the OM has been unresolved.

Taken together, our current and previous results suggest that TonB interacts with OM transporters as a monomer. First, H20A stalls TonB prior to or at stage I (26), and we showed here that it does so by preventing homodimerization through the carboxy terminus. This suggested that homodimerization of the carboxy terminus was necessary for one or more subsequent stages in energy transduction. Second because the H20A substitution prevents TonB from interacting with ExbD in stages II and III (26), TonB carboxy-terminal homodimerization appears to be necessary to form carboxy-terminal TonB-ExbD heterodimers of stage III. Third, because we discovered in this study that TonB carboxy-terminal homodimers cannot remain homodimerized and associate with the OM, homodimers must be resolved prior to interaction with a transporter in stage IV. Finally, the need for rehomodimerization of the carboxy terminus to initiate a new energy transduction cycle following stage IV suggested that the TonB carboxy terminus interacts with OM transporters as a monomer.

If one of the pair of amino-terminally homodimerized TonBs interacts with a single transporter, what might the other one do? Transporters themselves may form functionally significant oligomers. Dimerization of Cir has been recently shown to be necessary for colicin Ia uptake, so it is possible that other TonB-gated transporters also function as homodimers (71). FepA is known to formaldehyde cross-link both as a homodimer and a homotrimer *in vivo* (9, 48, 72), although it is not known if this multimerization is necessary for function.

(v) Another role for ExbB in energy transduction. We propose that the dynamic association and dissociation of $TonB_2$-$ExbB_4$ and $ExbD_2$-$ExbB_4$ complexes during energy transduction is accomplished through modulation of the ExbB cytoplasmic domains, which constitute the majority of its soluble residues (40, 73). The most speculative part of our model combines the $ExbB_4$ from $TonB_2$ and $ExbD_2$ into $ExbB_8$ as scaffolding for a dimer of carboxy-terminal heterodimers, since it appears that they subsequently revert back to their original condition as separate $TonB_2$-$ExbB_4$ and $ExbD_2$-$ExbB_4$ complexes to initiate a new cycle of energy transduction.

The soluble cytoplasmic domains of ExbB are important in allowing TonB and ExbD to form stage II heterodimers (Fig. 2) (23, 24). Eight out of nine 10-residue deletions in the ExbB cytoplasmic loop domain cause immediate growth arrest when they are expressed near chromosomal levels, suggesting that these regions bind important regulatory factors (25). Consistent with the possibility that ExbB and associated proteins are dynamically moved through the CM by unknown proteins within the CM, gliding motility in *Myxococcus xanthus* uses the *aglQRS* locus, which encodes one MotA/TolQ/ExbB homolog (AglR) and two MotB/TolR/ExbD homologs (AglQ and AglS), all of which participate in trafficking within the membrane for gliding motility (74). Such a complicated mechanism suggests the need for additional proteins to help run the cycle, and there is evidence that such currently unknown proteins exist. ExbB formaldehyde cross-links as a tetramer with 85 kDa of unknown protein (24, 75).

Results in the context of previous studies. Recently two models for TonB-dependent energy transduction have been proposed. They are both different from one another and from the model in this study (54, 76). Even though the three models use different techniques to address the mechanism, some commonalities emerge.

Jordan et al. used fluorescence polarization measurements to detect PMF-dependent movement of green fluorescent protein (GFP)-TonB (76). Although the authors speculate that the motion they detected was rotational, perhaps it might have reflected the movement of $ExbB_4$-$TonB_2$ in and out of association with $ExbB_4$-$ExbD_2$ proposed in our model. Consistent with that idea, the motion they detected was also dependent upon ExbB/ExbD, although they did not individually distinguish between effects of each protein. In our model, the absence of ExbB alone would have completely prevented the motion.

Sverzhinsky et al. reconstituted the first-ever observed tripartite complex of TonB, ExbB, and ExbD from purified components—a major accomplishment (54). Several aspects of their data are supportive of our model. In keeping with their *in vitro* results, we previously showed that ExbB was a dimer of homodimers *in vivo* (24). They purified a complex consisting of $ExbB_4$-$ExbD_1$-$TonB_1$, which could represent an intermediate that can form during the transition between stages II and III (Fig. 8). Their previous isolation of $ExbB_4$-$ExbD_2$ complexes *in vitro* (52) could reflect the pool of $ExbD_2$ homodimers that are not in contact with $TonB_2$ in Fig. 8. In addition, TonB-TonB homodimerization was not detected in their phage-panning assay, where the TonB TMD was absent, consistent with our results showing that TMD residue H20 was important for that homodimerization of the carboxy terminus. It would be interesting to know whether, in the $ExbB_4$-$ExbD_1$-$TonB_1$ complex of Sverzhinsky et al. (54), TonB and ExbD can form either a proteinase K-resistant conformation in micelles or the TonB-ExbD formaldehyde-cross-linked complex, which are hallmarks of TonB system activity *in vivo* (32).

In some areas, our findings diverge from those of Sverzhinsky et al. (54). Given our data showing that TonB is homodimeric throughout an energy transduction cycle, the purified $ExbB_4$-$ExbD_1$-$TonB_1$ complex is unlikely to represent the ExbB-ExbD-TonB complex characteristic of "functional" TonB. In addition, the model published by Sverzhinsky et al. (54) does not account for the cellular TonB-

ExbD-ExbB ratios of 1:2:7 seen *in vivo*. Moreover, their model is partially based on the idea that the TonB protein is proteolytically labile (54). However, when expressed to chromosomally encoded levels in a wild-type background, TonB is proteolytically stable over more than two cell doublings, as are ExbD, ExbB, and FepA (20, 77) (see Fig. S7 in the supplemental material). TonB is unstable only under non-wild-type conditions, such as when it is overexpressed and exceeds the capacity of ExbB and ExbD to stabilize it (57), or when ExbB and ExbD are absent (78).

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