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ExbD Mutants Define Initial Stages in TonB Energization

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Abstract

Cytoplasmic membrane (CM) proteins ExbB and ExbD of the *Escherichia coli* TonB system couple CM protonmotive force (pmf) to TonB. TonB transmits this energy to high-affinity outer membrane active transporters. ExbD is proposed to catalyze TonB conformational changes during energy transduction. Here, the effect of ExbD mutants and changes in pmf on TonB proteinase K sensitivity in spheroplasts was examined. Spheroplasts supported the pmf-dependent formaldehyde crosslink between periplasmic domains of TonB and ExbD, indicating that they constituted a biologically relevant *in vivo* system to study changes in TonB proteinase K sensitivity. Three stages in TonB energization were identified. In Stage I, ExbD L123Q or TonB H20A prevented proper interaction between TonB and ExbD, rendering TonB sensitive to proteinase K. In Stage II, ExbD D25N supported conversion of TonB to a proteinase K resistant form, but not energization of TonB or formation of the pmf-dependent formaldehyde crosslink. Addition of protonophores had the same effect as ExbD D25N. This suggested the existence of a pmf-independent association between TonB and ExbD. TonB proceeded to Stage III when pmf was present, again becoming proteinase K sensitive, but now able to form the pmf-dependent crosslink to ExbD. Absence or presence of pmf toggled TonB between Stage II and Stage III conformations, which were also detected in wild-type cells. ExbD also underwent pmf-dependent conformational changes that were interdependent with TonB. These observations supported the hypothesis that ExbD couples TonB to the pmf, with concomitant transitions of ExbD and TonB periplasmic domains from unenergized to energized heterodimers.

Introduction

The TonB system of Gram-negative bacteria solves the problems of nutrient acquisition created by their diffusion-limited outer membranes (OM) [1]. In *Escherichia coli* K12, it energizes active transport of iron-siderophore complexes and vitamin B12 across the OM through high affinity transporters. In other Gram-negative bacteria, many of which have multiple TonB systems, it energizes transport of diverse substrates such as heme, maltodextrin, sucrose, and nickel [2; 3; 4; 5; 6]. In *Escherichia coli*, OM transporters also serve as receptors for a variety of colicins and bacteriophages, many of which require the TonB system to enter cells [7; 8; 9]

TonB system proteins are found in both the cytoplasmic membrane (CM) and OM, as a variety of relatively substrate-specific OM TonB-gated transporters (TGTs) and three

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integral CM proteins, TonB, ExbB, and ExbD. TonB/ExbB/ExbD appear to form a complex. Together ExbB and ExbD are proposed to harvest the protonmotive force (pmf) energy which is then transmitted by TonB to drive active transport across the OM. Thus the limitations of the OM, which lacks ion gradients or access to ATP, are circumvented [For recent reviews see [9; 10; 11; 12]]. Active transport of TonB-dependent ligands across the OM is prevented in the presence of protonophores such as carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and dinitrophenol (DNP) that collapse the CM proton gradient [13]. Ligands are still able to bind their respective OM transporters in protonophore-treated cells [14; 15].

ExbD (141 amino acids) and TonB (239 amino acids) have identical membrane topologies of a single transmembrane domain, with the majority of each protein occupying the periplasmic space [16; 17; 18]. ExbB (244 amino acids) has 3 transmembrane domains, with the majority of its soluble domains localized to the cytoplasm [19; 20]. While the relative stoichiometry of TonB:ExbD:ExbB in the cell is 1:2:7, the stoichiometry of an active TonB-ExbD-ExbB complex remains unknown [21].

Because TonB is a stable protein, remains associated with the CM throughout energy transduction, and is limiting relative to the number of OM transporters, it is clear that the TonB periplasmic domain must undergo cyclic contact and dissociation from the OM during energy transduction [21; 22; 23]. Three different conformations of TonB have been identified in vivo, indicating that TonB is conformationally dynamic. Known conformational changes of TonB require the presence of pmf, ExbB, and ExbD, along with a functional TonB transmembrane domain (TMD) [24; 25; 26]. The TonB TMD was recently shown to play only a structural role and is not directly part of a proton translocation pathway. TonB H20, is the only TonB TMD residue that cannot be functionally substituted with ala [27]. H20A inactivity, however, is not due to lack of a protonatable side chain, as H20 can be fully substituted with non-protonatable asn [28]. This suggests the connection between TonB periplasmic domain conformational changes and the pmf is likely indirect. The precise details of this energy coupling process remain unknown.

Two point mutations in separate domains of ExbD render it inactive—D25N in the TMD and L132Q in the periplasmic domain [29]. D25 is the sole charged residue in the ExbD TMD and is highly conserved. ExbD D25 and the corresponding asp residues in ExbD paralogues TolR and MotB are candidate residues for proton binding as part of putative proton channels in these systems [30; 31]. The potential role of L132 is unknown.

The precise role of ExbD within this system is unknown. Current data suggest that ExbD converts TonB to the active conformation that initiates substrate transport through the OM transporters [26; 27; 32; 33]. A pmf-dependent interaction between the periplasmic domains of ExbD and TonB can be trapped by formaldehyde crosslinking *in vivo*. This energized TonB-ExbD complex is no longer observed when TonB H20A or ExbD D25N is present. While this result indicates that the formaldehyde crosslinkable residues in the two proteins are not in correct apposition to form the crosslink, it does not indicate that TonB and ExbD no longer interact at all. ExbD and ExbD D25N can also be trapped in pmf-independent formaldehyde crosslinked complexes with ExbB or with another ExbD [32].

Here we demonstrate that spheroplasts represent a valid *in vivo* system for definition of TonB conformational changes, based on pmf changes and effects of ExbD mutants. Using changes in TonB and ExbD proteinase K sensitivity in spheroplasts, we show for the first time that ExbD conformation is pmf-dependent, and define three different stages in TonB energization by ExbD. The ExbD carboxy-terminus (L132) and the TonB TMD (H20) were important for staging initial ExbD-TonB interaction. A wild-type TonB-ExbD complex

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could be subsequently toggled (reversibly switched) between pmf-independent and pmf-dependent conformations with the ExbD TMD (residue D25) required to mediate this conformational switch.

Results and Discussion

Loss of protonmotive force reversibly stalls TonB conformational changes

In spheroplasts, TonB is completely sensitive to exogenous proteinase K, as might be expected for a periplasmically exposed protein (Fig. 1A, lane 4). However, as observed previously, collapsing the pmf by addition of protonophores to spheroplasts renders the amino terminal 2/3 of TonB resistant to exogenous proteinase K, resulting in an ~23 kDa fragment referred to from here on as the proteinase K resistant form or fragment of TonB [[25] and Fig. 1A, lane 4]. The identity of this fragment was previously established by its apparent molecular mass and by mapping with a set of monoclonal antibodies for which the epitopes are known [25]. It was not known, however, if this conformation was a “dead-end” representing a now permanently inactivated TonB or a temporary stall with the potential of resuming its energy transduction cycle. To distinguish between these possibilities, we examined the reversibility of the TonB proteinase K resistant conformation by washing away the previously added protonophore prior to proteinase K treatment. A “dead end” conformation would still be present after re-establishing pmf, while a stalled conformation would resume the cycle of TonB conformational changes and once again become susceptible to proteinase K.

As observed previously [25], TonB in whole cells was not accessible to proteinase K (Figs. 1A and B, lane 2) but was fully accessible and proteolytically degraded in spheroplasts (Fig. 1A, lane 4). In spheroplasts where pmf was collapsed by addition of CCCP, treatment with proteinase K resulted in detection of the previously observed proteinase K resistant conformation of TonB (Fig. 1A, lane 6 and 1B, lane 4). When CCCP was washed away and pmf restored, TonB again became completely sensitive to proteinase K (Fig. 1B, lane 5), like TonB in spheroplasts (Fig. 1A, lane 4). To determine if a functional conformation of TonB had indeed been restored, washed spheroplasts were re-treated with CCCP and then with proteinase K. Notably, the proteinase K resistant form of TonB was again detected (Fig. 1B, lane 6). These results indicated that spheroplasts retained pmf during their preparation, and after treatment with protonophores, could regenerate pmf when it was washed away. More importantly they indicated that the conformation of TonB was reversibly stalled in the absence of pmf and could recover when pmf was restored. ExbD also showed a reversible pmf-dependent change in proteinase K sensitivity (Fig. 1B, lanes 4 through 6), and these results are discussed below.

A pmf-sensitive conformational switch could be triggered by cycles of TMD residue protonation/deprotonation events. In this scenario, collapse of the pmf by addition of CCCP or mutants with an inability to respond to pmf could equate to an inability to change the protonation state of a protein. In the ATP synthase complex, protonation/deprotonation of an essential carboxyl residue in subunit *c* promotes mechanical rotation, and protonation of a TMD asp residue in the AcrB efflux pump is proposed to cause observed large structural changes in the periplasmic domain [reviewed in [34; 35]]. While there is currently no direct evidence of TMD residue protonation in the TonB system, candidate residues, such as ExbD D25, exist. In addition, we note recent results which indicate that *E. coli* normally experiences strong fluctuations in pmf [36]. The ability of TonB to switch back and forth between conformations may be an important part of coping with such fluctuations.

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In vivo pmf-dependent TonB-ExbD interaction also occurs in spheroplasts

Spheroplast generation leaves the CM intact but, through disruption of the OM and hydrolysis of the peptidoglycan layer, exposes the periplasmic domains of CM proteins such as TonB and ExbD to solution. While the proteinase K assay takes advantage of this fact, this also equates to a non-native environment for these proteins, raising the question of whether this change in environment alters the native behavior or conformations of the solution-exposed domains.

To address whether the periplasmic domains of TonB and ExbD in spheroplasts exhibit native conformations and interactions, spheroplasts were generated from a wild-type (W3110) strain and crosslinked with formaldehyde in the presence or absence of pmf (presence of CCCP). Formaldehyde is a conformation-sensitive crosslinking agent that results in formation of a methylene (CH_2) bridge between reactive residues, which must be in close association to crosslink. As observed previously for ExbD in whole cells [32], ExbD in spheroplasts crosslinked into homodimers and heterodimeric complexes with TonB or ExbB (Fig. 2, αExbD, sph). The TonB-ExbD complex in spheroplasts was also pmf-dependent, providing confidence in the validity of those findings by demonstrating that complex formation in spheroplasts had the same determinants as in whole cells (Fig. 2, compare sph to sph + CCCP). The same held true for the immunoblot assessing TonB interactions, where all known complexes detected in formaldehyde-treated whole cells were also detected in spheroplasts (Fig. 2, αTonB). This even included the TonB complexes with the OM proteins FepA and Lpp, likely due to fragments of the OM still attached to spheroplasts [37; 38].

The possibility that, for this set of experiments, spheroplasts did not form, was ruled out by prominent detection of the TonB proteinase K resistant form in a portion of the same CCCP-treated spheroplasts that were crosslinked with formaldehyde after proteinase K treatment (Fig. 2, αTonB, sph + CCCP + PK). Two higher bands were also observed in this sample. One migrated at the apparent molecular mass of full-length TonB (36 kDa) and was likely residual, undigested TonB. The highest band migrated at approximately 47 kDa. Based on its similar abundance to the TonB-ExbB crosslink, this was potentially a partially digested form of that complex. Its identity was not confirmed.

The TonB proteinase K resistant conformation is a normal part of its energy transduction cycle

Because the proteinase K resistant conformation of TonB depended on a pmf-dependent toggle switch and was not a dead-end conformation, it should exist in wild-type bacteria. We reasoned that the population of TonBs in energized spheroplasts would be constantly transitioning from one conformation to another. Since at least one conformational state of TonB was sensitive to proteinase K, the total population of resistant TonB might decrease over time, and thus the proteinase K resistant conformation may be detectable only at early time points of treatment. To search for the proteinase K resistant conformation of TonB, we treated wild-type energized spheroplasts with proteinase K for a time-course of 2 min through the standard 15 min. As in the standard assay, by 15 min proteinase K treatment time, TonB in the presence of wild-type ExbD and the pmf was fully sensitive (Fig. 3A, sph, lane 5). However, at the 2 min time point, a low level of the proteinase K resistant fragment was detected (Fig. 3A, sph, lane 2), indicating that this was a normal conformation of TonB that occurred during the energy transduction cycle. We suggest that as time moved on in spheroplasts with pmf, all of the proteinase K resistant TonB eventually transitioned to a conformation that was fully degraded by proteinase K by 15 min; hence this population did not accumulate.

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However, when pmf was collapsed, stable accumulation of the TonB proteinase K resistant conformation was observed across the time-course (Fig. 3A, sph + CCCP, lanes 2 through 6). This suggested that following CCCP addition, all the TonBs not already at the proteinase K resistant stage of the cycle either reverted, or proceeded in the cycle to that point and became unable to proceed further. TonB was rapidly stalled in this conformation, possibly as soon as the pmf was collapsed.

These results raised the likelihood that the proteinase K resistant conformation of TonB, first observed in previous studies using a 15 min proteinase K treatment, was not, as originally proposed, a pmf-dependent TonB conformational response [25]. Instead these studies, which probed early times of proteinase K treatment for the first time, revealed that the TonB proteinase K resistant conformation was pmf-independent because it existed whether or not pmf was present. Based on this new information, it seemed logical that the TonB proteinase K resistant conformation must have occurred at a point in the energy transduction cycle prior to the pmf-dependent energization of TonB, which subsequently converted TonB from proteinase K resistant to proteinase K sensitive (see Fig. 3A, lanes 2 through 5, where the proteinase K resistant conformation of TonB at 2 min becomes sensitive by 15 min only in sph where pmf is present).

In the absence of ExbD, TonB was fully sensitive to proteinase K over the time-course, whether or not pmf was collapsed, and did not form the ~23 kDa proteinase K resistant fragment even at the shortest time-point [[39] and Fig. 3B, lanes 5 and 6]. From these studies two proteinase K sensitive conformations of TonB became apparent—one formed by TonB in the absence of ExbD interaction and another after response of TonB to pmf (compare Fig. 3A, sph, lane 5 and Fig. 3B, sph, lane 6). Therefore, including the TonB proteinase K resistant conformation, three *in vivo* conformations of TonB were evident. Because pmf establishes a specific formaldehyde crosslinkable interaction between the TonB and ExbD periplasmic domains [32], the TonB proteinase K sensitive conformation in fully energized wild-type spheroplasts did not simply reflect lack of interaction with ExbD.

Formation of the TonB proteinase K resistant fragment requires a wild-type ExbD periplasmic domain

Based on the identity of the proteinase K resistant fragment, about 60% of the TonB periplasmic domain (residues 33- 156) becomes resistant to proteolysis after collapse of the pmf [25]. This ExbD-dependent resistance could be the result of either a conformational change of TonB in the presence of ExbD or direct protection of this portion of the TonB periplasmic domain by the ExbD periplasmic domain. To examine the role of ExbD in the formation of the TonB proteinase K resistant conformation, the effects of two known ExbD missense mutants, L132Q in the periplasmic domain and D25N in the TMD, were analyzed. Like wild-type ExbD, both are stable proteins, capable of forming ExbD homodimers and heterodimers with ExbB, suggesting these inactivating point mutations do not significantly alter native ExbD conformation. They differ from wild-type ExbD only in their inability to form the pmf-dependent formaldehyde crosslinked TonB-ExbD heterodimer [[32] and Fig. S1]. That result means that relationships between crosslinkable residues in ExbD or TonB alter when pmf is collapsed, but does not rule out contact through different interfaces.

Periplasmic domain mutant ExbD L132Q did not support the proteinase K resistant conformation of TonB, showing the same results as the Δ*exbD* strain (Fig. 3B, compare lane 14 to lane 2). This result suggested that the L132Q mutation prevented interaction between ExbD and TonB periplasmic domains. In contrast, TMD mutant ExbD D25N supported formation of the TonB proteinase K resistant conformation, with almost full conversion detected at the 2 min time point (Fig. 3A, lane 7). Taken together these results suggested that

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a wild-type ExbD periplasmic domain was both necessary and sufficient to support formation of the TonB proteinase K resistant conformation.

The differences in the abilities of ExbD D25N or L132Q to support formation of the TonB proteinase K resistant conformation indicated that while both ExbD missense mutants were inactive, it was for different reasons. Perhaps most importantly, it also indicated that, ExbD TMD residue D25 was not important for TonB to form the pmf-independent proteinase K resistant conformation—the functional role of D25 appeared to occur at a later stage of the energy transduction cycle. The fact that formation of the TonB proteinase K resistant conformation required a native ExbD periplasmic domain but neither pmf nor ExbD activity suggested formation was dependent on an initial assembly of TonB with ExbD (Fig. 3A, lane 7). Pmf-independent assembly between TonB and ExbD had not been detected previously because it did not lead to a formaldehyde crosslinked complex [32]. Resistance to proteolysis induced by the presence of another protein suggests direct interaction between those proteins even when a heteromeric complex has not been observed, such as in the MacA/B-TolC system [40].

In contrast to the ExbD TMD, a wild-type TonB TMD was required for formation of the TonB proteinase K resistant conformation. TonB H20A was unable to form the wild-type proteinase K resistant fragment, suggesting that ExbD and TonB TMDs had distinct functional roles (Fig. 3B, lane 11). A low level of a faster migrating, ~21 kDa, proteinase K resistant TonB H20A fragment was detected at 2 min. Detection of this fragment was not dependent on the pmf, and it was not characterized further.

At the early time points of proteinase K treatment, an ~28 kDa TonB fragment was apparent to varying degrees in the presence of mutants ExbD D25N or L132Q and TonB H20A (Fig. 3A lane 7 and 3B lanes 11 and 14). Because this ~28 kDa fragment was also present in the Δ*exbD* (Fig. 3B, lane 5) but not $ExbD^+$ background, it appeared to be a form of TonB that arises independent of ExbD conformation. It was not characterized further.

ExbD D25N prevents pmf-dependent conformational changes of TonB

In wild type spheroplasts energized by the pmf, the low level of proteinase K resistant TonB appeared to reflect the proportion of TonB at a certain stage of the energy transduction cycle. In contrast, the effect of the ExbD D25N mutation was to convert the majority of TonB to the proteinase K resistant form, even when pmf was present (Fig. 3A, sph, lane 7), with the degree of formation equal to a wild-type strain after collapse of pmf (compare to Fig. 3A, lanes 2 and 7)). Thus the ExbD D25N TMD mutation had the same immediate effect on TonB conformation as collapse of the pmf. Consistent with that idea, the degree of conversion supported by ExbD D25N was unchanged after collapse of pmf (Fig. 3A, compare sph to sph + CCCP, lanes 7 through 10). ExbD D25, a protonatable residue, was therefore identified as necessary for the conformational response to the pmf that rendered TonB sensitive to proteinase K. While it is currently unknown if ExbD D25 is protonated, such an event could mediate the observed pmf-toggled conformational changes. The ExbD TMD is thus far unique in that respect because there appear to be no TonB TMD residues that could participate directly in proton translocation [28]. It is not yet clear whether ExbB TMD residues play a role in utilization of the pmf. Overall, these results pinpointed ExbD, not pmf, as the direct regulator of TonB conformational changes, where the inability of ExbD to respond to pmf (collapse of pmf or presence of D25N), stalled TonB in the proteinase K resistant conformation, dependent on an assembly of the periplasmic domains of TonB and ExbD.

It is unknown why the interaction between TonB and ExbD D25N became sensitive to proteinase K at a faster rate than with wild-type ExbD, even after collapse of pmf (Fig. 3A,

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sph + CCCP, compare lanes 2 through 5 to lanes 7 through 10). The high level of the TonB proteinase K resistant conformation supported by ExbD D25N at 2 min suggested this was not normally an unstable or transient interaction because the majority of the TonB population was in that conformation. A short-lived interaction would have resulted in only a small population of TonB remaining associated with ExbD D25N, in the proteinase K resistant conformation, at one time. The initial high level of proteinase K resistance followed by the rapid conversion to proteinase K sensitive may reflect a dissociation of the TonB-ExbD D25N complex over the time-course or greater accessibility of ExbD D25N compared to wild-type ExbD to proteinase K.

ExbD conformation changes when pmf is collapsed

Because ExbD was clearly involved in TonB conformational changes, we wanted to characterize the conformational behavior of ExbD, which had not been examined previously, except for a demonstrated sensitivity to proteinase K or trypsin treatment for 15 min in energized spheroplasts [41]. When Fig. 1 samples from the pmf-reversibility studies of TonB conformational changes were immunoblotted with ExbD-specific antibodies, the ExbD conformational response manifested as a change from almost full sensitivity in the presence of pmf (Fig. 1B, lane 5) to the full length of ExbD becoming resistant to proteinase K when pmf was collapsed (Fig. 1B, lane 4). As seen for TonB, these pmf-dependent conformational changes of ExbD were reversible (Fig. 1B, αExbD, compare lanes 4, 5, and 6). A proteinase K treatment time-course revealed that a relatively high level of proteinase K resistant wild-type ExbD was detected at 2 min in the presence of pmf and transitioned to a proteinase K sensitive form and was degraded by 15 min (Fig. 4A, sph, lanes 2 through 5). Like TonB, after collapse of the pmf, ExbD remained stalled in the proteinase K resistant conformation (Fig. 4A, sph + CCCP, lanes 2 through 5).

D25 in the ExbD TMD is a prime candidate through which direct response of ExbD to the pmf could be initiated. Consistent with that idea, no conformational response of ExbD D25N to pmf was observed—it remained sensitive to proteinase K whether or not pmf was present (Fig. 4A, lanes 7 through 10, compare sph to sph + CCCP). Homologous asp residues in the Tol and Mot protein systems also influence protein conformation. TolR D23, predicted to be on the TolQR ion pathway, plays a role in conformational changes of the TolR periplasmic domain, with D23A mimicking the effect of CCCP on TolR conformation [42]. To date, changes in the MotA cytoplasmic domain have been documented when MotB D32 is mutated [43], and current models also place D32 on a proton pathway [44].

TonB is required for pmf-dependent ExbD conformational change

Three different conditions prevented formation of the ExbD proteinase K resistant form when pmf was collapsed:Δ *tonB*, (Fig. 4B, lane 6), ExbD L132Q (Fig. 4B, lane 12), and TonB H20A (Fig. 4B, lane 15). These results suggested that the TonB and ExbD proteinase K resistant conformations were interdependent, and supported the idea that ExbD L132Q did not properly assemble with TonB; likewise TonB H20A also appeared to prevent proper interaction with ExbD. Because the pmf-dependent TonB-ExbD formaldehyde crosslink did not form in ExbD L132Q or TonB H20A mutants, this initial proper assembly appeared to be a prerequisite for subsequent energization of TonB ([32] and Fig. S1). Because the TonB transmembrane domain is not directly part of a proton translocation pathway [28] and yet TonB H20A did not support ExbD pmf responsiveness, it may mean that the TonB TMD is required for proper assembly of ExbD into an energy transduction complex. Alternatively, assembly of TonB with ExbD might initiate harnessing of the pmf by ExbB/ExbD, and trigger pmf-dependent conformational changes of ExbD only when required to energize TonB.

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A model for early stages in TonB energization

Based on these data, TonB appears to have three stages on the pathway to becoming "energized" by the pmf, which correspond to our finding of two stages that are sensitive to proteinase K and one stage that is resistant. TonB remains in the first stage if its periplasmic domain cannot properly assemble with ExbD (Fig. 5, Stage I). This can be either because the TonB TMD carries a mutation (H20A) that prevents proper TonB assembly with ExbD or because an ExbD periplasmic domain mutation (L132Q) prevents proper direct interaction with the TonB periplasmic domain. TonB stalled at Stage I is sensitive to proteinase K degradation whether or not the pmf is present (all mutant strains in Fig. 3B, for example). Consistent with its inability to respond to the pmf due to the absence of initial correct interactions, TonB stalled at Stage I also does not formaldehyde crosslink to ExbD ([32] and Fig. S1).

In the second stage, TonB and ExbD come together in such a way that both proteins become proteinase K resistant. This represents an initial assembly of TonB and ExbD prior to employing pmf energy for function (Fig. 5, Stage II). This stage is characterized by the proteinase K resistant conformation of TonB that is detected when pmf is present, but only significantly accumulates when TonB is stalled at this stage by collapse of pmf or when TonB conformation presumably cannot be coupled to the pmf, i.e. when ExbD D25N is present (see Fig. 3A, ExbD sph + CCCP or ExbD D25N). Consistent with this idea, TonB and ExbD D25N do not formaldehyde crosslink through their periplasmic domains *in vivo* [32]. Stage II contact between TonB and ExbD periplasmic domains is almost certainly mediated through interaction with ExbB, since ExbB appears to be the scaffold that stabilizes both proteins, and TonB remains at what we now call Stage I if ExbB is deleted [32; 39; 41].

Pmf energizes the TonB-ExbD transition to Stage III (Fig. 5, Stage III). This conformation is represented by the pmf-dependent formaldehyde crosslinked TonB-ExbD periplasmic domain interaction and the sensitivity to proteinase K that both occur only in wild-type *E. coli* (see Fig. 3A, ExbD sph and Fig. S1 wild type lane). Because the pmf acts as a toggle switch between Stages II and III, the TonB-ExbD complex that has been stalled at Stage II by addition of CCCP can proceed to Stage III once CCCP is washed away. The TonB-ExbD complex at Stage III can be sent back to Stage II by addition of CCCP (Fig. 1B, compare lanes 4, 5, and 6).

While it does not focus on TonB-TGT (TonB-gated transporter) interactions, this model raises some interesting possibilities in terms of how ExbD-TonB interactions might fit into the cycle of TonB-TGT interactions at the OM. In the absence of both ExbB/D and paralogues TolQ/R, which can otherwise partially substitute for ExbB/D, TonB is found predominantly in OM sucrose density gradient fractions [33; 39; 45; 47]. Because it is clear that TonB does not shuttle to the OM during energy transduction [23], this observation suggests that TonB interactions at the OM occur with sufficiently high affinity to pull TonB out of its CM location during the fractionation process. Known interactions of TonB with TGTs occur even in the absence of pmf [45; 46, data not shown]. Perhaps the observed TonB-TGT interactions represent TonB at Stages I and II in this model, with TonB spanning the periplasm, in contact with OM proteins and sensitive to proteinase K, but in Stage II, ExbD interaction at a more amino-terminal region of TonB protects that region, generating a proteinase K resistant fragment. It may be that energy of the pmf is used by ExbD to release this high affinity TonB-TGT interaction. Alternatively, it may be that TonB at Stage III represents the initial actual energy transducing contact with the TGT. In that case predominance of TonB at the OM in the absence of ExbB/D and TolQ/R would represent TonB whose disordered conformation could not be regulated and which therefore cannot result in an energy transduction event. It will be important not only to further define an

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energy transduction event, but to characterize the *in vivo* conformation(s) of the TonB carboxy terminus when it interacts with the TGTs or other OM proteins.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. A Δ*exbD*, Δ*tolQR* strain (RA1045) was used as the Δ*exbD* background and to express plasmid-encoded ExbD variants to avoid phenotypes attributable to ExbD paralogue TolR, which can partially substitute for ExbD function [33; 47]. pKP1333, pExbD(L132Q), was a derivative of pKP999 (*exbD* in pPro24). The L132Q substitution was generated using 30-cycle extra-long PCR. Forward and reverse primers were designed with the desired base change flanked on both sides by 12–15 homologous bases (primer sequences available upon request). DpnI digestion was used to remove the template plasmid. To ensure no unintended base changes were present, the sequence of the entire *exbD* gene was verified by DNA sequencing at the Penn State Genomics Core Facility – University Park, PA.

Media and culture conditions

Luria-Bertani (LB) and M9 minimal salts were prepared as previously described [48]. Liquid cultures and agar plates were supplemented with 34 μg ml^{-1} chloramphenicol or 100 μg ml^{-1} ampicillin and plasmid-specific levels of sodium propionate or L-arabinose (percent as w/v), as needed for expression of ExbD and TonB proteins from plasmids. M9 salts were supplemented with 0.5% glycerol, 0.4 μg ml^{-1} thiamine, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.2% casamino acids, 40 μg ml^{-1} tryptophan, and 1.85μM or 37 μM $FeCl_3 \cdot 6H_2O$. Cultures were grown with aeration at 37°C.

Proteinase K accessibility assays

For reversibility assays, spheroplasts were generated from wild-type cells (W3110) and treated with CCCP to collapse pmf. Identical results were observed when spheroplasts were treated with the protonophore DNP (data not shown). Samples were harvested and treated with proteinase K or left untreated. Those samples represented the end point of the previous proteinase K assays, where treatment with proteinase K resulted in detection of the novel proteinase K resistant form of TonB. To re-establish pmf for the remaining sample, the proteinase K untreated, CCCP-treated spheroplasts were subsequently pelleted, washed, and resuspended in buffer without CCCP. These washed spheroplasts were divided in half. One sample was re-treated with CCCP, and one was treated with solvent only (DMSO). Both were then treated with proteinase K. As a control, whole cells were also treated with proteinase K or left untreated. All manipulations occurred at 4 ° C.

For standard assays, spheroplasts were prepared and treated with proteinase K as described previously [24; 25]. For time-course studies, spheroplasts were treated for 2, 5, 10, or 15 min with proteinase K. The effect of protonophores was examined by proteinase K treatment after treatment of spheroplasts with 60μM carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) or 10mM DNP compared to an equal volume of solvent only, dimethyl sulfoxide (DMSO) for 5 min. After limited proteolysis, TCA precipitated samples were visualized on immunoblots of 11% or 15% SDS polyacrylamide gels with TonB-specific monoclonal antibodies or ExbD-specific polyclonal antibodies, respectively. All manipulations occurred at 4 ° C.

Formaldehyde crosslinking in spheroplasts

Spheroplasts were prepared as above for standard assays and treated with DMSO solvent or 60μM CCCP. A CCCP-treated sample was also treated with proteinase K for 15 min as

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described above. For formaldehyde crosslinking, spheroplasts were pelleted and resuspended in 100mM sodium phosphate buffer, pH 6.8 containing 0.25M sucrose and 2mM $MgSO_4$. For CCCP-treated spheroplasts, CCCP was maintained in the crosslinking buffer. Samples were treated with 1% monomeric paraformaldehyde (inverting to mix) for 15 min at room temperature. Pellets were solubilized at 60ºC in Laemmli sample buffer [49].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CCCP	carbonylcyanide-*m*-chlorophenylhydrazone
CM	cytoplasmic membrane
DMSO	dimethyl sulfoxide
DNP	dinitrophenol
OM	outer membrane
pmf	protonmotive force
sph	spheroplasts
TGT	TonB-gated transporter
TMD	transmembrane domain

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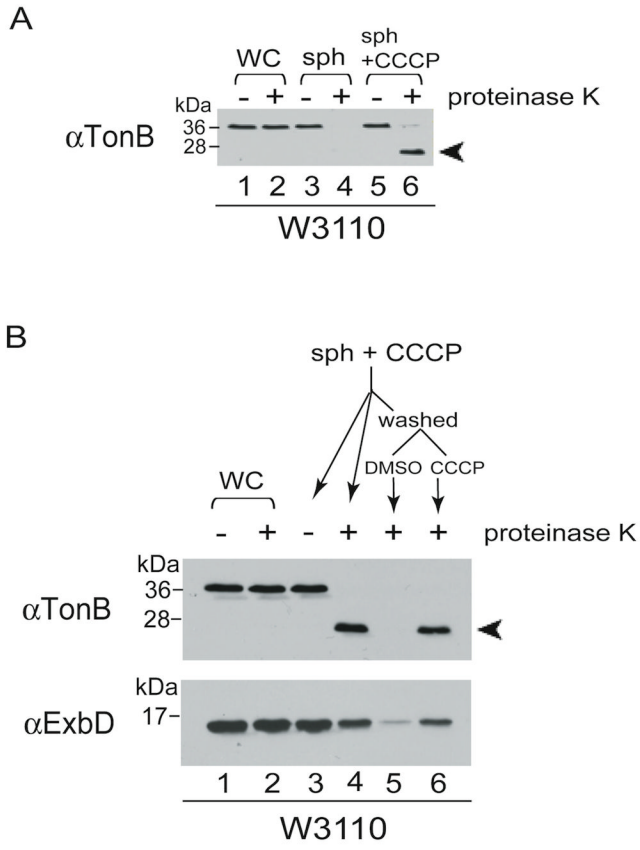

Fig. 1.

Pmf is the toggle switch for TonB and ExbD conformational changes. Spheroplasts were generated with a wild-type strain (W3110). A, For a standard assay, whole cells (WC), spheroplasts (sph) or spheroplasts treated with CCCP (sph + CCCP) were treated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) proteinase K for 15 min, as described in Materials and Methods. B, For a reversibility assay, spheroplast preparations were treated with CCCP (sph + CCCP) and divided in half. From one half, samples were treated with (lane 4) or without (lane 3) proteinase K as described in Materials and Methods. The remaining half was washed then resuspended in buffer without CCCP (washed). Then half was re-treated with CCCP (lane 6) and half with solvent only (DMSO, lane 5). Both samples were then treated with proteinase K as above. Whole cells (WC) were also treated with (lane 2) or without (lane 1) proteinase K. For all, TCA precipitated samples were resolved on 11% or 15% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal or ExbD-specific polyclonal antibodies, respectively. Data shown are representative immunoblots from triplicate assays. Samples shown, immunoblotted with two antibodies, came from the same assay. “+” or “−” above each lane indicates presence or absence, respectively, of added proteinase K. Positions of molecular mass markers are indicated on the left. “ ◂” indicates the ~23 kDa proteinase K resistant fragment of TonB.

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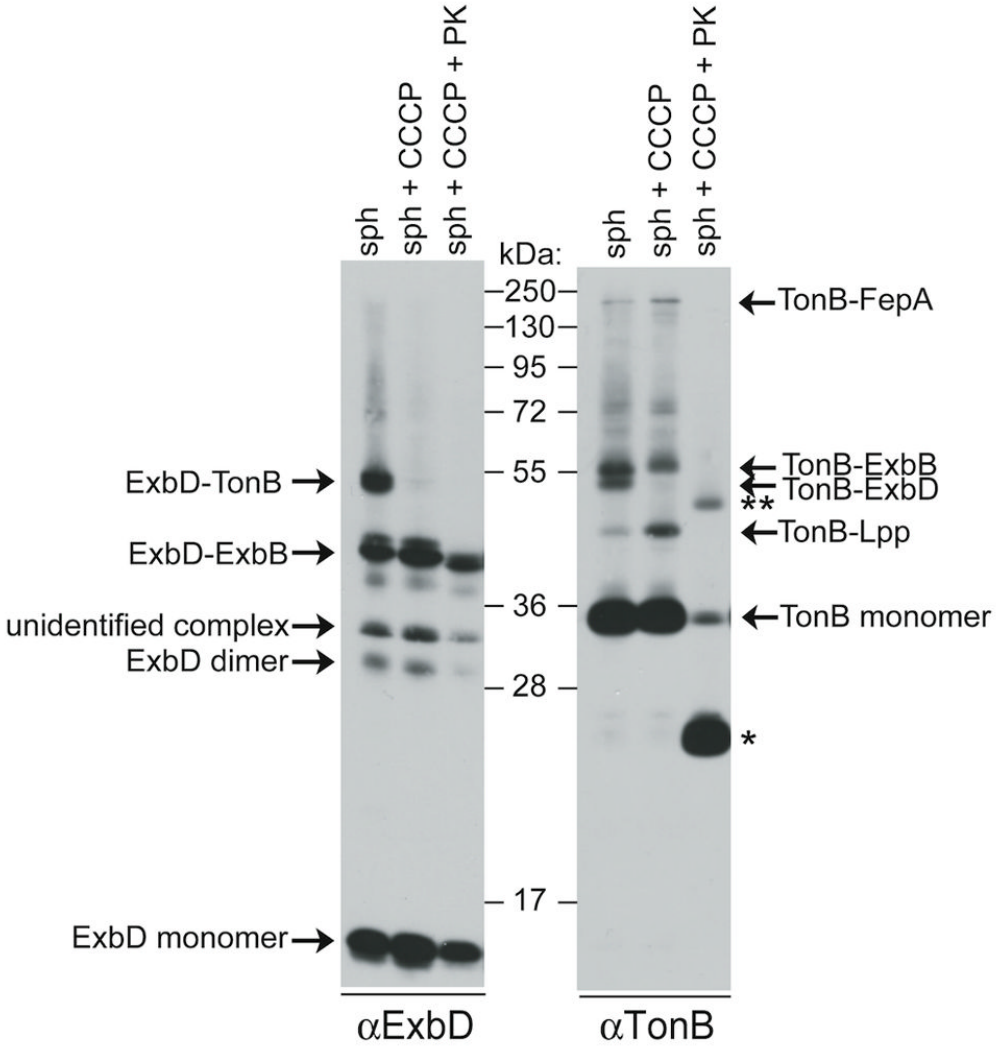

Fig. 2.

The pmf-dependent TonB-ExbD formaldehyde crosslink forms in spheroplasts. Spheroplasts were generated with wild-type strain (W3110) and treated with DMSO solvent (sph) or CCCP (sph + CCCP). One set of CCCP-treated spheroplasts was also treated with proteinase K. All were then crosslinked with formaldehyde as described in Materials and Methods. Samples were divided and duplicate sets resolved on the same 13% SDS-polyacrylamide gel. The duplicate sets were separately immunoblotted with either ExbD- or TonB-specific antibodies. Positions of ExbD- or TonB-specific complexes and the monomeric protein are indicated on the sides. Positions of molecular mass standards are indicated in the center. "*" indicates the ~ 23 kDa proteinase K resistant fragment of TonB. "**" indicates an unidentified crosslinked complex of the TonB proteinase K resistant fragment.

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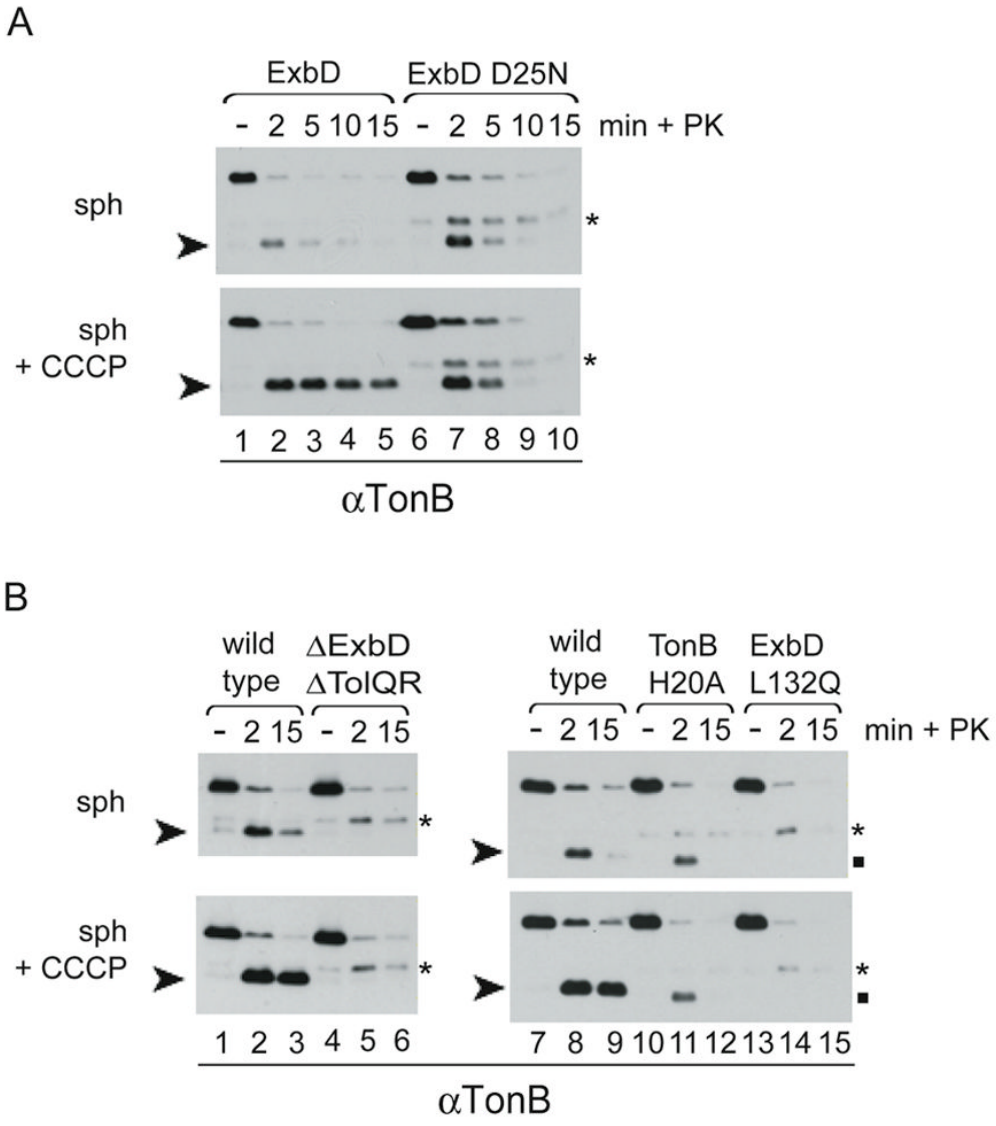

Fig. 3.

Mutations cause variations in pmf-responsive conformations of TonB. A. Spheroplasts were generated with a Δ*exbD*, Δ*tolQR* (RA1045) strain expressing plasmid-encoded wild-type ExbD (RA1045/pKP999, 3mM sodium propionate) or ExbD D25N (RA1045/pKP1064, no inducer). Spheroplasts treated with DMSO (sph) or CCCP (sph +CCCP) were treated without (−) or with proteinase K for a time-course of 2, 5, 10, and 15 minutes as described in Materials and Methods. B, Spheroplasts were generated with a wild-type strain (W3110), a Δ*exbD*, Δ*tolQR* (RA1045) strain, a *tonB::blaM* strain (KP1344) expressing plasmid-encoded TonB H20A (KP1344/pKP381, 0.001% arabinose), or a Δ*exbD*, Δ*tolQR* (RA1045) strain expressing plasmid-encoded ExbD L132Q (RA1045/pKP1333, 0.8mM sodium propionate). Spheroplasts treated with DMSO (sph) or CCCP (sph +CCCP) were treated without (−) or with proteinase K for 2 and 15 minutes. For all, TCA precipitated samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal antibody. "➤" indicates the ~ 23 kDa proteinase K resistant fragment of TonB. "*" indicates the ~28 kDa TonB fragment. "■" indicates the ~21 kDa TonB H20A fragment.

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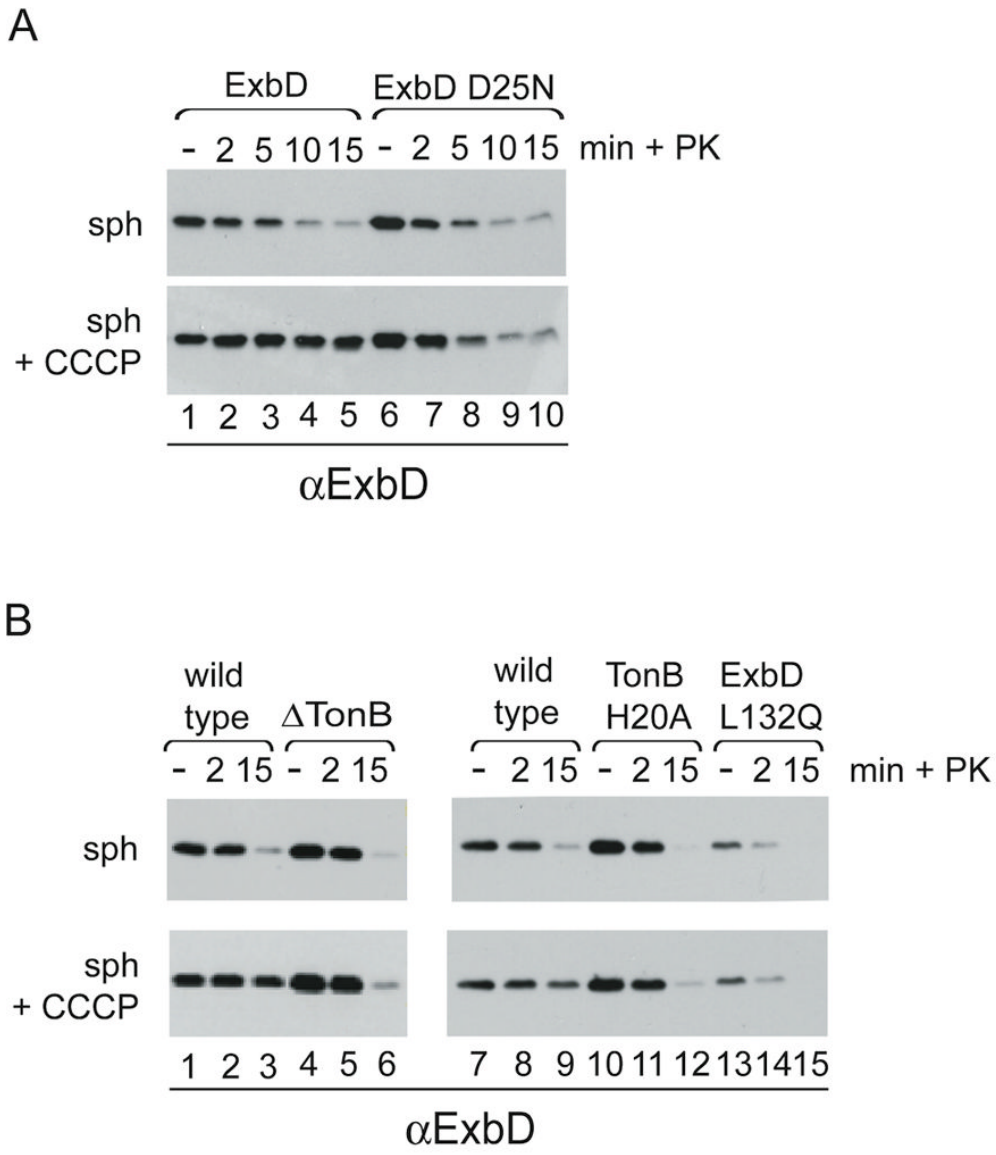

Fig. 4.

ExbD mutants alter its conformational response to changes in pmf. A. Spheroplasts were generated with a Δ*exbD*, Δ*tolQR* (RA1045) strain expressing plasmid-encoded wild-type ExbD (RA1045/pKP999, 3mM sodium propionate) or ExbD D25N (RA1045/pKP1064, no inducer). Spheroplasts treated with DMSO (sph) or CCCP (sph +CCCP) were treated without (−) or with proteinase K for a time-course of 2, 5, 10, and 15 minutes as described in Materials and Methods. B, Spheroplasts were generated with a wild-type strain (W3110), a "ΔTonB" *tonB::blaM* strain (KP1344), a *tonB::blaM* strain (KP1344) expressing plasmid-encoded TonB H20A (KP1344/pKP381, 0.001% arabinose), or a Δ*exbD*, Δ*tolQR* (RA1045) strain expressing plasmid-encoded ExbD L132Q (RA1045/pKP1333, 0.8mM sodium propionate). Spheroplasts treated with DMSO (sph) or CCCP (sph +CCCP) were treated without (−) or with proteinase K for 2 and 15 minutes. For all, TCA precipitated samples were resolved on 15% SDS-polyacrylamide gels and immunoblotted with ExbD-specific monoclonal antibody. All immunoblots shown in this figure came from the same samples immunoblotted with different antibody in Figure 3.

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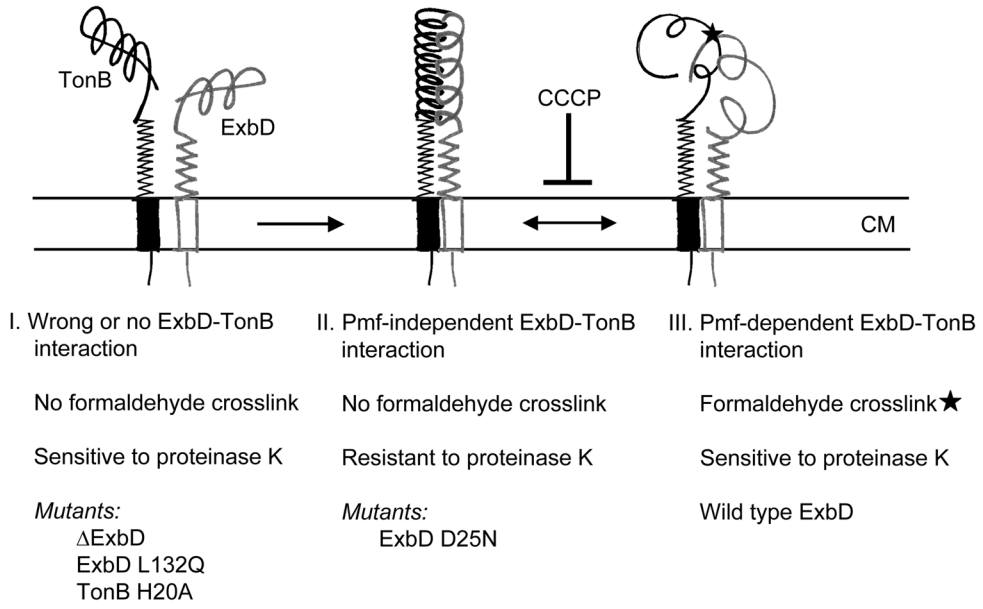

Fig. 5.

Model for initial stages in TonB energization. Three sequential stages in TonB energization in the cytoplasmic membrane (CM) are shown from left to right. ExbB, assumed to be present for all stages, is not shown. Black constructs with filled transmembrane domains represent TonB; gray constructs with empty transmembrane domains represent ExbD. Jagged regions represent disordered domains. This model is not drawn to scale and represents a conceptual framework only. Mutants that stall TonB at each stage are listed below the stage. Stage I is a theoretical possibility not demonstrated to exist for wild-type strains but invoked due to the behavior of ExbD periplasmic mutants and TonB in the absence of ExbD or vice versa. In Stage I, ExbD and TonB periplasmic domains are not in detectable contact. They cannot proceed to Stage II if ExbD is absent or when present, carries the periplasmic L132Q mutation. In this condition, TonB is fully sensitive to proteinase K. TonB carrying the H20A mutation also appears to be stalled at this Stage. In Stage II, the periplasmic domains of TonB and ExbD interact in a configuration that does not require the pmf. This configuration becomes detectable when TonB fails to proceed further to Stage III and remains stalled at Stage II, the hallmark of which is proteinase K resistance of the amino terminal 2/3 of TonB and of ExbD. Collapse of the pmf by CCCP or the D25N mutation in the ExbD amino terminus prevent ExbD and TonB from proceeding to Stage III. In Stage III, the conformational relationship between the TonB and ExbD periplasmic domains has changed such that formaldehyde crosslinkable residues in the periplasmic domains of both proteins move into close proximity (star). This new conformational relationship is also marked by complete TonB sensitivity to proteinase K. The transition between Stages II and III is reversible, with presence or absence of pmf acting as the toggle switch.

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Table 1

Strains and plasmids used in this study.

Strain or Plasmid	Genotype or Phenotype	Reference
Strains		
W3110	F^- IN*(rrnD-rrnE)1*	49
RA1045	W3110, Δ*exbD*, Δ*tolQR*	33
KP1344	W3110 *tonB*, *P14::blaM*	25
Plasmids		
pPro24	sodium propionate (2-methyl citrate)-inducible, pBR322 *ori*	50
pKP381	TonB H20A	27
pKP999	*exbD* in pPro24	32
pKP1064	ExbD D25N	32
pKP1333	ExbD L132Q	Present study